Supporting information

Automated chemoenzymatic modular synthesis of human milk oligosaccharides on a digital microfluidic platform

Yiran Wu, †[abc] Yunze Sun, †[abc] Caixia Pei, [abd] Xinlv Peng, [abc] Xianming Liu, [e] Eika Qian, [d] Yuguang Du, *[ab] and Jian-Jun Li *[ab]

[a.] *State Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, China. E-mail: jjli@ipe.ac.cn; ygdu@ipe.ac.cn.*

[b.] *Key Laboratory of Biopharmaceutical Preparation and Delivery, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, China.*

[c.] *School of Chemical Engineering, University of Chinese Academy of Sciences, Beijing 100049, China*

[d.] *Graduate School of Bio-Applications and Systems Engineering, Tokyo University of Agriculture and Technology, Tokyo 184-8588, Japan.*

[e.] *Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China*

† Equal contribution.

Correspondence:

Jianjun Li, Institute of Process Engineering, Chinese Academy of Sciences. E-mail: jjli@ipe.ac.cn; Yuguang Du, Institute of Process Engineering, Chinese Academy of Sciences. E-mail: ygdu@ipe.ac.cn.

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Table S8. Comparison of the performance of the various modules

Figure S1. A schematic representation on how the system works

2. Enzyme modules-catalyzed reactions and components of each module

1.1 Enzyme modules 1: β1,4-galactosylasation module

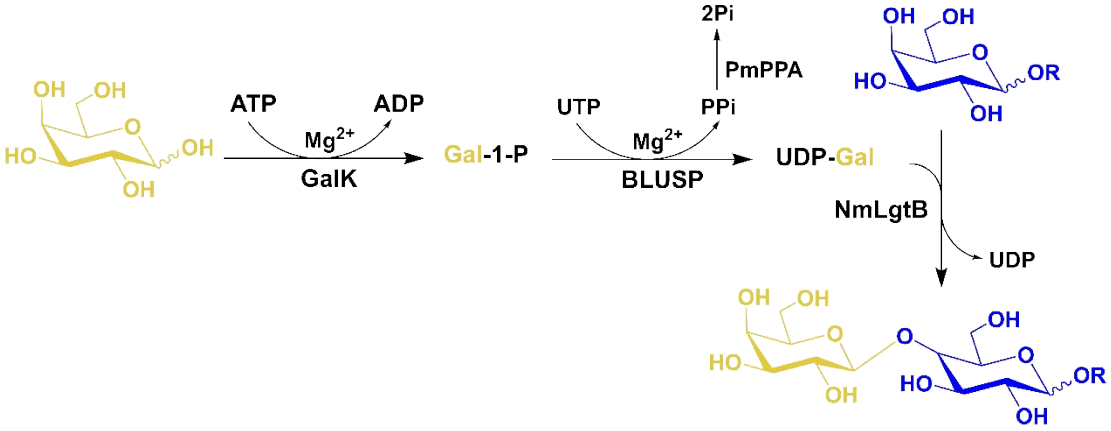


Table S1 Components of β-1,4-galactosylation module

components	final concentration
Receptor	3 mM
Gal	3.6 mM
ATP	3.6 mM
UTP	3.6 mM
$MgCl_2$	20 mM
Tris-HCl buffer (pH 7.0)	100 mM
GalK	0.25 mg/mL
BLUSP	0.5 mg/mL
PPA	0.25 mg/mL
NmLgtB	0.5 mg/mL

1.2 Enzyme modules 2: α2,6-sialylation module

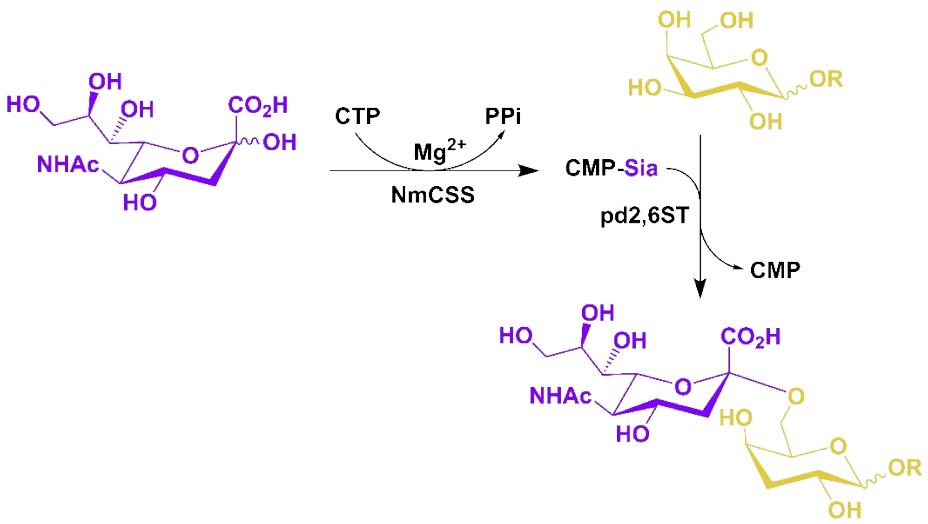


Table S2 Components of α-2,6-sialylation module

components	final concentration
receptor	3 mM
Neu5Ac	3.6 mM
CTP	3.6 mM
$MgCl_2$	10 mM
Tris-HCl buffer (pH 8.5)	100 mM
NmCSS	0.25 mg/mL
Pd2,6ST	0.5 mg/mL

1.3 Enzyme modules 3: α2,3 sialylation module

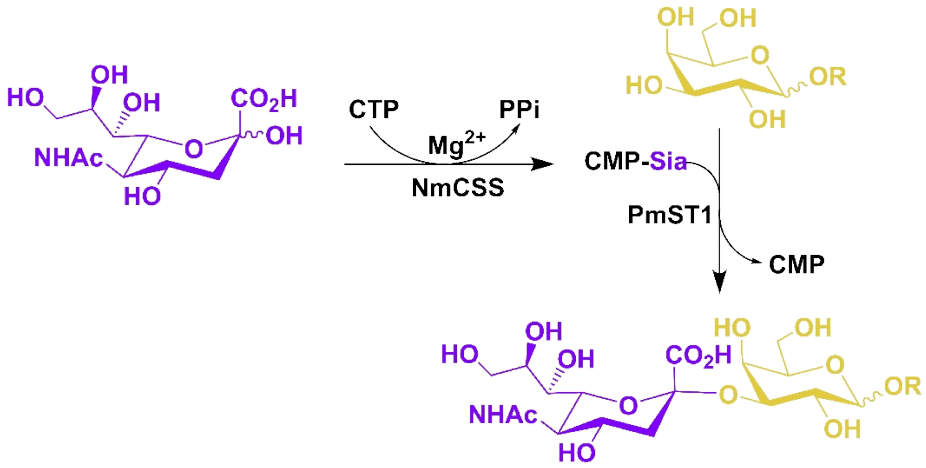


Table S3 Components of α-2,3-sialylation module

components	final concentration
receptor	3 mM
Neu5Ac	3.6 mM
CTP	3.6 mM
$MgCl_2$	10 mM
Tris-HCl buffer (pH 8.5)	100 mM
NmCSS	0.25 mg/mL
PmST1	0.5 mg/mL

1.4 Enzyme modules 4: α1,3-fucosylation module

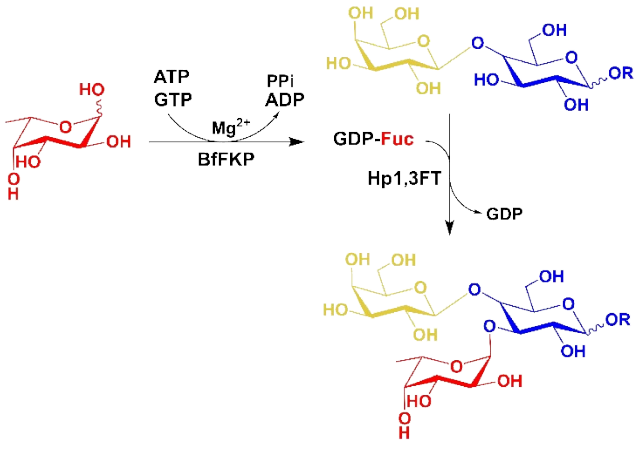


Table S4 Components of α-1,3-fucosylation module

components	final concentration
receptor	3 mM
Fuc	3.6 mM
ATP	3.6 mM
$MgCl_2$	10 mM
GTP	3.6 mM
Tris-HCl buffer (pH 7.5)	100 mM
BfFKP	0.25 mg/mL
PPA	0.25 mg/mL
Hp1,3FT	0.5 mg/mL

1.5 Enzyme modules 5: α1,3-fucosylation module

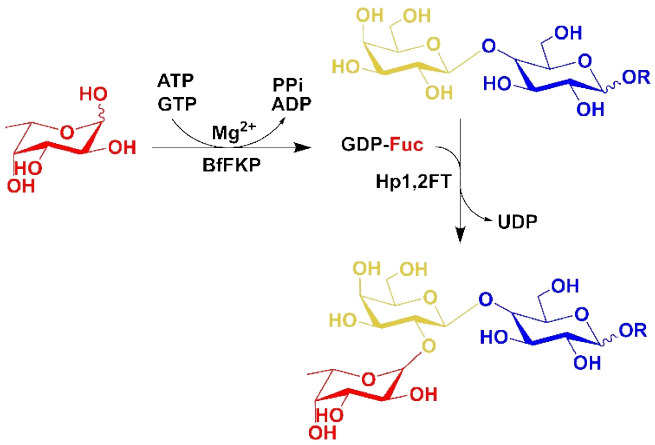


Table S5 Components of α-1,2-fucosylation module

components	final concentration
receptor	3 mM
Fuc	3.6 mM
ATP	3.6 mM

$MgCl_2$	10 mM
GTP	3.6 mM
Tris-HCl buffer (pH 7.5)	100 mM
BfFKP	0.25 mg/mL
PPA	0.25 mg/mL
Hp1,2FT	0.5 mg/mL

1.6 Enzyme modules 6: β1,3-N-acetylglucosamination

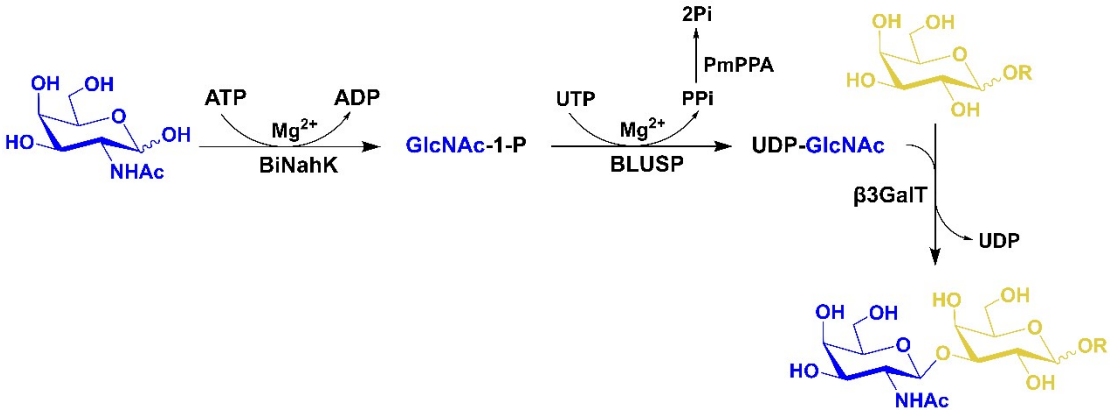


Table S6 Components of β-1,3-*N*-acetylglucosaminylation module

components	final concentration
receptor	3 mM
GlcNAc	3.6 mM
ATP	3.6 mM
UTP	3.6 mM
$MgCl_2$	20 mM
Tris-HCl buffer (pH 7.5)	100 mM
BiNahK	0.25 mg/mL
PmGlmU	0.5 mg/mL

PPA	0.25 mg/mL
LgtA	0.5 mg/mL

2.7 Enzyme modules 7: β1,3-galactosylation module

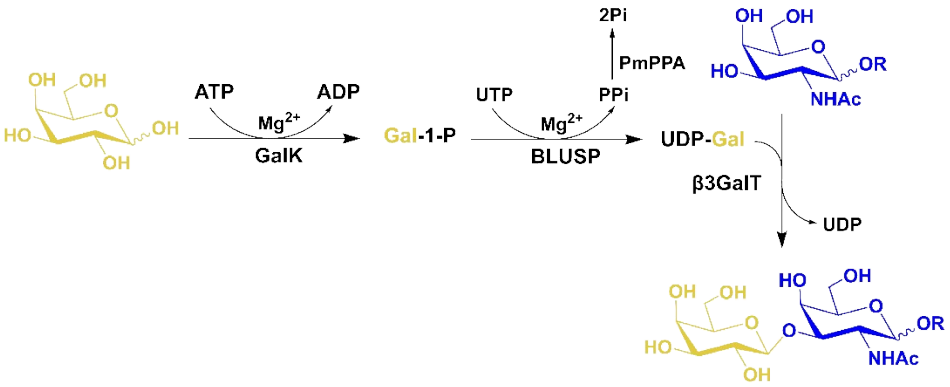
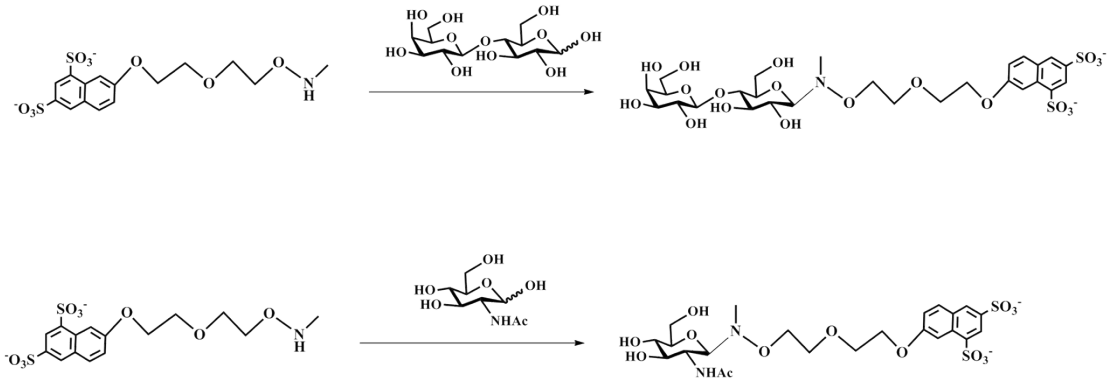


Table S7 Components of β-1,4-galactosylation module

system component	final concentration
receptor	3 mM
Gal	3.6 mM
ATP	3.6 mM
UTP	3.6 mM
$MgCl_2$	20 mM
Tris-HCl buffer (pH 7.5)	100 mM
GalK	0.25 mg/mL
BLUSP	0.5 mg/mL
PPA	0.25 mg/mL
β3GalT	0.5 mg/mL

2. Synthesis of tag-labelled saccharides



2.1 Synthesis of Lac-tag

Tag (6 mg, 0.016 mmol) was dissolved in NaOAc solution (100 mM, pH 6.0, 200 μL). The pH of the resulting solution was adjusted to 4.2. Subsequently, lactose (Lac, 54.8 mg, 0.16 mmol) was added, and the final volume was adjusted to 300 μL using NaOAc buffer (100 mM, pH 4.2). The reaction mixture was incubated on a shaking incubator at 37°C for 72 hr, and the reaction was stopped when no tag was detected by TLC (thin layer chromatography). The resulting product was purified on Bio-Gel P-2 and freeze-dried, yielding a white amorphous solid weighing 8.67 mg, with a yield of 73%. MS (ESI): *m/z* 371.68 calcd for $[Lac\text{-}tag]^{2-}$, found: 371.80.

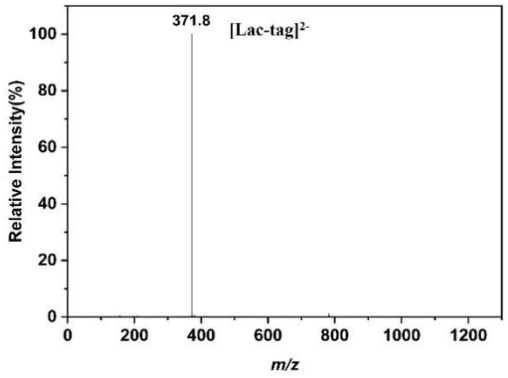


Figure S1. MS of Lac-tag

2.2 Synthesis of GlcNAc-tag

Tag (6 mg, 0.016 mmol), was dissolved in a NaOAc solution (100 mM, pH 6.0, 200 μL). The pH of the resulting solution was adjusted to 4.2. Subsequently, *N*-

acetylglucosamine (GlcNAc, 34 mg, 0.16 mmol) was added, and the final volume was adjusted to 300 μL using NaOAc buffer (100 mM, pH 4.2). The reaction mixture was incubated on a shaking incubator at 37°C for 72 hr, and the reaction was stopped when no tag was detected by TLC. The resulting product was purified on Bio-Gel P-2 and freeze-dried, yielding a white amorphous solid weighing 5.09 mg with a yield of 76%. MS (ESI): *m/z* 311.12 calcd for $[GlcNAc\text{-}tag]^{2-}$, found: 311.20.

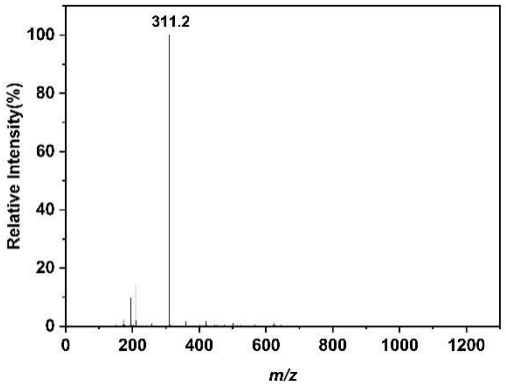


Figure S2. MS of GlcNAc-tag

4. Procedures for automatic enzymatic synthesis of HMOs on the DMF device

3.1 Automatic chemoenzymatic modular synthesis of LNT II

Enzymatic synthesis of tagged LNT II

Lactose modified with tag2 (Lac-tag) at a concentration of 3 mM was loaded in the first droplet. Enzymatic module 6 (Table S6), 10 mM $MgCl_2$, 100 mM Tris-HCl, 3 mM *N*-acetylglucosamine, 3.6 mM ATP and 3.6 mM UTP were loaded in another droplet.

On the DMF platform, two droplets were mixed evenly and left for 1 hr for enzymatic synthesis. Then the reaction mixture was mixed with DEAE magnetic beads in one droplet. The DEAE magnetic beads were suspended in the droplet to capture

products modified with tag. The DEAE magnetic beads and supernatants were separated by a magnet, and the supernatant including enzymes, excess (sugar) nucleotides, $MgCl_2$ and NH_4HCO_3 was taken out and discarded. Subsequently, DEAE magnetic beads were washed in turn with water and 60 mM NH_4HCO_3. Next, captured product LNT II-tag on DEAE magnetic beads were released by washing with 0.3 M NH_4HCO_3, and the eluent obtained was transferred and characterized by MS. MS (ESI): *m/z* 473.15 calcd for $[LNT\ II\text{-}tag]^{2-}$, found: 473.10.

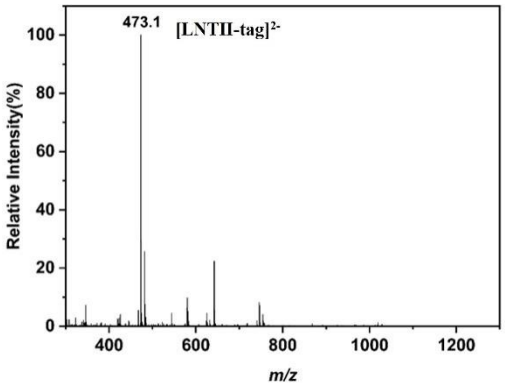


Figure S3. MS of LNT II-tag

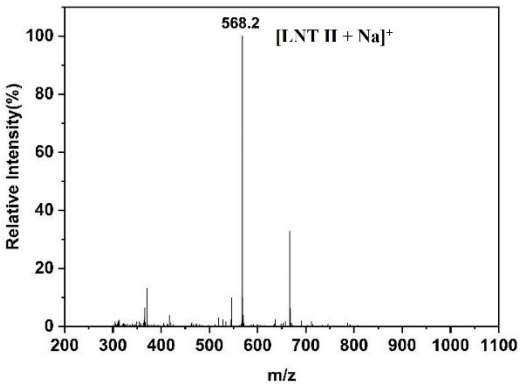


Figure S4. MS of LNT II

Cleavage of tag

The eluent containing the product LNT II-tag was mixed with a droplet (2 μL) of trifluoroacetic acid (TFA) (0.25%, v/v) on the DMF platform. The reaction mixture was agitated at room temperature for 2 hr. After the reaction, the reaction mixture was mixed with a droplet of 50 mM NH_4HCO_3 to adjust pH. Then, DEAE magnetic beads were used for capturing sulfonate tags removed from LNT II-tag, and the supernatant including LNT II was taken out and characterized by MS. MS (ESI): *m/z* 568.47 calcd for $[LNT\ II + Na]^+$, found: 567.90.

3.2 Automatic chemoenzymatic modular synthesis of LNT and LNnT

Enzymatic reactions were conducted using LNT II-tag as the substrate to synthesize LNT-tag and LNnT-tag through enzymatic modules 1 (Table S1) and 6 (Table S6), respectively. Similar reaction conditions and work-up to synthesis of LNT II-tag were followed. The released product in 0.3 M NH_4HCO_3 elution buffer was captured using DEAE magnetic beads and subjected to MS analysis. MS (ESI): *m/z* calcd 554.52 for $[LNT\text{-}tag]^{2-}$, found: 554.6; *m/z* 554.52 calcd for $[LNnT\text{-}tag]^{2-}$, found: 554.40; LNT and LNnT were obtained by cleaving the tag using 0.25% TFA. MS (ESI): *m/z*729.63 calcd for $[LNT + Na]^+$, found: 730.2; *m/z*729.63 calcd for $[LNnT + Na]^+$, found: 730.2.

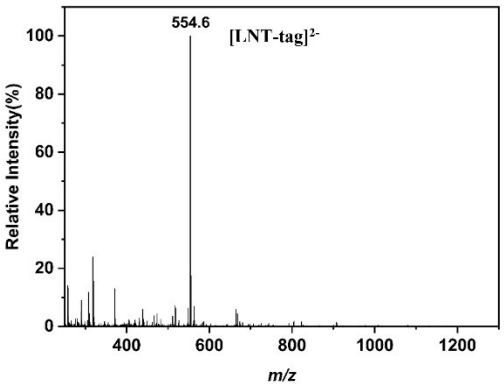


Figure S5. MS of LNT-tag

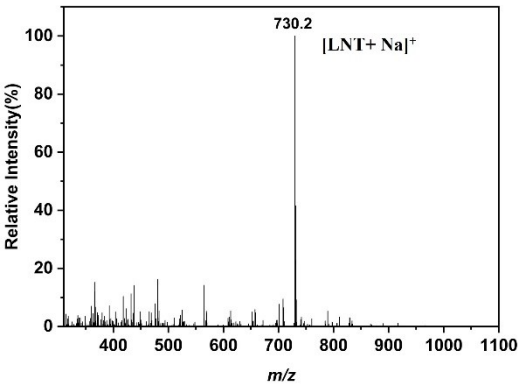


Figure S6. MS of LNT

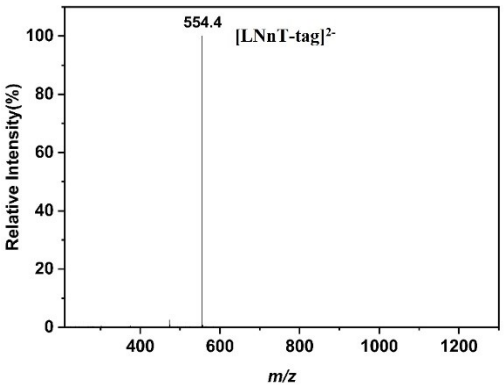


Figure S7. MS of LNnT-tag

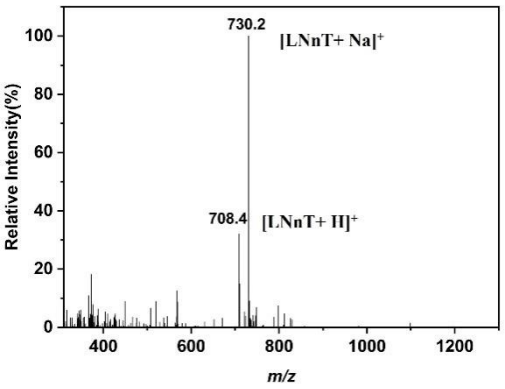


Figure S8. MS of LNnT

3.3 Automatic chemoenzymatic modular synthesis of fucosylated HMOs

Chemoenzymatic modular synthesis of LeX triaose

Starting from GlcNAc-tag, enzymatic reactions were conducted consecutively on the DMF platform using GlcNAc-tag as the substrate with enzymatic modules 1 (Table S1) and 4 (Table S4) to synthesize LeX triaose-tag. Similar reaction conditions and work-up to synthesis of LNT II-tag were followed. Upon completion of the enzymatic reactions in the enzymatic modules, the tag was cleaved using 0.25% TFA to obtain the LeX triaose product. MS (ESI): *m/z* 456.10 calcd for [LeX triaose-tag]$^{2-}$, found:456.10. *m/z* 552.40 calcd for [LeX triaose + Na]$^{+}$, found:551.90.

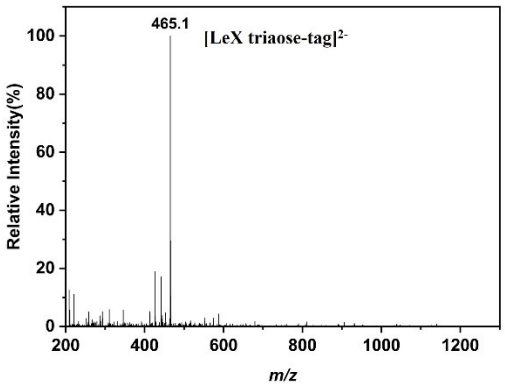


Figure S9. MS of LeX tiraose-tag

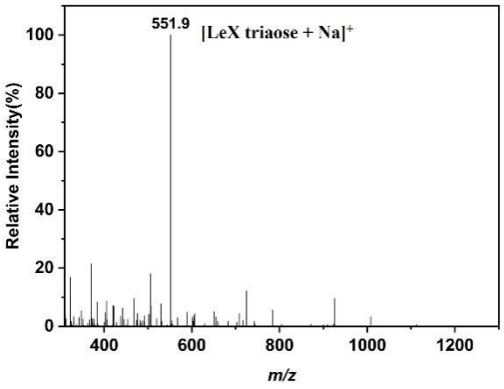


Figure S10. MS of LeX tiraose

Chemoenzymatic modular synthesis of 3-FL and 2'-FL

Starting from Lac-tag, enzymatic reactions were carried out separately using enzymatic modules 4 (Table S4) and 5 (Table S5) to synthesize 3-FL-tag and 2'FL-tag, respectively. Similar reaction conditions and work-up to synthesis of LNT II-tag were followed. After completion of the enzymatic reactions in the enzymatic modules, the tag was removed by cleavage using 0.25% TFA to obtain 3-FL and 2'-FL. MS (ESI): *m/z* 444.74 calcd for [3-FL-tag]$^{2-}$, found: 444.60; *m/z* 444.74 calcd for[2'-FL-tag]$^{2-}$, found: 444.50; *m/z* 510.40 calcd for [3-FL + Na]$^{+}$, found: 510.60; *m/z* 510.40 calcd for

[2'-FL + Na]$^+$, found: 510.60.

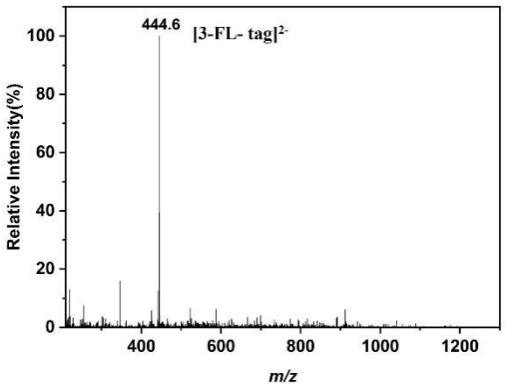


Figure S11. MS of 3-FL-tag

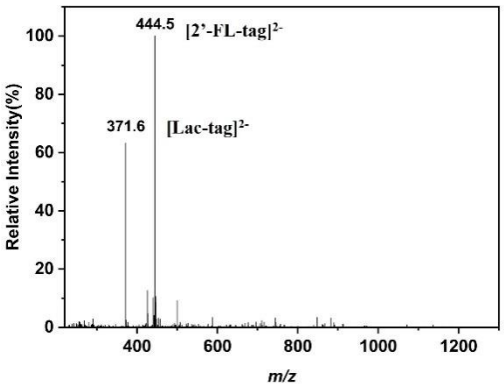


Figure S12. MS of 2'-FL-tag

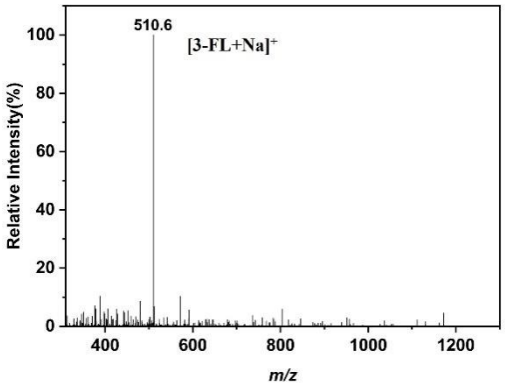


Figure S13. MS of 3-FL

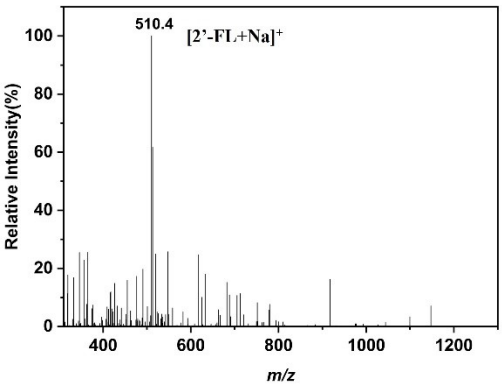


Figure S14. MS of 2'-FL

Chemoenzymatic modular synthesis of LNFP III [1]

Starting from Lac-tag, enzymatic reactions were sequentially carried out using enzymatic modules 3 (Table S3), 7 (Table S7), and 4 (Table S4) to synthesize LNFP III-tag. Similar reaction conditions and work-up to synthesis of LNT II-tag were followed. After the final step reaction, the released product was captured on DEAE magnetic beads and washed with 0.3 M NH_4HCO_3 eluent for subsequent MS analysis. Upon completion of the enzymatic reactions in the enzymatic modules, the tag was removed using 0.25% TFA to obtain the LNFP III product. MS (ESI): *m/z* 627.21 calcd for [LNFP Ⅲ-tag]$^{2-}$, found: 627.10. Simultaneously, *m/z* [LNnT-tag]$^{2-}$ (calcd for

554.52; found: 554.60) was observed. MS (ESI): *m/z* 853.83 calcd for [LNFP III + Na]$^{+}$, found: 853.60.

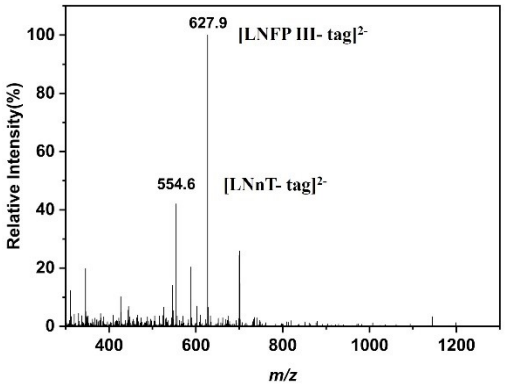


Figure S15. MS of LNFP III-tag

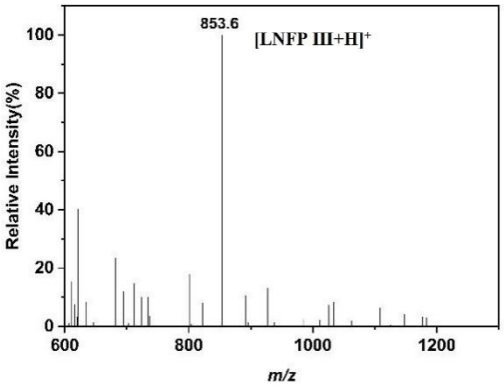


Figure S16. MS of LNFP III

3.4 Automatic enzymatic synthesis of sialylated HMOs

Chemoenzymatic modular synthesis of 6'/3'-SLN

Starting from GlcNAc-tag, enzymatic reactions were conducted using enzymatic module 3 to synthesize LN-tag. Subsequently, LN-tag was subjected to separate enzymatic reactions using enzymatic modules 3 (Table S3) and 2 (Table S2) to synthesize 3'-SLN-tag and 6'-SLN-tag, respectively. Similar reaction conditions and work-up to synthesis of LNT II-tag were followed. The released product was captured on DEAE magnetic beads and washed with 0.3 M NH_4HCO_3 eluent for subsequent MS analysis. The tags were removed using 0.25% TFA to obtain the 3'-SLN and 6'-SLN products. MS (ESI): *m/z* 358.6 calcd for [3'-SLN-tag]$^{3-}$, found: 358.30; *m/z* 358.6 calcd for [6'-SLN-tag]$^{3-}$, found: 358.40; *m/z* 673.58 calcd for [3'-SLN - H]$^{-}$, found: 673.20; *m/z* 673.58 calcd for[6'-SLN - H]$^{-}$, found: 673.20.

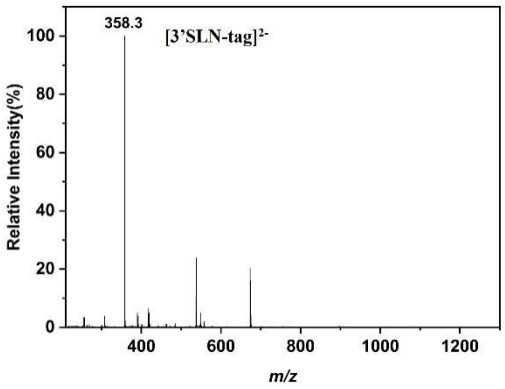


Figure S17. MS of 3'-SLN-tag

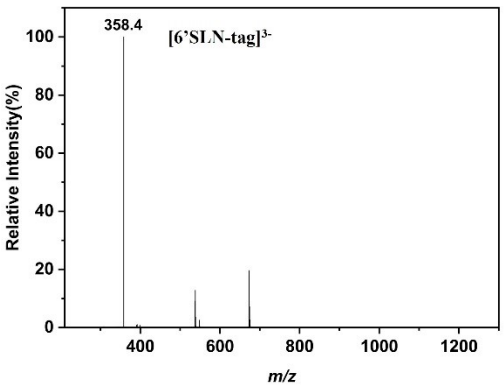


Figure S18. MS of 6'-SLN-tag

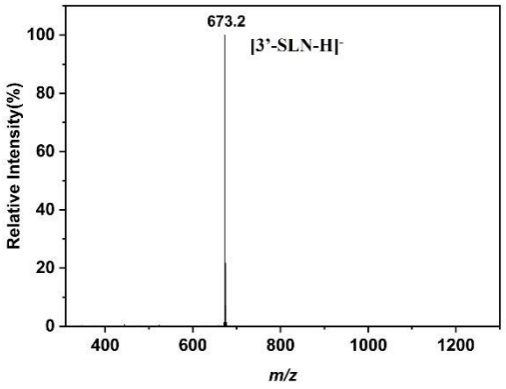


Figure S19. MS of 3'-SLN

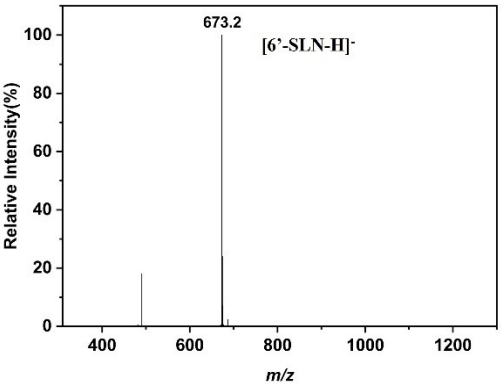


Figure S20. MS of 6'-SLN

Chemoenzymatic modular synthesis of 6'/3'-SL

Starting from Lac-tag, enzymatic reactions were sequentially conducted using enzymatic modules 3 (Table S3) and 2 (Table S2) to synthesize 3'-SL-tag and 6'-SL-tag, respectively. Similar reaction conditions and work-up to synthesis of LNT II-tag were followed. The released product was captured on DEAE magnetic beads and washed with 0.3 M NH_4HCO_3 eluent for subsequent MS analysis. The tags were removed using 0.25% TFA to obtain the 3'-SL and 6'-SL products. MS (ESI): *m/z* 344.6 calcd for [3'-SL-tag]$^{3-}$, found: 344.85; *m/z* 344.6 calcd for [6'-SL-tag]$^{3-}$, found: 344.60; *m/z* 632.55 calcd for[3'-SL - H]$^{-}$, found: 632.20; *m/z* 632.55 calcd for [6'-SL - H]$^{-}$, found: 632.17.

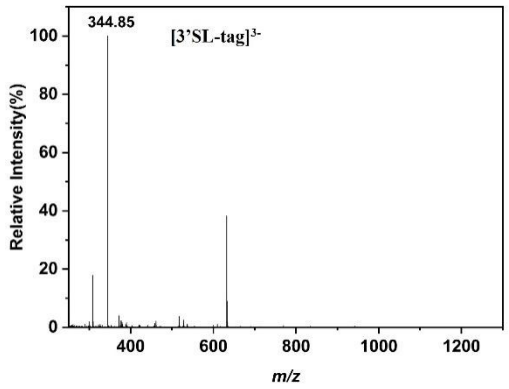


Figure S21. MS of 3'-SL-tag

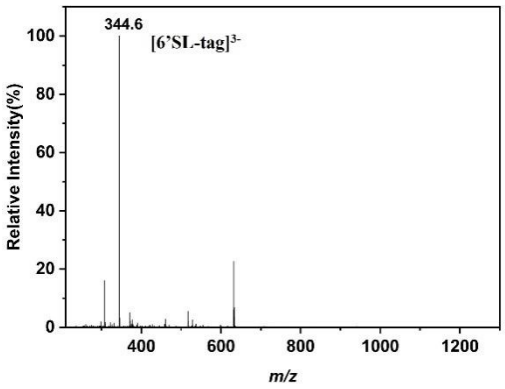


Figure S22. MS of 6'-SL-tag

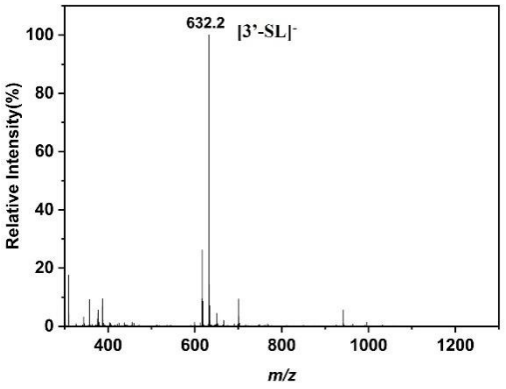


Figure S23. MS of 3'-SL

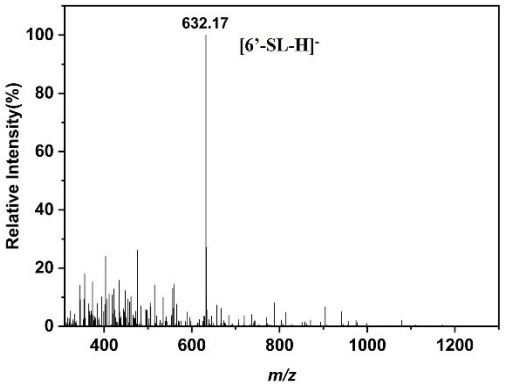


Figure S24. MS of 6'-SL

Chemoenzymatic modular synthesis of LSTa

After synthesizing the neutral HMOs backbone on the DMF platform, further modifications were performed on the LNT-tag through sialylation. Enzymatic module 3 (Table S3) was utilized to catalyze the synthesis of monosialylated LSTa-tag. Similar reaction conditions and work-up to synthesis of LNT II-tag were followed. The released product was captured on DEAE magnetic beads and washed with 0.3 M NH_4HCO_3 eluent for subsequent MS analysis. The tag was removed using 0.25% TFA, resulting in the formation of LSTa product. MS (ESI): *m/z* 466.4 calcd for $[LSTa\ -tag]^{3-}$, found: 466.4; *m/z* 997.9 calcd for $[LSTa - H]^-$, found: 997.8.

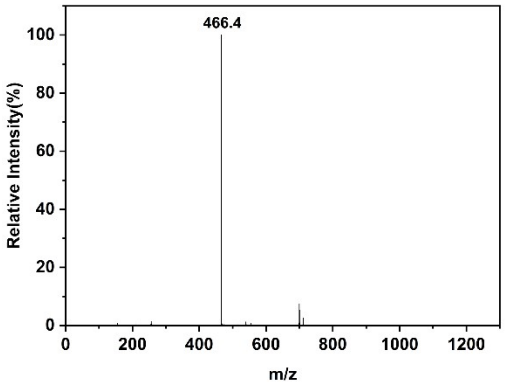


Figure S25. MS of LSTa-tag

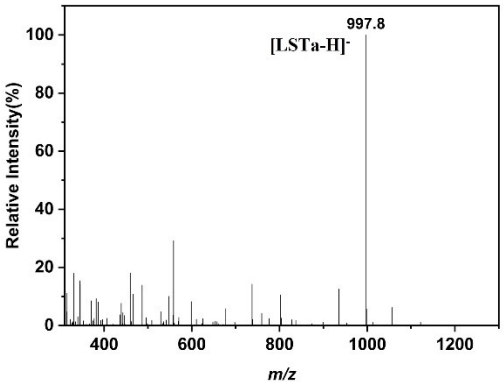
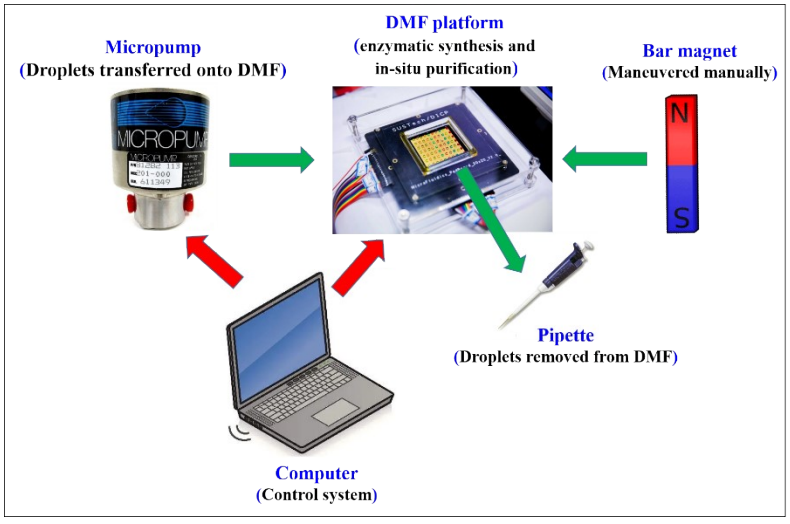


Figure S26. MS of LSTa

Table S8. Comparison of the performance of the various modules

Modules	**Yields (%)**
Module 1 (β-1,4-galactosylation)	> 90%
Module 2 (α2,6-sialylation)	> 90%
Module 3 (α-2,3-sialylation)	> 90%
Module 4 (α-1,3-fucosylation)	> 90% (3-FL), > 63% (LNFP III-tag)
Module 5 (α-1,2-fucosylation)	> 63%
Module 6 (β-1,3-*N*-acetylglucosaminylation)	> 90%
Module 7 (β-1,3-galacosylation)	> 90%

Figure S1. A schematic representation on how the system works



References

[1] Chen, C., Zhang, Y., Xue, M., Liu, X. W., *et al.*, Sequential one-pot multienzyme (OPME) synthesis of lacto-N-neotetraose and its sialyl and fucosyl derivatives. *Chem Commun (Camb)* 2015, *51*, 7689-7692.