Supplementary Information

Applied Microbiology and Biotechnology

Enzyme Cascades for Nucleotide Sugar Regeneration in Glycoconjugate Synthesis

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Abbreviations:

Sucrose synthases:

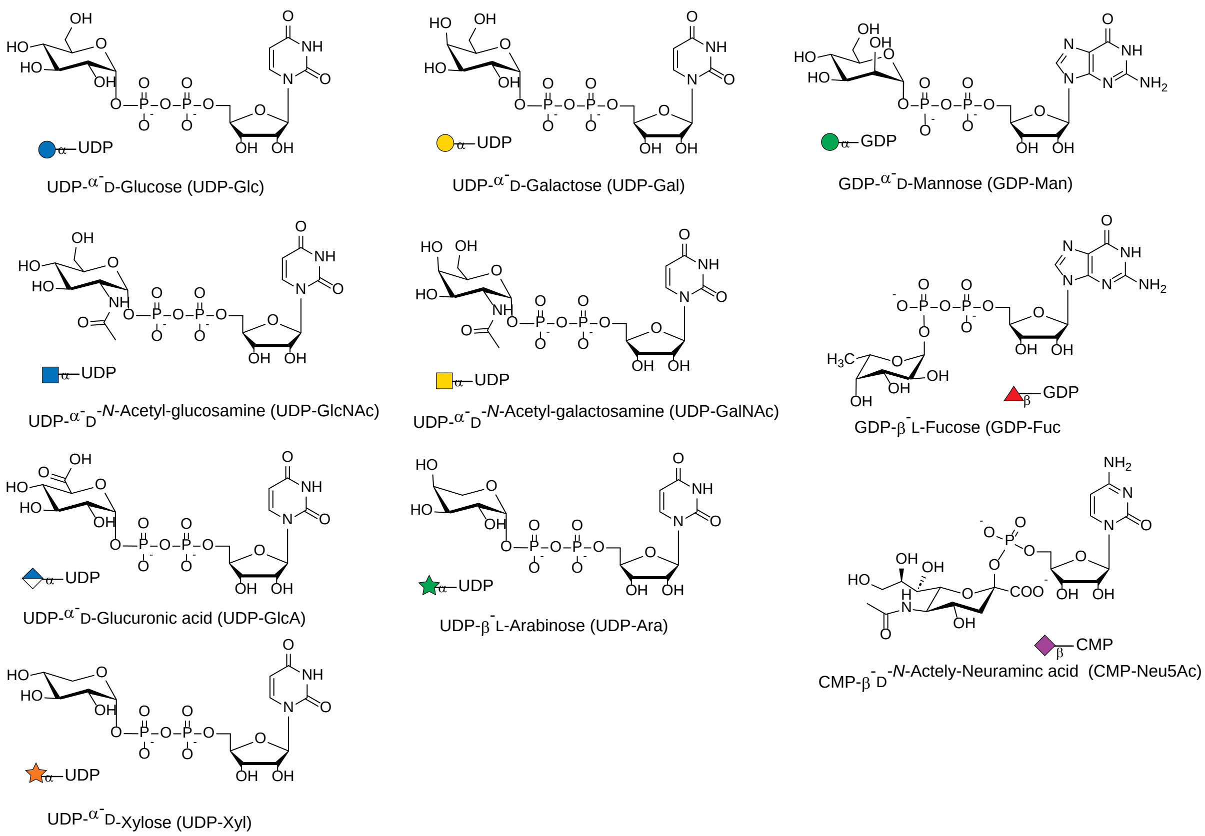
***Ac*SuSy**: SuSy from *Acidithiobacillus caldus;* ***At*SuSy1**: SuSy1 from *Arabidopsis thaliana*; ***Gm*SuSy**: SuSy from soybean (*Glycine max*); ***Gu*SUS1**: SuSy1 from licorice (*Glycyrrhiza uralensis)*; **mbSUS**: SuSy from *Vigna radiata*; ***Mc*SuSy**: SuSy from *Micractinium conductrix*; ***Os*SuSy**: SuSy from rice (*Oryzae sativa*); ***St*SuSy1/*St*SUS1**: SuSy1 from potato (*Solanum tuberosum*);

Enzymes for NDP-sugar recycling:

At*UGDH3**: UDP-Glc dehydrogenase from *Arabidopsis thaliana;* ***At*USP**: UDP-sugar pyrophosphorylase from *Arabidopsis thaliana*; ***At*RHM1**: UDP-rhamnose synthase from *Arabidopsis thaliana*; ***At*UX3**: UDP-GlcA decarboxylase from *Arabidopsis thaliana*; ***At*GalE:** UDP-Glc 4'-epimerase; ***Bf*FKP**: fucokinase/ GDP-fucose pyrophosphorylase from *Bacillus fragilis*; ***Bl*NahK**: *N*-acetyl-hexosamine kinase from *Bifidobacterium longum ssp. infantis*; **CMPK/CMK**: CMP kinase; **CSS**: CMP-Neu5Ac synthetase; ***Ec*CMK**: Cytidin-5'-monophospahte kinase from *Escherichia coli*; ***Ec*GalK**: galactokinase from *Escherichia coli*; ***Ec*GlmU**: UDP-GlcNAc/GalNAc pyrophosphorylase from *Escherichia coli;* ***Ec*PK**: Pyruvate kinase from *Escherichia coli*; **FDH**: formate dehydrogenase; **GalU**: UDP-Gal uridylyltransferase; ***Lb*NOX**: NADH oxidase from *Lactobacilllus brevis*; **NRS/ER**: UDP-4-keto-6-deoxy-D-glucose 3,5-epimerase/UDP-4-keto-rhamnose 4-keto-reductase; ***Ps*UGE2**: UDP-Glc 4'-epimerase from *Pisum sativum*; **PGM**: phosphoglucomutase; **PK**: pyruvate kinase; ***Pm*CSS**: CMP-Neu5Ac synthetase from *Pasteurella multocida*; **PPK**: polyP$_n$ kinase; **RmlB**: dTDP-Glc 4,6-dehydratase; **RmlC**: dTDP-4-keto-6-deoxy-Glc 3,5-epimerase, **RmlD**: dTDP-4-keto-6-deoxy-L-mannose 4-ketoreductase; **UGDH**: UDP-Glc dehydrogenase; **UDP-Glc PP*: UDP-Glc pyrophosphorylase; **USP**: UDP-sugar pyrophosphorylase; ***Vv*RHM**: UDP-rhamnose synthase from *Vitis vinifera.*

Leloir-Glycosyltransferases:

***As*UGT99D1**: UDP-arabinosyltransferase from *Avena strigose*; ***At*UGT73C5**: UDP-glucosyltransferase from *Arabidopsis thaliana*; ***At*UGT78D1**: UDP-rhamnose flavonol glycosyltransferase from *Arabidopsis thaliana*; ***At*UGT78D2**: UDP-glucosyltransferase from *Arabidopsis thaliana*; ***As*UGT99D1**: UDP-arabinosyltransferase from *Avena strigose;* ***Bs*YjiC**: UDP-glucosyltransferase from *Bacillus subtilis* 168; ***Ca*UGT3**: 1,6-glucosyltransferase from *Catharanthus roseus*; ***Ca*UGT73A15**: UDP-glucosyltransferase from *Catharanthus roseus*; ***Ct*UGT71E5**: UDP-glucosyltransferase from safflower (*Carthamus tinctorius*); ***Cr*UGT2**: UDP-glucosyltransferase from *Catharanthus roseus*; **F7GT**: flavonoid 7-O-glucosyltransferase from *S. baicalensis*; ***Gu*UGT73C11**: UDP-sugar glycosyltransferase from *Glycyrrhiza uralensis*; ***Gu*UGT73F24m**: I23G/L84N mutant UDP-glucosyltransferase from *Glycyrrhiza uralensis*; ***Hi*LgtD**: β1,3GalNAcT from *Haemophilus influenzae*; ***Hp*FutC**: α1,2Fucosyltransferase from *Helicobacter pylori*; ***Hs*β3GlcAT-P**: β3-glucuronosyltransferase P from *Homo sapiens*; ***Hs*β4GalT1**: β4-galactosyltransferase 1 from *Homo sapiens*; ***It*UGT2**: UDP-glucosyltransferase from *Indigofera tinctorial*; ***Mm*α3GalT**: α3GalT from mouse (*Mus musculus*); ***Md*UGT71A15**: UGT from *Malus domestica*; ***Nm*LgtC**: α1,4Galactosyltransferase from *Neisseria meningitidis*; **OleD**: UDP-glucosyltransferase from *Streptomyces antibioticus*; ***Os*CGT**: C-glucosyltransferase from rice (*Oryza sativa*); ***Os*UGT79**: UDP-glucosyltransferase from rice (*Oryza sativa);* ***Pc*OGT**: UDP-glucosyltransferase from pear (*Pyrus communis*); ***Sc*Sorf**: Sorf from *Sorangium cellulosum*; ***Sr*UGT76G1/S195Q**: UDP-glucosyltransferase from *S. rebaudiana*; ***Sr*UGT76G1** :UDP-sugar glycosyl transferase from *Stevia rebaudiana Bertoni*; **Sia T**: sialyltransferase; **UGTs**: UDP-glucosyltransferases from plants; **UGT$_{\mathbf{BL}}$1**: Microbial glycosyltransferase; **VspJT-FAJ-16**: α2,3Sialyltransferase; **UGTSL2**: UDP-sugar glycosyltransferase from *Solanum lycopersicum.*



SHAPE	White (Generic)	Blue	Green	Yellow	Orange	Pink	Purple	Light Blue	Brown	Red
Filled Circle	Hexose	Glc	Man	Gal	Gul	Alt	All	Tal	Ido	
Filled Square	HexNAc	GlcNAc	ManNAc	GalNAc	GulNAc	AltNAc	AllNAc	TalNAc	IdoNAc	
Crossed Square	Hexosamine	GlcN	ManN	GalN	GulN	AltN	AllN	TalN	IdoN	
Divided Diamond	Hexuronate	GlcA	ManA	GalA	GulA	AltA	AllA	TalA	IdoA	
Filled Triangle	Deoxyhexose	Qui	Rha		6dGul	6dAlt		6dTal		Fuc
Divided Triangle	DeoxyhexNAc	QuiNAc	RhaNAc			6dAltNAc		6dTalNAc		FucNAc
Flat Rectangle	Di-deoxyhexose	Oli	Tyv		Abe	Par	Dig	Col		
Filled Star	Pentose		Ara	Lyx	Xyl	Rib				
Filled Diamond	3-deoxy-nonulosonic acids		Kdn				Neu5Ac	Neu5Gc	Neu	Sia
Flat Diamond	3,9-dideoxy-nonulosonic acids		Pse	Leg		Aci		4eLeg		
Flat Hexagon	Unknown	Bac	LDmanHep	Kdo	Dha	DDmanHep	MurNAc	MurNGc	Mur	
Pentagon	Assigned	Api	Fru	Tag	Sor	Psi				

Scheme S1 Chemical Structures, symbol presentation, names, and abbreviations of common nucleotide sugars (Frohnmeyer, Elling 2023). Common nucleotide sugars are depicted according to the symbol nomenclature for graphical representations of glycans (Varki et al. 2015).

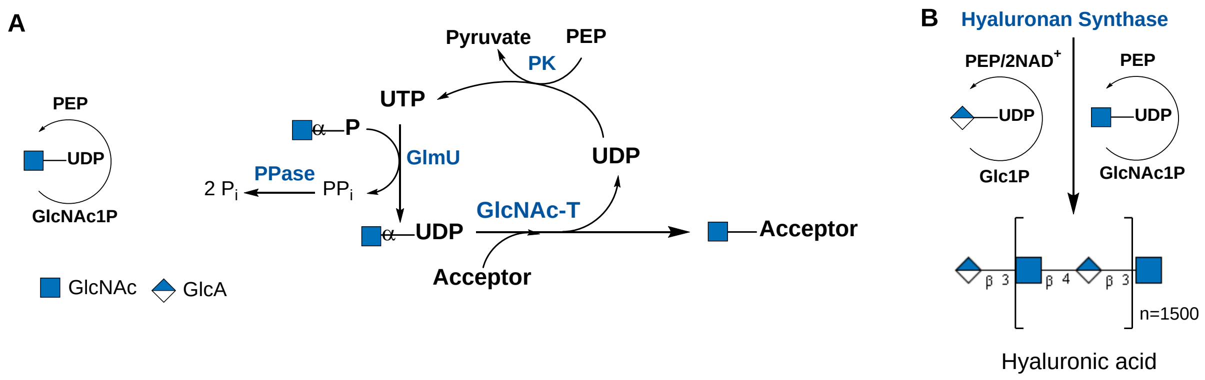


Figure S1 A Regeneration cycle for UDP-GlcNAc and synthesis of hyaluronic acid with *in situ* regeneration of UDP-GlcA and UDP-GlcNAc (De Luca et al. 1995).

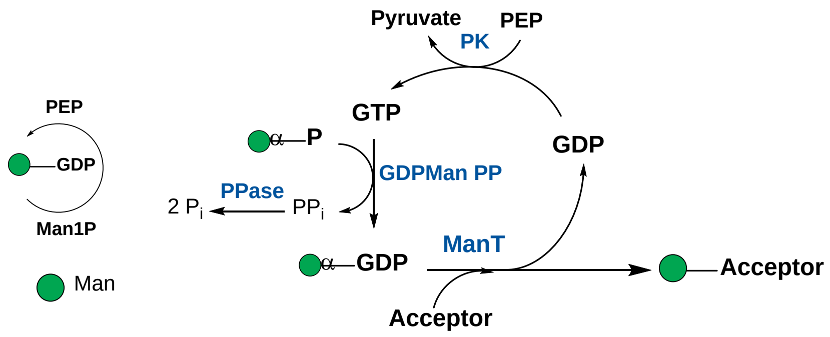


Figure S2 Regeneration cycle for GDP-Man (Wang et al. 1993).

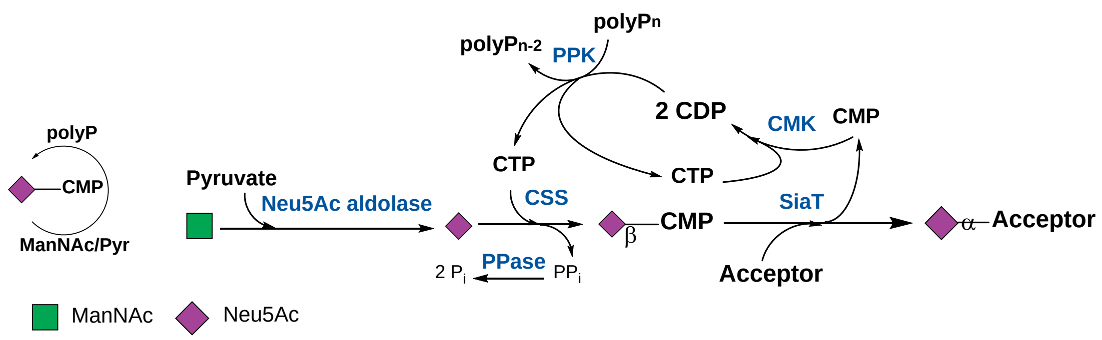


Figure S3 Regeneration of CMP-Neu5Ac from ManNAc/pyruvate with polyP/PPK (Nahálka, Pätoprstý 2009). CSS: CMP-Neu5Ac synthetase from calf brain or recombinant enzyme from *E. coli*; CMPK/CMK: CMP kinase; PPK: polyphosphate kinase, SiaT: sialyltransferase.

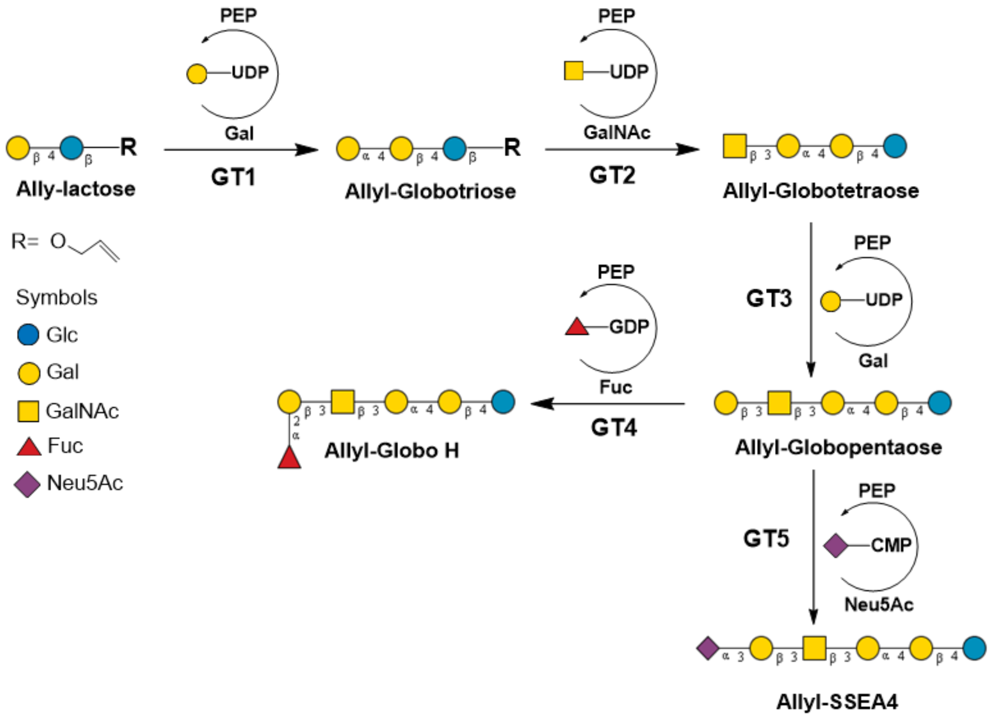


Figure S4 Multi-gram scale sequential synthesis of the tumor-associated antigens Globo H and SSEA4 with *in situ* nucleotide sugar regeneration (Tsai et al. 2013). UDP-Gal cycle with *Ec*GalK, *At*USP; UDP-GalNAc cycle with *Bl*NahK, *Ec*GlmU; GDP-Fuc cycle with *Bf*FKP; CMP-Neu5Ac cycle with *Ec*CMK, *Pm*CSS; *Ec*PK was used for nucleotide regeneration. GT1: α1,4GalT (*Nm*LgtC); GT2/GT3: β1,3GalNAcT (*Hi*LgtD); GT4: α1,2FucT (*Hp*FutC); GT5: α2,3SiaT (*Vsp*JT-FAJ-16) (see also **Table S1**; SI).

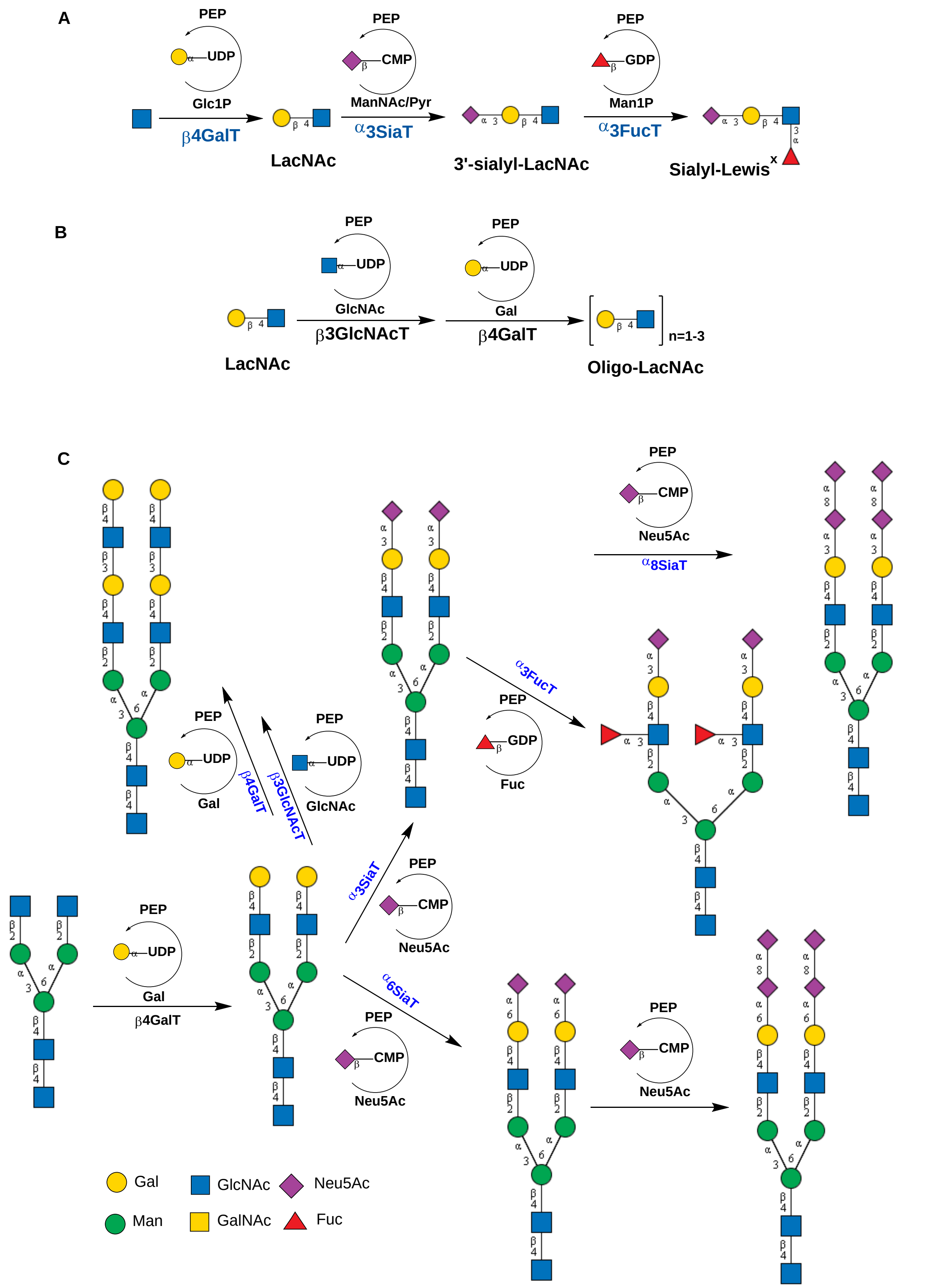


Figure S5 A One-pot synthesis of sialyl-Lewis^x with *in situ* regeneration of UDP-Gal, CMP-Neu5Ac and GDP-Fuc (Ichikawa et al. 1992b; Ichikawa et al. 1992a). **B** Sequential synthesis of oligo-LacNAc with *in situ* regeneration of UDP-GlcNAc and UDP-Gal (Wu et al. 2019). **C** Sequential synthesis of sialylated and fucosylated bi-antennary N-glycans (Anwar et al. 2022).

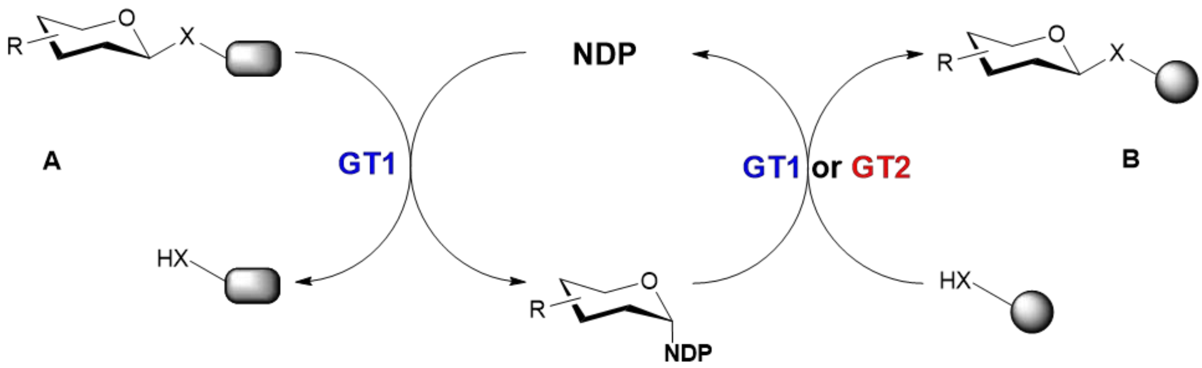


Figure S6 **A** Single GT-coupled and **B** dual GT-coupled aglycone exchange by reverse GT reactions (Minami et al. 2005; Gantt et al. 2011).

Table S1 Enzyme cascades for the *in situ* regeneration of nucleotide sugars

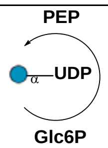
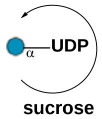
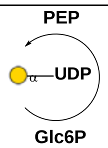
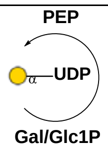
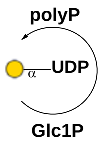
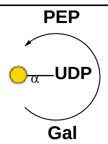
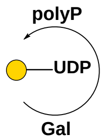
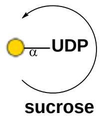
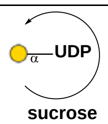
Regeneration cycle	Number of enzymes for regeneration of	Stoichiometric substrates or in excess	References
		UDP-Glc	
	**4**: PGM, UDP-Glc PP; PPase, PK	Glc6P, PEP	(Haynie, Whitesides 1990)
	**1**: SuSy	Sucrose	(Masada et al. 2007)
		UDP-Gal	
	**5:** PGM, UDP-Glc PP; PPase, GalE, PK	Glc6P, PEP	(Wong et al. 1992)
	**5:** GalK, UDP-Glc PP, PPase, GalU, PK	**Gal**, Glc1P, PEP	(Wong et al. 1992)
	**4:** UDP-Glc PP; PPase, GalE, PPK	Glc1P, polyP	(Noguchi, Shiba 1998)
	**4**: GalK, USP, PPase, PK	Gal, PEP	(Tsai et al. 2013; Wu et al. 2019)
	**4**: GalK, USP, PPase, PPK	Gal, polyP	(Jiao et al. 2024)
	**2**: SuSy, GalE	Sucrose	(Zervosen, Elling 1996; Hokke et al. 1996; Pei et al. 2017)
	**3**: SuSy, GalU	Sucrose, Gal1P	(Zervosen, Elling 1996)

Table S1 continued

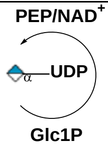
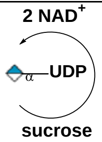
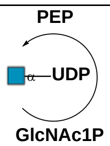
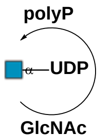
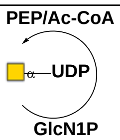
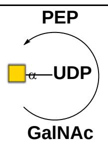
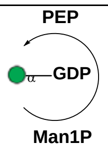
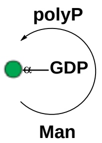
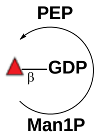
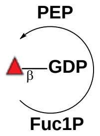
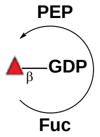
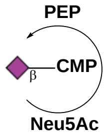
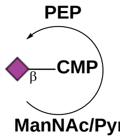
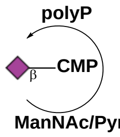
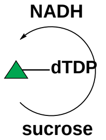
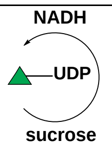
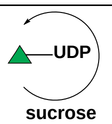
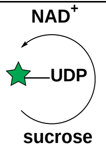
Regeneration cycle	Number of enzymes for regeneration of	Stoichiometric substrates or in excess	References
	UDP-GlcA		
	**4:** UDP-Glc PP; PPase, UGDH, PK **5:** 4+ enzyme for NAD^+ regeneration	Glc1P, PEP, (NAD^+)	(Gygax et al. 1991)
	**2**: SuSy, UGDH **3**: 2 + enzyme for NAD^+ regeneration	Sucrose, (NAD^+)	(Engels et al. 2015; Eisele et al. 2018)
	UDP-GlcNAc		
	**3**: GlmU, PPase, PK	GlcNAc1P, PEP	(De Luca et al. 1995)
	**4:** NaHK; GlmU or AGX1, PPase, PPK	GlcNAc, polyP	(Gottschalk et al. 2021)
	UDP-GalNAc		
	**5**: GlnM, GlmU, PPase, GalE, PK **6**: 5 + enzyme for Ac-CoA regeneration	GlcN1P, PEP, Ac-CoA, (acetyl-P)	(Shao et al. 2002)
	**4**: NaHK; GlmU or AGX1, PPase, PK	GalNAc, PEP	(Tsai et al. 2013)
	GDP-Man		
	**3**: GDP-Man PP (ManC), PPase, PK	Man1P, PEP	(Wang et al. 1993)
	**5**: GlcK, ManB, GDP-Man PP (ManC), PPase, PPK	Man, polyP	(Rexer et al. 2018)

Table S1 continued

Regeneration cycle	Number of enzymes for regeneration of	Stoichiometric substrates or in excess	References
GDP-Fuc			
	**5**: ManC, PPase, GMD, GDP-Fuc Synthetase, PK **6**: 5 + enzyme for NADH regeneration	Man1P, PEP	(Ichikawa et al. 1992b)
	**3**: FKP, PPase, PK	Fuc1P, PEP	(Ichikawa et al. 1992b)
	**3**: FKP, PPase, PK	Fuc, PEP	(Tsai et al. 2013)
CMP-Neu5Ac			
	**4**: CSS, PPase, CMPK, PK	Neu5Ac, PEP	(Ichikawa et al. 1991)
	**5**: Neu5Ac aldolase, CSS, PPase, CMPK, PK	ManNAc, PEP	(Liu et al. 1992; Ichikawa et al. 1992a)
	**5**: Neu5Ac aldolase, CSS, PPase, CMPK, PPK	ManNAc, Pyr, polyP	(Nahálka, Pätoprstý 2009)
dTDP-/UDP-L-Rha			
	**5 (6):** *St*SuSy, RmlB, RmlC, RmlD, (NOX for NAD^+ regeneration from NADH	sucrose	(Rupprath et al. 2007)
	**2:** *Gm*SuSy, *At*RHM1	sucrose	(Thapa et al. 2019)
	**3**: *Gm*SuSy, *Vv*RHM/AtNRS/ER	sucrose	(Pei et al. 2018)
UDP-L-Ara			
	**4**:*Gu*SuS1-Δ9, *At*UGDH3, *At*UX3, *Ps*UGE2	Sucrose, NAD^+	(Sun et al. 2023)

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