US 20090048440A1

(19) **United States**

(12) **Patent Application Publication** (10) **Pub. No.: US 2009/0048440 A1**

Felo et al. (43) **Pub. Date: Feb. 19, 2009**

(54) NUCLEOTIDE SUGAR PURIFICATION USING MEMBRANES

(75) Inventors: **Michael Felo**, Havertown, PA (US); **Shawn DeFrees**, North Wales, PA (US)

Correspondence Address:
MORGAN, LEWIS & BOCKIUS LLP (SF)
One Market, Spear Street Tower, Suite 2800
San Francisco, CA 94105 (US)

(73) Assignee: **Neose Technologies, Inc.**, Horsham, PA (US)

(21) Appl. No.: **12/092,563**

(22) PCT Filed: **Nov. 3, 2006**

(86) PCT No.: **PCT/US06/43048**

§ 371 (c)(1),
(2), (4) Date: **Jun. 18, 2008**

Related U.S. Application Data

(60) Provisional application No. 60/733,975, filed on Nov. 3, 2005, provisional application No. 60/796,281, filed on Apr. 28, 2006, provisional application No. 60/746,754, filed on May 8, 2006, provisional application No. 60/823,538, filed on Aug. 25, 2006, provisional application No. 60/829,242, filed on Oct. 12, 2006.

Publication Classification

(51) **Int. Cl.**
C07H 1/06 (2006.01)

(52) **U.S. Cl.** **536/55.3**; 536/127

(57) ABSTRACT

The invention provides methods of removing contaminants from a mixture of a desired product and contaminants by pH adjustments and molecular weight cut-offs. The contaminants include phosphate groups, magnesium sulfate, sodium pyruvate and tetrasodium pyrophosphate groups. The desired product includes nucleotide sugars, glycolipids, LnNT, sialyl lactose, and salts.

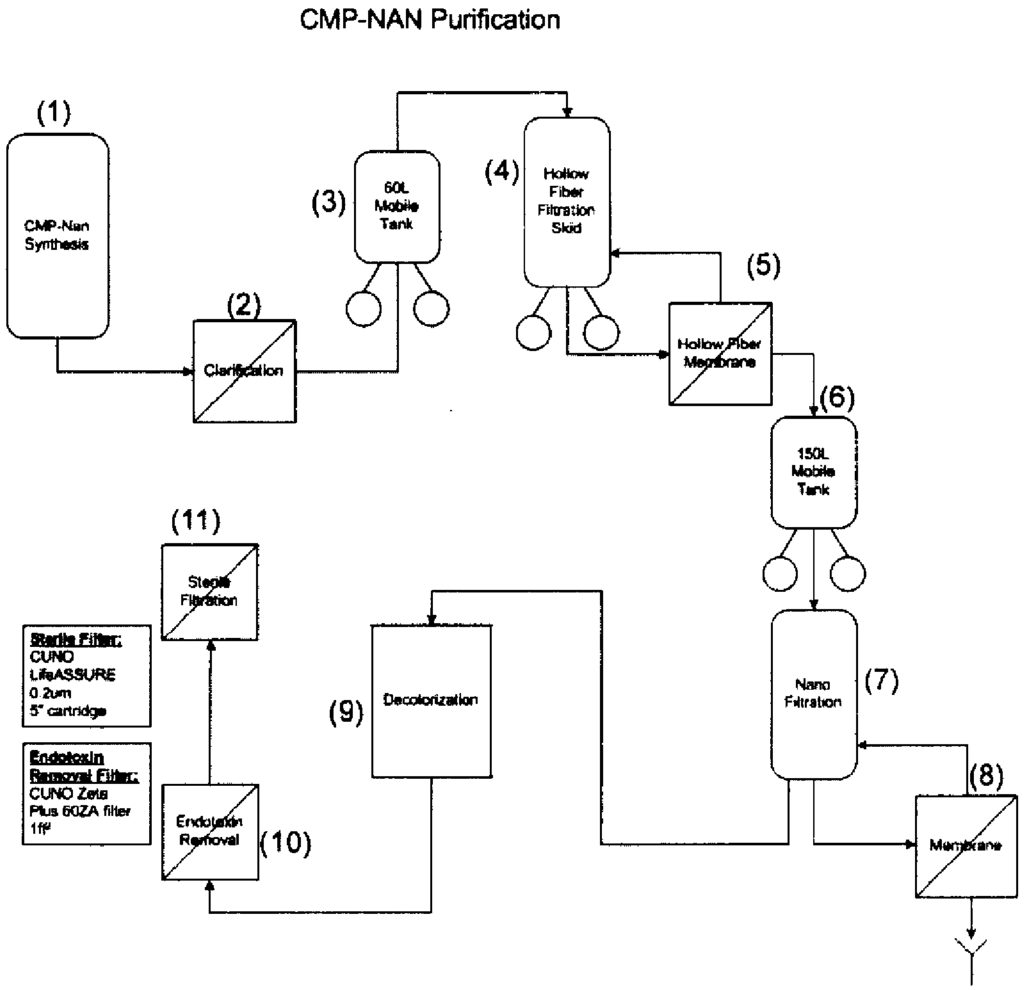


FIGURE 1

CMP-NAN Purification

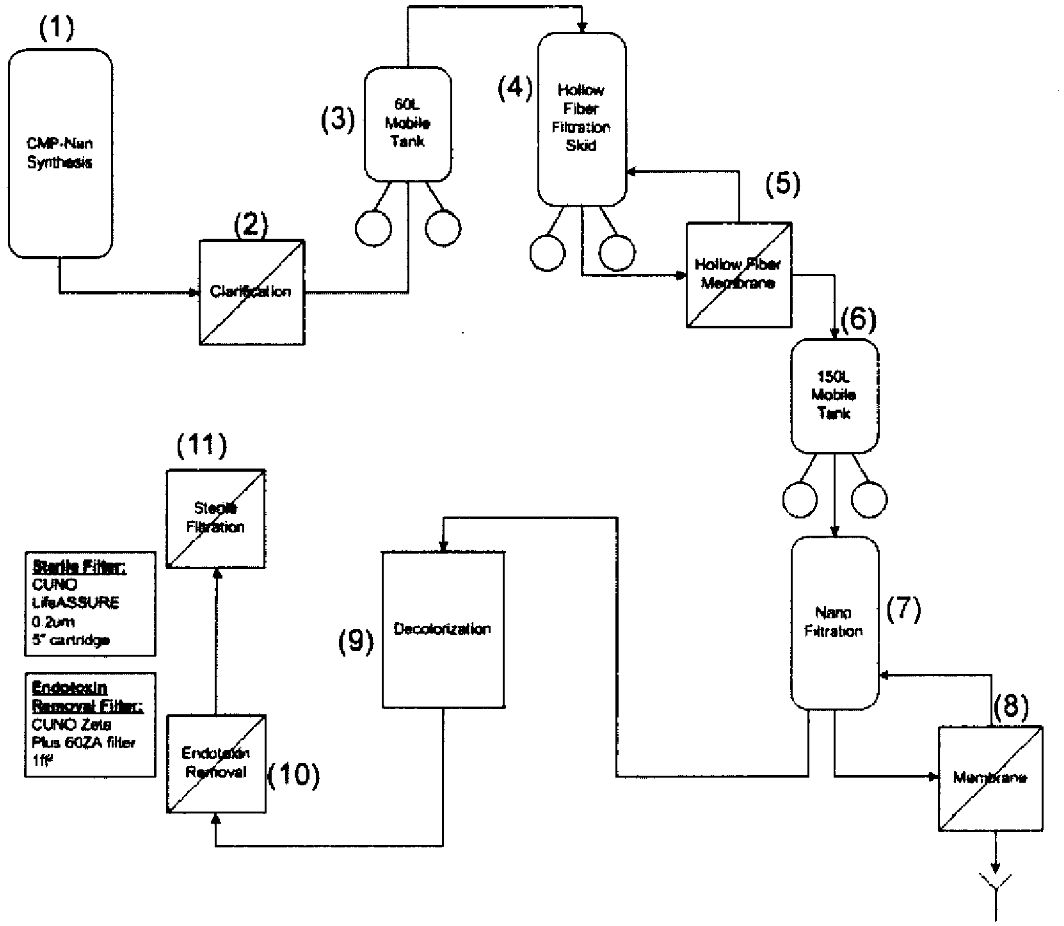


FIGURE 2

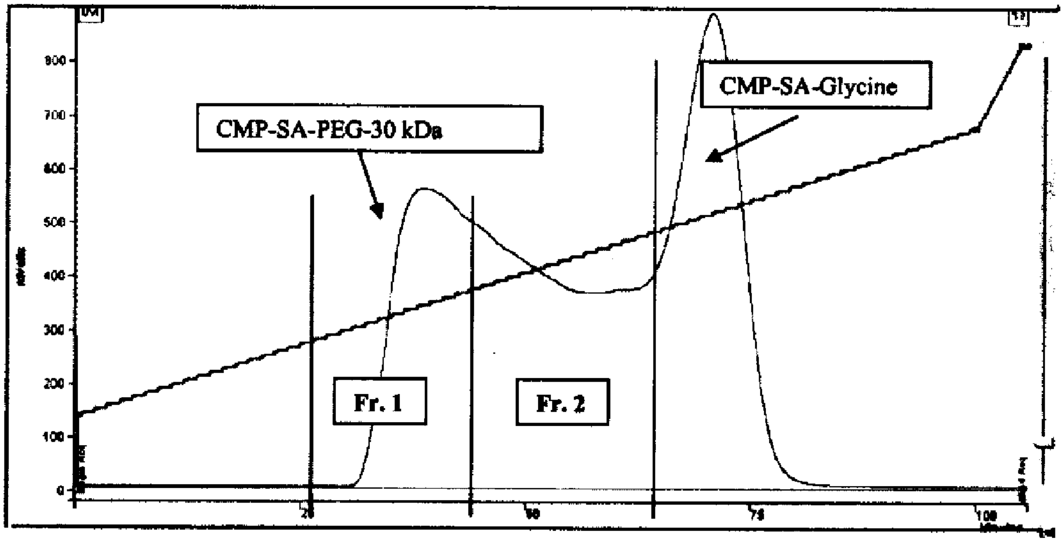

CMP-SA-PEG-30 kDa
CMP-SA-Glycine
Fr. 1
Fr. 2
800
700
600
500
400
300
200
100
0
25
50
75
100

FIGURE 3

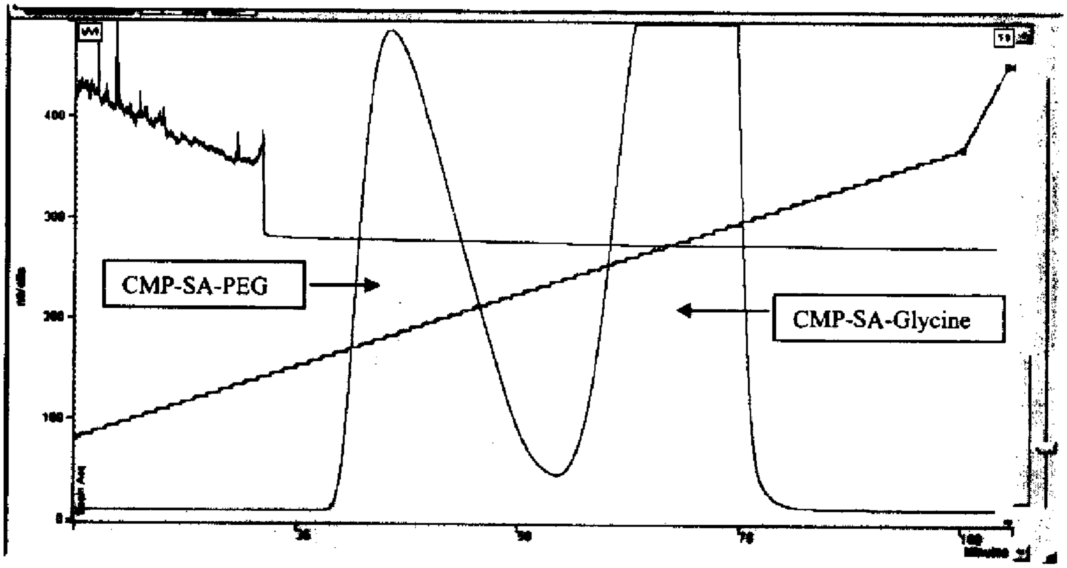


FIGURE 4

CMP-Glycyl Sialic Acid Process Flow Chart

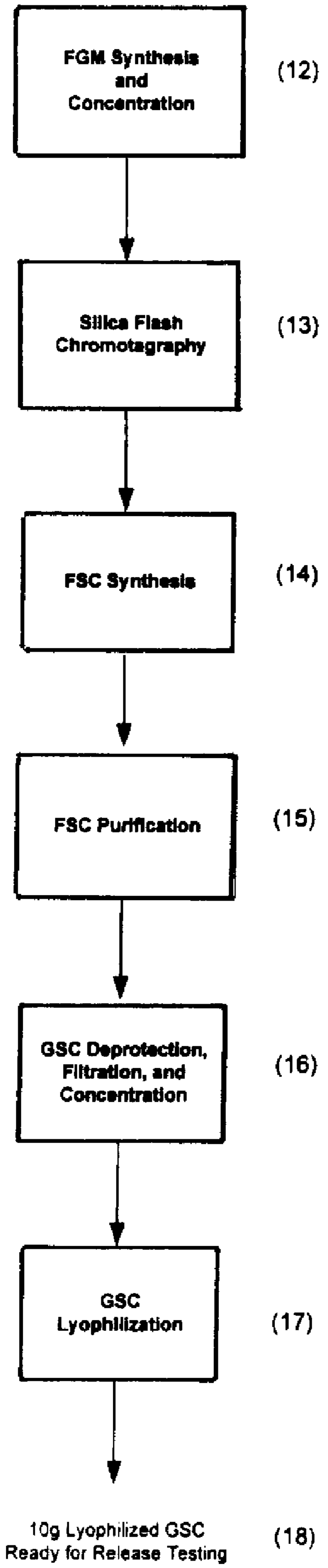


FIGURE 5

CMP-SA-PEG Process Flow Diagram

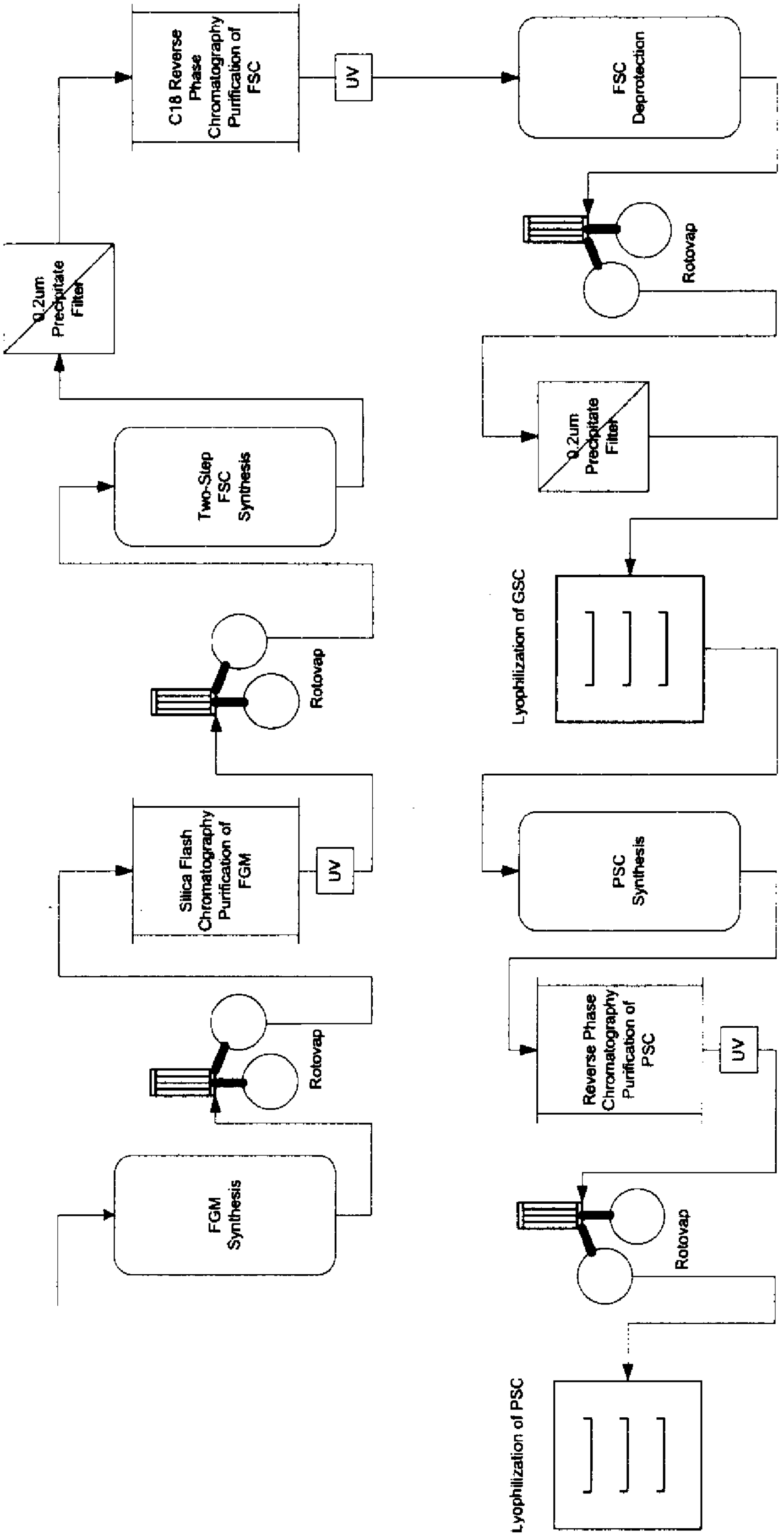
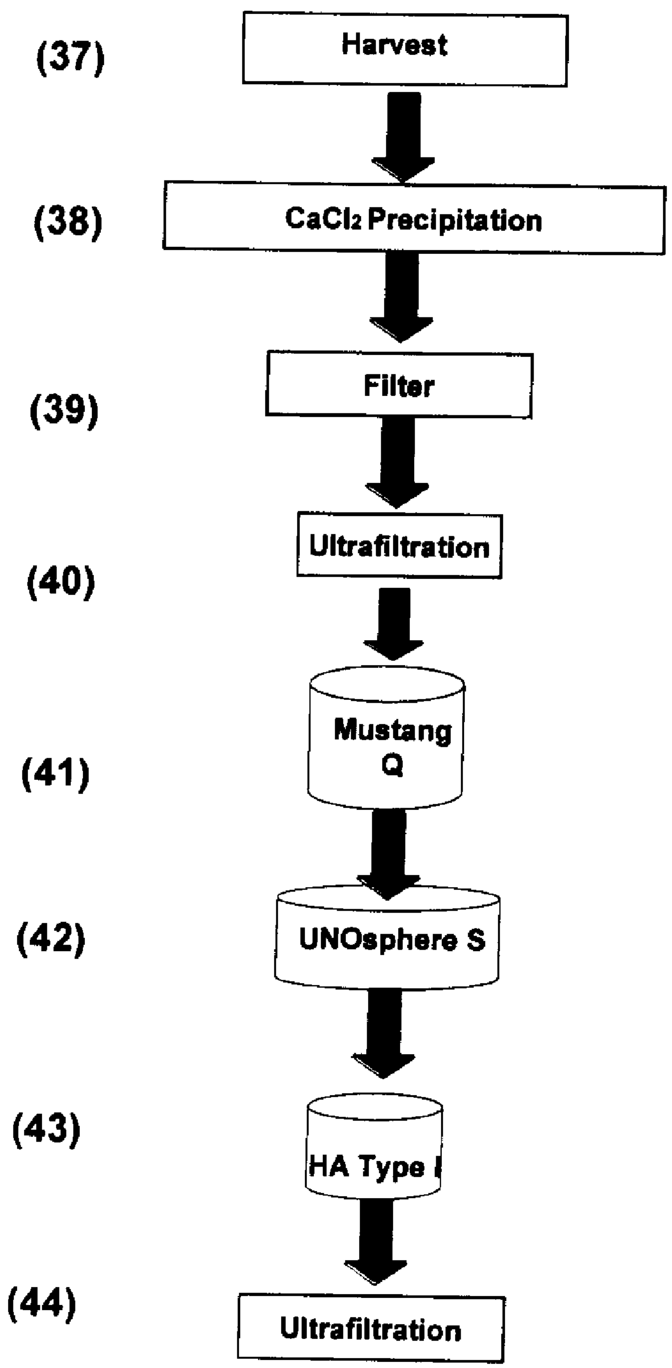


FIGURE 6



chST6GalNac Purification Process Overview

FIGURE 7A

Protein	Organism		EC#	GenBank / GenPept		SwissProt	PDB / 3D
At1g08280		*Arabidopsis thaliana*	n.d.	AC011438 BT004583 NC_003070	AAF18241.1 AAO42829.1 **NP_172305.1**	Q84W00 Q9SGD2	
At1g08660/F22O13.14		*Arabidopsis thaliana*	n.d.	AC003981 AY064135 AY124807 NC_003070 NM_180609	AAF99778.1 **AAL36042.1** AAM70516.1 NP_172342.1 NP_850940.1	Q8VZJ0 Q9FRR9	
At3g48820/T21J18_90		*Arabidopsis thaliana*	n.d.	AY080589 AY133816 AL132963 NM_114741	AAL85966.1 AAM91750.1 **CAB87910.1** NP_190451.1	Q8RY00 Q9M301	
α-2,3-sialyltransferase (ST3GAL-IV)		*Bos taurus*	n.d.	AJ584673	**CAE48298.1**		
α-2,3-sialyltransferase (St3Gal-V)		*Bos taurus*	n.d.	AJ585768	**CAE51392.1**		
α-2,6-sialyltransferase (Siat7b)		*Bos taurus*	n.d.	AJ620651	**CAF05850.1**		
α-2,8-sialyltransferase (SIAT8A)		*Bos taurus*	2.4.99.8	AJ699418	**CAG27880.1**		
α-2,8-sialyltransferase (Siat8D)		*Bos taurus*	n.d.	AJ699421	**CAG27883.1**		
α-2,8-sialyltransferase ST8Siα-III (Siat8C)		*Bos taurus*	n.d.	AJ704563	**CAG28696.1**		
CMP α-2,6-sialyltransferase (ST6Gal I)		*Bos taurus*	2.4.99.1	Y15111 NM_177517	CAA75385.1 NP_803483.1	O18974	
sialyltransferase 8 (fragment)		*Bos taurus*	n.d.	AF450088	**AAL47018.1**	Q8WN13	
sialyltransferase ST3Gal-II (Siat4B)		*Bos taurus*	n.d.	AJ748841	**CAG44450.1**		
sialyltransferase ST3Gal-III (Siat6)		*Bos taurus*	n.d.	AJ748842	**CAG44451.1**		
sialyltransferase ST3Gal-VI (Siat10)		*Bos taurus*	n.d.	AJ748843	**CAG44452.1**		
ST3Gal I		*Bos taurus*	n.d.	AJ305086	**CAC24698.1**	Q9BEG4	
St6GalNAc-VI		*Bos taurus*	n.d.	AJ620949	**CAF06586.1**		
CDS4		*Branchiostoma floridae*	n.d.	AF391289	**AAM18873.1**	Q8T771	
polysialyltransferase (PST) (fragment) ST8Sia IV		*Cercopithecus aethiops*	2.4.99.-	AF210729	**AAF17105.1**	Q9TT09	
polysialyltransferase (STX) (fragment) ST8Sia II		*Cercopithecus aethiops*	2.4.99.-	AF210318	**AAF17104.1**	Q9TT10	
α-2,3-sialyltransferase ST3Gal I (Siat4)		*Ciona intestinalis*	n.d.	AJ626815	**CAF25173.1**		
α-2,3-sialyltransferase ST3Gal I (Siat4)		*Ciona savignyi*	n.d.	AJ626814	**CAF25172.1**		
α-2,8-polysialyltransferase ST8Sia IV		*Cricetulus griseus*	2.4.99.-	- Z46801	AAE28634 CAA86822.1	**Q64690**	
Gal β-1,3/4-GlcNAc α-2,3-sialyltransferase St3Gal I		*Cricetulus griseus*	n.d.	AY266675	**AAP22942.1**	Q80WL0	
Gal β-1,3/4-GlcNAc α-2,3-sialyltransferase St3Gal II (fragment)		*Cricetulus griseus*	n.d.	AY266676	**AAP22943.1**	Q80WK9	
α-2,3-sialyltransferase ST3Gal I (Siat4)		*Danio rerio*	n.d.	AJ783740	**CAH04017.1**		
α-2,3-sialyltransferase ST3Gal II (Siat5)		*Danio rerio*	n.d.	AJ783741	**CAH04018.1**		

FIGURE 7B

Protein	Organism		EC#	GenBank / GenPept		SwissProt	PDB / 3D
α-2,3-sialyltransferase ST3Gal III (Siat6)		*Danio rerio*	n.d.	AJ626821	**CAF25179.1**		
α-2,3-sialyltransferase ST3Gal IV (Siat4c)		*Danio rerio*	n.d.	AJ744809	**CAG32845.1**		
α-2,3-sialyltransferase ST3Gal V-r (Siat5-related)		*Danio rerio*	n.d.	AJ783742	**CAH04019.1**		
α-2,6-sialyltransferase ST6Gal I (Siat1)		*Danio rerio*	n.d.	AJ744801	**CAG32837.1**		
α-2,6-sialyltransferase ST6GalNAc II (Siat7B)		*Danio rerio*	n.d.	AJ634459	**CAG25680.1**		
α-2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)		*Danio rerio*	n.d.	AJ646874	**CAG26703.1**		
α-2,6-sialyltransferase ST6GalNAc VI (Siat7F) (fragment)		*Danio rerio*	n.d.	AJ646883	**CAG26712.1**		
α-2,8-sialyltransferase ST8Sia I (Siat 8A) (fragment)		*Danio rerio*	n.d.	AJ715535	**CAG29374.1**		
α-2,8-sialyltransferase ST8Sia III (Siat 8C) (fragment)		*Danio rerio*	n.d.	AJ715543	**CAG29382.1**		
α-2,8-sialyltransferase ST8Sia IV (Siat 8D) (fragment)		*Danio rerio*	n.d.	AJ715545	**CAG29384.1**		
α-2,8-sialyltransferase ST8Sia V (Siat 8E) (fragment)		*Danio rerio*	n.d.	AJ715546	**CAG29385.1**		
α-2,8-sialyltransferase ST8Sia VI (Siat 8F) (fragment)		*Danio rerio*	n.d.	AJ715551	**CAG29390.1**		
β-galactosamide α-2,6-sialyltransferase II (ST6Gal II)		*Danio rerio*	n.d.	AJ627627	**CAF29495.1**		
N-glycan α-2,8-sialyltransferase		*Danio rerio*	n.d.	BC050483 AY055462 NM_153662	AAH50483.1 **AAL17875.1** NP_705948.1	Q7ZU51 Q8QH83	
ST3Gal III-related (siat6r)		*Danio rerio*	n.d.	BC053179 AJ626820 NM_200355	AAH53179.1 **CAF25178.1** NP_956649.1	Q7T3B9	
St3Gal-V		*Danio rerio*	n.d.	AJ619960	**CAF04061.1**		
st6GalNAc-VI		*Danio rerio*	n.d.	BC060932 AJ620947	**AAH60932.1** CAF06584.1		
α-2,6-sialyltransferase (CG4871) ST6Gal I		*Drosophila melanogaster*	2.4.99.1	AE003465 AF218237 AF397532 AE003465 NM_079129 NM_166684	AAF47256.1 **AAG13185.1** AAK92126.1 AAM70791.1 NP_523853.1 NP_726474.1	Q9GU23 Q9W121	
α-2,3-sialyltransferase (ST3Gal-VI)		*Gallus gallus*	n.d.	AJ585767 AJ627204	**CAE51391.1** CAF25503.1		
α-2,3-sialyltransferase ST3Gal I		*Gallus gallus*	2.4.99.4	X80503 NM_205217	**CAA56666.1** NP_990548.1	Q11200	
α-2,3-sialyltransferase ST3Gal IV (fragment)		*Gallus gallus*	2.4.99.-	AF035250	AAC14163.1	**O73724**	
α-2,3-sialytransferase (ST3GAL-II)		*Gallus gallus*	n.d.	AJ585761	**CAE51385.2**		
α-2,6-sialyltransferase (Siat7b)		*Gallus gallus*	n.d.	AJ620653	**CAF05852.1**		
α-2,6-sialyltransferase ST6Gal I		*Gallus gallus*	2.4.99.1	X75558 NM_205241	**CAA53235.1** **NP_990572.1**	Q92182	
α-2,6-sialyltransferase		*Gallus gallus*	2.4.99.3	-	AAE68028.1	Q92183	

FIGURE 7C

Protein	Organism		EC#	GenBank / GenPept		SwissProt	PDB / 3D
ST6GalNAc I				- X74946 NM_205240	AAE68029.1 **CAA52902.1** NP_990571.1		
α-2,6-sialyltransferase ST6GalNAc II		*Gallus gallus*	2.4.99.-	X77775 NM_205233	AAE68030.1 **CAA54813.1** NP_990564.1	Q92184	
α-2,6-sialyltransferase ST6GalNAc III (SIAT7C) (fragment)		*Gallus gallus*	n.d.	AJ634455	**CAG25677.1**		
α-2,6-sialyltransferase ST6GalNAc V (SIAT7E) (fragment)		*Gallus gallus*	n.d.	AJ646877	**CAG26706.1**		
α-2,8-sialyltransferase (GD3 Synthase) ST8Sia I		*Gallus gallus*	2.4.99.-	U73176	AAC28888.1	**P79783**	
α-2,8-sialyltransferase (SIAT8B)		*Gallus gallus*	n.d.	AJ699419	**CAG27881.1**		
α-2,8-sialyltransferase (SIAT8C)		*Gallus gallus*	n.d.	AJ699420	**CAG27882.1**		
α-2,8-sialyltransferase (SIAT8F)		*Gallus gallus*	n.d.	AJ699424	**CAG27886.1**		
α-2,8-syalyltransferase ST8Siα-V (SIAT8C)		*Gallus gallus*	n.d.	AJ704564	**CAG28697.1**		
β-galactosamide α-2,6-sialyltransferase II (ST6Gal II)		*Gallus gallus*	n.d.	AJ627629	**CAF29497.1**		
GM3 synthase (SIAT9)		*Gallus gallus*	2.4.99.9	AY515255	**AAS83519.1**		
polysialyltransferase ST8Sia IV		*Gallus gallus*	2.4.99.-	AF008194	AAB95120.1	**O42399**	
α-2,3-sialyltransferase ST3Gal I		*Homo sapiens*	2.4.99.4	L29555 AF059321 L13972 AF155238 AF186191 BC018357 NM_003033 NM_173344	AAA36612.1 AAC17874.1 AAC37574.1 AAD39238.1 **AAG29876.1** AAH18357.1 NP_003024.1 NP_775479.1	Q11201 O60677 Q9UN51	
α-2,3-sialyltransferase ST3Gal II		*Homo sapiens*	2.4.99.4	U63090 BC036777 X96667 NM_006927	AAB40389.1 **AAH36777.1** CAA65447.1 NP_008858.1	Q16842 O00654	
α-2,3-sialyltransferase ST3Gal III (SiaT6)		*Homo sapiens*	2.4.99.6	L23768 BC050380 AF425851 AF425852 AF425853 AF425854 AF425855 AF425856 AF425857 AF425858 AF425859 AF425860 AF425861 AF425862 AF425863 AF425864 AF425865 AF425866 AF425867 AY167992 AY167993 AY167994	AAA35778.1 AAH50380.1 AAO13859.1 AAO13860.1 AAO13861.1 AAO13862.1 AAO13863.1 AAO13864.1 AAO13865.1 AAO13866.1 AAO13867.1 AAO13868.1 AAO13869.1 **AAO13870.1** AAO13871.1 AAO13872.1 AAO13873.1 AAO13874.1 AAO13875.1 AAO38806.1 AAO38807.1 AAO38808.1	Q11203 Q86UR6 Q86UR7 Q86UR8 Q86UR9 Q86US0 Q86US1 Q86US2 Q8IX43 Q8IX44 Q8IX45 Q8IX46 Q8IX47 Q8IX48 Q8IX49 Q8IX50 Q8IX51 Q8IX52 Q8IX53 Q8IX54 Q8IX55 Q8IX56	

FIGURE 7D

Protein	Organism		EC#	GenBank / GenPept		SwissProt	PDB / 3D
				AY167995 AY167996 AY167997 AY167998 NM_006279 NM_174964 NM_174965 NM_174966 NM_174967 NM_174969 NM_174970 NM_174972	AAO38809.1 AAO38810.1 AAO38811.1 AAO38812.1 NP_006270.1 NP_777624.1 NP_777625.1 NP_777626.1 NP_777627.1 NP_777629.1 NP_777630.1 NP_777632.1	Q8IX57 Q8IX58	
α-2,3-sialyltransferase ST3Gal IV		*Homo sapiens*	2.4.99.-	L23767 AF035249 BC010645 AY040826 AF516602 AF516603 AF516604 AF525084 X74570 CR456858 NM_006278	AAA16460.1 AAC14162.1 **AAH10645.1** AAK93790.1 AAM66431.1 AAM66432.1 AAM66433.1 AAM81378.1 CAA52662.1 CAG33139.1 NP_006269.1	Q11206 O60497 Q96QQ9 Q8N6A6 Q8N6A7 Q8NFD3 Q8NFG7	
α-2,3-sialyltransferase ST3Gal VI		*Homo sapiens*	2.4.99.4	AF119391 BC023312 AB022918 AX877828 AX886023 NM_006100	**AAD39131.1** AAH23312.1 BAA77609.1 CAE89895.1 CAF00161.1 NP_006091.1	Q9Y274	
α-2,6-sialyltransferase (ST6Gal II ; KIAA1877)		*Homo sapiens*	n.d.	BC008680 AB058780 AB059555 AJ512141 AX795193 AX795193 NM_032528	AAH08680.1 **BAB47506.1** BAC24793.1 CAD54408.1 CAE48260.1 CAE48261.1 NP_115917.1	Q86Y44 Q8IUG7 Q96HE4 Q96JF0	
α-2,6-sialyltransferase (ST6GALNAC III)		*Homo sapiens*	n.d.	BC059363 AY358540 AK091215 AJ507291 NM_152996	AAH59363.1 AAQ88904.1 BAC03611.1 **CAD45371.1** NP_694541.1	Q8N259 Q8NDV1	
α-2,6-sialyltransferase (ST6GalNAc V)		*Homo sapiens*	n.d.	BC001201 AK056241 AL035409 AJ507292 NM_030965	**AAH01201.1** BAB71127.1 CAB72344.1 CAD45372.1 NP_112227.1	Q9BVH7	
α-2,6-sialyltransferase (SThM) ST6GalNAc II		*Homo sapiens*	2.4.99.-	U14550 BC040455 AJ251053 NM_006456	AAA52228.1 **AAH40455.1** CAB61434.1 NP_006447.1	Q9UJ37 Q12971	
α-2,6-sialyltransferase ST6Gal I		*Homo sapiens*	2.4.99.1	BC031476 BC040009 A17362 A23699 X17247 X54363 X62822 NM_003032 NM_173216	AAH31476.1 AAH40009.1 **CAA01327.1** CAA01686.1 CAA35111.1 CAA38246.1 CAA44634.1 NP_003023.1 NP_775323.1	P15907	
α-2,6-sialyltransferase ST6GalNAc I		*Homo sapiens*	2.4.99.3	BC022462 AY096001 AY358918 AK000113 Y11339	AAH22462.1 AAM22800.1 AAQ89277.1 BAA90953.1 **CAA72179.2**	Q8TBJ6 Q9NSC7 Q9NXQ7	

FIGURE 7E

Protein	Organism		EC#	GenBank / GenPept		SwissProt	PDB / 3D
				NM_018414	NP_060884.1		
α-2,8-polysialyltransferase ST8Sia IV		*Homo sapiens*	2.4.99.-	L41680 BC027866 BC053657 NM_005668	AAC41775.1 AAH27866.1 **AAH53657.1** NP_005659.1	Q8N1F4 Q92187 Q92693	
α-2,8-sialyltransferase (GD3 synthase) ST8Sia I		*Homo sapiens*	2.4.99.8	L32867 L43494 BC046158 - AY569975 D26360 X77922 NM_003034	AAA62366.1 **AAC37586.1** AAH46158.1 AAQ53140.1 AAS75783.1 BAA05391.1 CAA54891.1 NP_003025.1	Q86X71 Q92185 Q93064	
α-2,8-sialyltransferase ST8Sia II		*Homo sapiens*	2.4.99.-	L29556 U82762 U33551 BC069584 NM_006011	AAA36613.1 AAB51242.1 **AAC24458.1** AAH69584.1 NP_006002.1	Q92186 Q92470 Q92746	
α-2,8-sialyltransferase ST8Sia III		*Homo sapiens*	2.4.99.-	AF004668 AF003092 NM_015879	**AAB87642.1** AAC15901.2 NP_056963.1	O43173 Q9NS41	
α-2,8-sialyltransferase ST8Sia V		*Homo sapiens*	2.4.99.-	U91641 CR457037 NM_013305	**AAC51727.1** CAG33318.1 NP_037437.1	O15466	
ENSP00000020221 (fragment)			n.d.	AC023295	-		
lactosylceramide α-2,3-sialyltransferase (ST3Gal V)		*Homo sapiens*	2.4.99.9	AF105026 AF119415 BC065936 AY152815 AAP65066 AY359105 AB018356 AX876536 NM_003896	**AAD14634.1** AAF66146.1 AAH65936.1 AAO16866.1 AAP65066.1 AAQ89463.1 BAA33950.1 CAE89320.1 NP_003887.2	Q9UNP4 O94902	
N-acetylgalactosaminide α-2,6-sialyltransferase (ST6GalNAc VI)		*Homo sapiens*	2.4.99.-	BC006564 BC007802 BC016299 AY358672 AB035173 AK023900 AJ507293 AX880950 CR457318 NM_013443	AAH06564.1 AAH07802.1 AAH16299.1 AAQ89035.1 **BAA87035.1** BAB14715.1 CAD45373.1 CAE91145.1 CAG33599.1 NP_038471.2	Q969X2 Q9H8A2 Q9ULB8	
N-acetylgalactosaminide α-2,6-sialyltransferase IV (ST6GalNAc IV)		*Homo sapiens*	2.4.99.-	AF127142 BC036705 - AB035172 AK000600 Y17461 AJ271734 AX061620 AX068265 AX969252 NM_014403 NM_175039	AAF00102.1 AAH36705.1 AAP63349.1 **BAA87034**.1 BAA91281.1 CAB44354.1 CAC07404.1 CAC24981.1 CAC27250.1 CAF14360.1 NP_055218.3 NP_778204.1	Q9H4F1 Q9NWU6 Q9UKU1 Q9ULB9 Q9Y3G3 Q9Y3G4	
ST8SIA-VI (fragment)		*Homo sapiens*	n.d.	AJ621583 XM_291725	**CAF21722.1** XP_291725.2		
unnamed protein product		*Homo sapiens*	n.d.	AK021929 AX881696	**BAB13940.1** CAE91353.1	Q9HAA9	
Gal β-1,3/4-GlcNAc α-		*Mesocricetus*	2.4.99.6	AJ245699	**CAB53394.1**	Q9QXF6	

FIGURE 7F

Protein	Organism		EC#	GenBank / GenPept		SwissProt	PDB / 3D
2,3-sialyltransferase (ST3Gal III)		*auratus*					
Gal β-1,3/4-GlcNAc α-2,3-sialyltransferase (ST3Gal IV)		*Mesocricetus auratus*	2.4.99.6	AJ245700	**CAB53395.1**	Q9QXF5	
GD3 synthase (fragment) ST8Sia I		*Mesocricetus auratus*	n.d.	AF141657	**AAD33879.1**	Q9WUL1	
polysialyltransferase (ST8Sia IV)		*Mesocricetus auratus*	2.4.99.-	AJ245701	**CAB53396.1**	Q9QXF4	
α-2,3-sialyltransferase ST3Gal I	*St3gal1*	*Mus musculus*	2.4.99.4	AF214028 AK031344 AK078469 X73523 NM_009177	AAF60973.1 BAC27356.1 BAC37290.1 CAA51919.1 NP_033203.1	**P54751** **Q11202** Q9JL30	
α-2,3-sialyltransferase ST3Gal II	*St3gal2*	*Mus musculus*	2.4.99.4	BC015264 BC066064 AK034554 AK034863 AK053827 X76989 NM_009179 NM_178048	**AAH15264.1** AAH66064.1 BAC28752.1 BAC28859.1 BAC35543.1 CAA54294.1 NP_033205.1 NP_835149.1	Q11204 Q8BPL0 Q8BSA0 Q8BSE9 Q91WH6	
α-2,3-sialyltransferase ST3Gal III	*St3gal3*	*Mus musculus*	2.4.99.-	BC006710 AK005053 AK013016 X84234 NM_009176	AAH06710.1 **BAB23779.1** BAB28598.1 CAA59013.1 NP_033202.2	P97325 Q922X5 Q9CZ48 Q9DBB6	
α-2,3-sialyltransferase ST3Gal IV	*St3gal4*	*Mus musculus*	2.4.99.4	BC011121 BC050773 D28941 AK008543 AB061305 X95809 NM_009178	AAH11121.1 **AAH50773.1** BAA06068.1 BAB25732.1 BAB47508.1 CAA65076.1 NP_033204.2	P97354 Q61325 Q91Y74 Q921R5 Q9CVE8	
α-2,3-sialyltransferase ST3Gal VI	*St3gal6*	*Mus musculus*	2.4.99.4	AF119390 BC052338 AB063326 AK033562 AK041173 NM_018784	AAD39130.1 AAH52338.1 **BAB79494.1** BAC28360.1 BAC30851.1 NP_061254	Q80UR7 Q8BLV1 Q8VIB3 Q9WVG2	
α-2,6-sialyltransferase ST6GalNAc II	*St6galnac2*	*Mus musculus*	2.4.99.-	NM_009180 BC010208 AB027198 AK004613 X93999 X94000 NM_009180	6677963 AAH10208.1 BAB00637.1 BAB23410.1 CAA63821.1 CAA63822.1 NP_033206.2	**P70277** Q9DC24 Q9JJM5	
α-2,6-sialyltransferase ST6Gal I	*St6gal1*	*Mus musculus*	2.4.99.1	- BC027833 D16106 AK034768 AK084124 NM_145933	AAE68031.1 AAH27833.1 BAA03680.1 BAC28828.1 BAC39120.1 NP_666045.1	**Q64685** Q8BM62 Q8K1L1	
α-2,6-sialyltransferase ST6Gal II	*St6gal2*	*Mus musculus*	n.d.	AK082566 AB095093 AK129462 NM_172829	BAC38534.1 **BAC87752.1** BAC98272.1 NP_766417.1	Q8BUU4	
α-2,6-sialyltransferase ST6GalNAc I	*St6galnac1*	*Mus musculus*	2.4.99.3	Y11274 NM_011371	**CAA72137.1** NP_035501.1	Q9QZ39 Q9JJP5	
α-2,6-sialyltransferase ST6GalNAc III	*St6galnac3*	*Mus musculus*	n.d.	BC058387 AK034804 Y11342 Y11343	AAH58387.1 BAC28836.1 CAA72181.2 **CAB95031.1**	Q9WUV2 Q9JHP5	

FIGURE 7G

Protein	Organism		EC#	GenBank / GenPept		SwissProt	PDB / 3D
				NM_011372	NP_035502		
α-2,6-sialyltransferase ST6GalNAc IV	*St6galnac4*	*Mus musculus*	2.4.99.7	BC056451 AK085730 AJ007310 Y15779 Y15780 Y19055 Y19057 NM_011373	AAH56451.1 BAC39523.1 CAA07446.1 CAB43507.1 **CAB43514.1** CAB93946.1 CAB93948.1 NP_035503.1	Q8C3J2 Q9JHP2 Q9R2B6 O88725 Q9JHP0 Q9QUP9 Q9R2B5	
α-2,8-sialyltransferase (GD3 synthase) ST8Sia I	*St8sia1*	*Mus musculus*	2.4.99.8	L38677 BC024821 AK046188 AK052444 X84235 AJ401102 NM_011374	AAA91869.1 **AAH24821**.1 BAC32625.1 BAC34994.1 CAA59014.1 CAC20706.1 NP_035504.1	Q64468 Q64687 Q8BL76 Q8BWI0 Q8K1C1 Q9EPK0	
α-2,8-sialyltransferase (ST8Sia VI)	*St8sia6*	*Mus musculus*	n.d.	AB059554 AK085105 NM_145838	**BAC01265**.1 BAC39367.1 NP_665837.1	Q8BI43 Q8K4T1	
α-2,8-sialyltransferase ST8Sia II	*St8sia2*	*Mus musculus*	2.4.99.-	X83562 X99646 X99647 X99648 X99649 X99650 X99651 NM_009181	CAA58548.1 CAA67965.1 CAA67965.1 CAA67965.1 CAA67965.1 CAA67965.1 CAA67965.1 NP_033207.1	**O35696**	
α-2,8-sialyltransferase ST8Sia IV	*St8sia4*	*Mus musculus*	2.4.99.8	BC060112 AK003690 AK041723 AJ223956 X86000 Y09484 NM_009183	AAH60112.1 BAB22941.1 BAC31044.1 **CAA11685**.1 CAA59992.1 CAA70692.1 NP_033209.1	Q64692 Q8BY70	
α-2,8-sialyltransferase ST8Sia V	*St8sia5*	*Mus musculus*	2.4.99.-	BC034855 AK078670 X98014 X98014 X98014 NM_013666 NM_153124 NM_177416	AAH34855.1 BAC37354.1 **CAA66642.1** CAA66643.1 CAA66644.1 NP_038694.1 NP_694764.1 NP_803135.1	P70126 P70127 P70128 Q8BJW0 Q8JZQ3	
α-2,8-sialytransferase ST8Sia III	*St8sia3*	*Mus musculus*	2.4.99.-	BC075645 AK015874 X80502 NM_009182	AAH75645.1 BAB30012.1 CAA56665.1 NP_033208.1	**Q64689** Q9CUJ6	
GD1 synthase (ST6GalNAc V)	*St6galnac5*	*Mus musculus*	n.d.	BC055737 AB030836 AB028840 AK034387 AK038434 AK042683 NM_012028	**AAH55737**.1 BAA85747.1 BAA89292.1 BAC28693.1 BAC29997.1 BAC31331.1 NP_036158.2	Q8CAM7 Q8CBX1 Q9QYJ1 Q9R0K6	
GM3 synthase (α-2,3-sialyltransferase) ST3Gal V	*St3gal5*	*Mus musculus*	2.4.99.9	AF119416 - AB018048 AB013302 AK012961 Y15003 NM_011375	**AAF66147.1** AAP65063.1 BAA33491.1 BAA76467.1 BAB28571.1 CAA75235.1 NP_035505.1	O88829 Q9CZ65 Q9QWF9	
N-acetylgalactosaminide α-2,6-sialyltransferase (ST6GalNAc VI)	*St6galnac6*	*Mus musculus*	2.4.99.-	BC036985 AB035174 AB035123 AK030648	**AAH36985**.1 BAA87036.1 BAA95940.1 BAC27064.1	Q8CDC3 Q8JZW3 Q9JM95 Q9R0G9	

FIGURE 7H

Protein	Organism		EC#	GenBank / GenPept		SwissProt	PDB / 3D
				NM_016973	NP_058669.1		
M138L		*Myxoma virus*	n.d.	U46578 AF170726 NC_001132	AAD00069.1 AAE61323.1 AAE61326.1 **AAF15026.1** NP_051852.1		
α-2,3-sialyltransferase (St3Gal-I)		*Oncorhynchus mykiss*	n.d.	AJ585760	**CAE51384.1**		
α-2,6-sialyltransferase (Siat1)		*Oncorhynchus mykiss*	n.d.	AJ620649	**CAF05848.1**		
α-2,8-polysialyltransferase IV (ST8Sia IV)		*Oncorhynchus mykiss*	n.d.	AB094402	**BAC77411.1**	Q7T2X5	
GalNAc α-2,6-sialyltransferase (RtST6GalNAc)		*Oncorhynchus mykiss*	n.d.	AB097943	**BAC77520.1**	Q7T2X4	
α-2,3-sialyltransferase ST3Gal IV		*Oryctolagus cuniculus*	2.4.99.-	AF121967	**AAF28871.1**	Q9N257	
OJ1217_F02.7		*Oryza sativa (japonica cultivar-group)*	n.d.	AP004084	**BAD07616.1**		
OSJNBa0043L24.2 or OSJNBb0002J11.9		*Oryza sativa (japonica cultivar-group)*	n.d.	AL731626 AL662969	**CAD41185.1** CAE04714.1		
P0683f02.18 or P0489B03.1		*Oryza sativa (japonica cultivar-group)*	n.d.	AP003289 AP003794	**BAB63715.1** BAB90552.1		
α-2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)		*Oryzias latipes*	n.d.	AJ646876	**CAG26705.1**		
α-2,3-sialyltransferase ST3Gal I (Siat4)		*Pan troglodytes*	n.d.	AJ744803	**CAG32839.1**		
α-2,3-sialyltransferase ST3Gal II (Siat5)		*Pan troglodytes*	n.d.	AJ744804	**CAG32840.1**		
α-2,3-sialyltransferase ST3Gal III (Siat6)		*Pan troglodytes*	n.d.	AJ626819	**CAF25177.1**		
α-2,3-sialyltransferase ST3Gal IV (Siat4c)		*Pan troglodytes*	n.d.	AJ626824	**CAF25182.1**		
α-2,3-sialyltransferase ST3Gal VI (Siat10)		*Pan troglodytes*	n.d.	AJ744808	**CAG32844.1**		
α-2,6-sialyltransferase (Sia7A)		*Pan troglodytes*	n.d.	AJ748740	**CAG38615.1**		
α-2,6-sialyltransferase (Sia7B)		*Pan troglodytes*	n.d.	AJ748741	**CAG38616.1**		
α-2,6-sialyltransferase ST6GalNAc III (Siat7C)		*Pan troglodytes*	n.d.	AJ634454	**CAG25676.1**		
α-2,6-sialyltransferase ST6GalNAc IV (Siat7D) (fragment)		*Pan troglodytes*	n.d.	AJ646870	**CAG26699.1**		
α-2,6-sialyltransferase ST6GalNAc V (Siat7E)		*Pan troglodytes*	n.d.	AJ646875	**CAG26704.1**		
α-2,6-sialyltransferase ST6GalNAc VI (Siat7F) (fragment)		*Pan troglodytes*	n.d.	AJ646882	**CAG26711.1**		
α-2,8-sialyltransferase 8A (Siat8A)		*Pan troglodytes*	2.4.99.8	AJ697658	**CAG26896.1**		
α-2,8-sialyltransferase 8B (Siat8B)		*Pan troglodytes*	n.d.	AJ697659	**CAG26897.1**		
α-2,8-sialyltransferase 8C (Siat8C)		*Pan troglodytes*	n.d.	AJ697660	**CAG26898.1**		
α-2,8-sialyltransferase 8D (Siat8D)		*Pan troglodytes*	n.d.	AJ697661	**CAG26899.1**		
α-2,8-sialyltransferase		*Pan troglodytes*	n.d.	AJ697662	**CAG26900.1**		

FIGURE 7I

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB / 3D
8E (Siat8E)						
α-2,8-sialyltransferase 8F (Siat8F)	*Pan troglodytes*	n.d.	AJ697663	**CAG26901.1**		
β-galactosamide α-2,6-sialyltransferase I (ST6Gal I; Siat1)	*Pan troglodytes*	2.4.99.1	AJ627624	**CAF29492.1**		
β-galactosamide α-2,6-sialyltransferase II (ST6Gal II)	*Pan troglodytes*	n.d.	AJ627625	**CAF29493.1**		
GM3 synthase ST3Gal V (Siat9)	*Pan troglodytes*	n.d.	AJ744807	**CAG32843.1**		
S138L	*Rabbit fibroma virus Kasza*	n.d.	NC_001266	**NP_052025**		
α-2,3-sialyltransferase ST3Gal III	*Rattus norvegicus*	2.4.99.6	M97754 NM_031697	AAA42146.1 NP_113885.1	**Q02734**	
α-2,3-sialyltransferase ST3Gal IV (Siat4c)	*Rattus norvegicus*	n.d.	AJ626825	**CAF25183.1**		
α-2,3-sialyltransferase ST3Gal VI	*Rattus norvegicus*	n.d.	AJ626743	**CAF25053.1**		
α-2,6-sialyltransferase ST3Gal II	*Rattus norvegicus*	2.4.99.-	X76988 NM_031695	CAA54293.1 NP_113883.1	**Q11205**	
α-2,6-sialyltransferase ST6Gal I	*Rattus norvegicus*	2.4.99.1	M18769 M83143	AAA41196.1 AAB07233.1	**P13721**	
α-2,6-sialyltransferase ST6GalNAc I (Siat7A)	*Rattus norvegicus*	n.d.	AJ634458	**CAG25684.1**		
α-2,6-sialyltransferase ST6GalNAc II (Siat7B)	*Rattus norvegicus*	n.d.	AJ634457	**CAG25679.1**		
α-2,6-sialyltransferase ST6GalNAc III	*Rattus norvegicus*	2.4.99.-	L29554 BC072501 NM_019123	AAC42086.1 AAH72501.1 NP_061996.1	**Q64686**	
α-2,6-sialyltransferase ST6GalNAc IV (Siat7D) (fragment)	*Rattus norvegicus*	n.d.	AJ646871	**CAG26700.1**		
α-2,6-sialyltransferase ST6GalNAc V (Siat7E)	*Rattus norvegicus*	n.d.	AJ646872	**CAG26701.1**		
α-2,6-sialyltransferase ST6GalNAc VI (Siat7F) (fragment)	*Rattus norvegicus*	n.d.	AJ646881	**CAG26710.1**		
α-2,8-sialyltransferase (GD3 synthase) ST8Sia I	*Rattus norvegicus*	2.4.99.-	U53883 D45255	AAC27541.1 BAA08213.1	**P70554** **P97713**	
α-2,8-sialyltransferase (SIAT8E)	*Rattus norvegicus*	n.d.	AJ699422	**CAG27884.1**		
α-2,8-sialyltransferase (SIAT8F)	*Rattus norvegicus*	n.d.	AJ699423	**CAG27885.1**		
α-2,8-sialyltransferase ST8Sia II	*Rattus norvegicus*	2.4.99.-	L13445 NM_057156	AAA42147.1 NP_476497.1	**Q07977** **Q64688**	
α-2,8-sialyltransferase ST8Sia III	*Rattus norvegicus*	2.4.99.-	U55938 NM_013029	AAB50061.1 NP_037161.1	**P97877**	
α-2,8-sialyltransferase ST8Sia IV	*Rattus norvegicus*	2.4.99.-	U90215	AAB49989.1	**O08563**	
β-galactosamide α-2,6-sialyltransferase II (ST6Gal II)	*Rattus norvegicus*	n.d.	AJ627626	**CAF29494.1**		
GM3 synthase ST3Gal V	*Rattus norvegicus*	n.d.	AB018049 NM_031337	BAA33492.1 NP_112627.1	**O88830**	

FIGURE 7J

Protein	Organism		EC#	GenBank / GenPept		SwissProt	PDB / 3D
sialyltransferase ST3Gal-I (Siat4A)		*Rattus norvegicus*	n.d.	AJ748840	CAG44449.1		
α-2,3-sialyltransferase (St3Gal-II)		*Silurana tropicalis*	n.d.	AJ585763	CAE51387.1		
α-2,6-sialyltransferase (Siat7b)		*Silurana tropicalis*	n.d.	AJ620650	CAF05849.1		
α-2,6-sialyltransferase (St6galnac)		*Strongylocentrotus purpuratus*	n.d.	AJ699425	CAG27887.1		
α-2,3-sialyltransferase (ST3GAL-III)		*Sus scrofa*	n.d.	AJ585765	CAE51389.1		
α-2,3-sialyltransferase (ST3GAL-IV)		*Sus scrofa*	n.d.	AJ584674	CAE48299.1		
α-2,3-sialyltransferase ST3Gal I		*Sus scrofa*	2.4.99.4	M97753	AAA31125.1	Q02745	
α-2,6-sialyltransferase (fragment) ST6Gal I		*Sus scrofa*	2.4.99.1	AF136746	AAD33059.1	Q9XSG8	
β-galactosamide α-2,6-sialyltransferase (ST6GalNAc-V)		*Sus scrofa*	n.d.	AJ620948	CAF06585.2		
sialyltransferase (fragment) ST6Gal I		*sus scrofa*	n.d.	AF041031	AAC15633.1	O62717	
ST6GALNAC-V		*Sus scrofa*	n.d.	AJ620948	CAF06585.1		
α-2,3-sialyltransferase (Siat5-r)		*Takifugu rubripes*	n.d.	AJ744805	CAG32841.1		
α-2,3-sialyltransferase ST3Gal I (Siat4)		*Takifugu rubripes*	n.d.	AJ626816	CAF25174.1		
α-2,3-sialyltransferase ST3Gal II (Siat5) (fragment)		*Takifugu rubripes*	n.d.	AJ626817	CAF25175.1		
α-2,3-sialyltransferase ST3Gal III (Siat6)		*Takifugu rubripes*	n.d.	AJ626818	CAF25176.1		
α-2,6-sialyltransferase ST6Gal I (Siat1)		*Takifugu rubripes*	n.d.	AJ744800	CAG32836.1		
α-2,6-sialyltransferase ST6GalNAc II (Siat7B)		*Takifugu rubripes*	n.d.	AJ634460	CAG25681.1		
α-2,6-sialyltransferase ST6GalNAc II B (Siat7B-related)		*Takifugu rubripes*	n.d.	AJ634461	CAG25682.1		
α-2,6-sialyltransferase ST6GalNAc III (Siat7C) (fragment)		*Takifugu rubripes*	n.d.	AJ634456	CAG25678.1		
α-2,6-sialyltransferase ST6GalNAc IV (siat7D) (fragment)		*Takifugu rubripes*	2.4.99.3	Y17466 AJ646869	CAB44338.1 CAG26698.1	Q9W6U6	
α-2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)		*Takifugu rubripes*	n.d.	AJ646873	CAG26702.1		
α-2,6-sialyltransferase ST6GalNAc VI (Siat7F) (fragment)		*Takifugu rubripes*	n.d.	AJ646880	CAG26709.1		
α-2,8-sialyltransferase ST8Sia I (Siat 8A) (fragment)		*Takifugu rubripes*	n.d.	AJ715534	CAG29373.1		
α-2,8-sialyltransferase ST8Sia II (Siat 8B) (fragment)		*Takifugu rubripes*	n.d.	AJ715538	CAG29377.1		
α-2,8-sialyltransferase ST8Sia III (Siat 8C) (fragment)		*Takifugu rubripes*	n.d.	AJ715541	CAG29380.1		
α-2,8-sialyltransferase ST8Sia IIIr (Siat 8Cr)		*Takifugu rubripes*	n.d.	AJ715542	CAG29381.1		
α-2,8-sialyltransferase ST8Sia V (Siat 8E)		*Takifugu rubripes*	n.d.	AJ715547	CAG29386.1		

FIGURE 7K

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB / 3D
(fragment)						
α-2,8-sialyltransferase ST8Sia VI (Siat 8F) (fragment)	*Takifugu rubripes*	n.d.	AJ715549	**CAG29388.1**		
α-2,8-sialyltransferase ST8Sia VIr (Siat 8Fr)	*Takifugu rubripes*	n.d.	AJ715550	**CAG29389.1**		
α-2,3-sialyltransferase (Siat5-r)	*Tetraodon nigroviridis*	n.d.	AJ744806	**CAG32842.1**		
α-2,3-sialyltransferase ST3Gal I (Siat4)	*Tetraodon nigroviridis*	n.d.	AJ744802	**CAG32838.1**		
α-2,3-sialyltransferase ST3Gal III (Siat6)	*Tetraodon nigroviridis*	n.d.	AJ626822	**CAF25180.1**		
α-2,6-sialyltransferase ST6GalNAc II (Siat7B)	*Tetraodon nigroviridis*	n.d.	AJ634462	**CAG25683.1**		
α-2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)	*Tetraodon nigroviridis*	n.d.	AJ646879	**CAG26708.1**		
α-2,8-sialyltransferase ST8Sia I (Siat 8A) (fragment)	*Tetraodon nigroviridis*	n.d.	AJ715536	**CAG29375.1**		
α-2,8-sialyltransferase ST8Sia II (Siat 8B) (fragment)	*Tetraodon nigroviridis*	n.d.	AJ715537	**CAG29376.1**		
α-2,8-sialyltransferase ST8Sia III (Siat 8C) (fragment)	*Tetraodon nigroviridis*	n.d.	AJ715539	**CAG29378.1**		
α-2,8-sialyltransferase ST8Sia IIIr (Siat 8Cr) (fragment)	*Tetraodon nigroviridis*	n.d.	AJ715540	**CAG29379.1**		
α-2,8-sialyltransferase ST8Sia V (Siat 8E) (fragment)	*Tetraodon nigroviridis*	n.d.	AJ715548	**CAG29387.1**		
α-2,3-sialyltransferase (St3Gal-II)	*Xenopus laevis*	n.d.	AJ585762	CAE51386.1		
α-2,3-sialyltransferase (St3Gal-VI)	*Xenopus laevis*	n.d.	AJ585766	**CAE51390.1**		
α-2,3-sialyltransferase St3Gal-III (Siat6)	*Xenopus laevis*	n.d.	AJ585764 AJ626823	**CAE51388.1** CAF25181.1		
α-2,8-polysialyltransferase	*Xenopus laevis*	2.4.99.-	AB007468	BAA32617.1	**O93234**	
α-2,8-sialyltransferase ST8Siα-I (Siat8A;GD3 synthase)	*Xenopus laevis*	n.d.	AY272056 AY272057 AJ704562	AAQ16162.1 AAQ16163.1 **CAG28695.1**		
Unknown (protein for MGC:81265)	*Xenopus laevis*	n.d.	BC068760	**AAH68760.1**		
α-2,3-sialyltransferase (3Gal-VI)	*Xenopus tropicalis*	n.d.	AJ626744	**CAF25054.1**		
α-2,3-sialyltransferase (Siat4c)	*Xenopus tropicalis*	n.d.	AJ622908	**CAF22058.1**		
α-2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)	*Xenopus tropicalis*	n.d.	AJ646878	**CAG26707.1**		
α-2,8-sialyltransferase ST8Sia III (Siat 8C) (fragment)	*Xenopus tropicalis*	n.d.	AJ715544	**CAG29383.1**		
β-galactosamide α-2,6-sialyltransferase II (ST6Gal II)	*Xenopus tropicalis*	n.d.	AJ627628	**CAF29496.1**		
sialytransferase St8Sial	*Xenopus tropicalis*	n.d.	AY652775	**AAT67042**		
poly-α-2,8-sialosyl sialyltransferase (NeuS)	*Escherichia coli K1*	2.4.-.-	M76370 X60598	AAA24213.1 CAA43053.1	**Q57269**	
polysialyltransferase	*Escherichia coli K92*	2.4.-.-	M88479	**AAA24215.1**	Q47404	

FIGURE 7L

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB / 3D
α-2,8 polysialyltransferase SiaD	*Neisseria meningitidis B1940*	2.4.-.-	M95053 X78068	AAA20478.1 CAA54985.1	**Q51281** **Q51145**	
SynE	*Neisseria meningitidis FAM18*	n.d.	U75650	**AAB53842.1**	O06435	
polysialyltransferase (SiaD)(fragment)	*Neisseria meningitidis M1019*	n.d.	AY234192	**AAO85290.1**		
SiaD (fragment)	*Neisseria meningitidis M209*	n.d.	AY281046	**AAP34769.1**		
SiaD (fragment)	*Neisseria meningitidis M3045*	n.d.	AY281044	**AAP34767.1**		
polysialyltransferase (SiaD)(fragment)	*Neisseria meningitidis M3315*	n.d.	AY234191	**AAO85289.1**		
SiaD (fragment)	*Neisseria meningitidis M3515*	n.d.	AY281047	**AAP34770.1**		
polysialyltransferase (SiaD)(fragment)	*Neisseria meningitidis M4211*	n.d.	AY234190	**AAO85288.1**		
SiaD (fragment)	*Neisseria meningitidis M4642*	n.d.	AY281048	**AAP34771.1**		
polysialyltransferase (SiaD)(fragment)	*Neisseria meningitidis M5177*	n.d.	AY234193	**AAO85291.1**		
SiaD	*Neisseria meningitidis M5178*	n.d.	AY281043	**AAP34766.1**		
SiaD (fragment)	*Neisseria meningitidis M980*	n.d.	AY281045	**AAP34768.1**		
NMB0067	*Neisseria meningitidis MC58*	n.d.	NC_003112	**NP_273131**		
Lst	*Aeromonas punctata Sch3*	n.d.	AF126256	**AAS66624.1**		
ORF2	*Haemophilus influenzae A2*	n.d.	M94855	**AAA24979.1**		
HI1699	*Haemophilus influenzae Rd*	n.d.	U32842 NC_000907	**AAC23345.1** NP_439841.1	Q48211	
α-2,3-sialyltransferase	*Neisseria gonorrhoeae F62*	2.4.99.4	U60664	**AAC44539.1** AAE67205.1	P72074	
α-2,3-sialyltransferase	*Neisseria meningitidis 126E, NRCC 4010*	2.4.99.4	U60662	**AAC44544.2**		
α-2,3-sialyltransferase	*Neisseria meningitidis 406Y, NRCC 4030*	2.4.99.4	U60661	**AAC44543.1**		
α-2,3-sialyltransferase (NMB0922)	*Neisseria meningitidis MC58*	2.4.99.4	U60660 AE002443 NC_003112	AAC44541.1 AAF41330.1 **NP_273962.1**	P72097	
NMA1118	*Neisseria meningitidis Z2491*	n.d.	AL162755 NC_003116	CAB84380.1 NP_283887.1	**Q9JUV5**	
PM0508	*Pasteurella multocida PM70*	n.d.	AE006086 NC_002663	**AAK02592.1** NP_245445.1	Q9CNC4	
WaaH	*Salmonella enterica SARB25*	n.d.	AF519787	**AAM82550.1**	Q8KS93	
WaaH	*Salmonella enterica SARB3*	n.d.	AF519788	**AAM82551.1**	Q8KS92	
WaaH	*Salmonella enterica SARB39*	n.d.	AF519789	**AAM82552.1**		
WaaH	*Salmonella enterica SARB53*	n.d.	AF519790	**AAM82553.1**		
WaaH	*Salmonella enterica SARB57*	n.d.	AF519791	**AAM82554.1**	Q8KS91	
WaaH	*Salmonella enterica SARB71*	n.d.	AF519793	**AAM82556.1**	Q8KS89	
WaaH	*Salmonella enterica*	n.d.	AF519792	**AAM82555.1**	Q8KS90	

FIGURE 7M

Protein	Organism		EC#	GenBank / GenPept		SwissProt	PDB / 3D
		SARB8					
WaaH		*Salmonella enterica SARC10V*	n.d.	AF519779	**AAM88840.1**	Q8KS99	
WaaH (fragment)		*Salmonella enterica SARC12*	n.d.	AF519781	**AAM88842.1**		
WaaH (fragment)		*Salmonella enterica SARC13I*	n.d.	AF519782	**AAM88843.1**	Q8KS98	
WaaH (fragment)		*Salmonella enterica SARC14I*	n.d.	AF519783	**AAM88844.1**	Q8KS97	
WaaH		*Salmonella enterica SARC15II*	n.d.	AF519784	**AAM88845.1**	Q8KS96	
WaaH		*Salmonella enterica SARC16II*	n.d.	AF519785	**AAM88846.1**	Q8KS95	
WaaH (fragment)		*Salmonella enterica SARC3I*	n.d.	AF519772	**AAM88834.1**	Q8KSA4	
WaaH (fragment)		*Salmonella enterica SARC4I*	n.d.	AF519773	**AAM88835.1**	Q8KSA3	
WaaH		*Salmonella enterica SARC5IIa*	n.d.	AF519774	**AAM88836.1**		
WaaH		*Salmonella enterica SARC6IIa*	n.d.	AF519775	**AAM88837.1**	Q8KSA2	
WaaH		*Salmonella enterica SARC8*	n.d.	AF519777	**AAM88838.1**	Q8KSA1	
WaaH		*Salmonella enterica SARC9V*	n.d.	AF519778	**AAM88839.1**	Q8KSA0	
UDP-glucose : α-1,2-glucosyltransferase (WaaH)		*Salmonella enterica subsp. arizonae SARC 5*	2.4.1.-	AF511116	**AAM48166.1**		
bifunctional α-2,3/-2,8-sialyltransferase (Cst-II)		*Campylobacter jejuni ATCC 43449*	n.d.	AF401529	**AAL06004.1**	Q93CZ5	
Cst		*Campylobacter jejuni 81-176*	n.d.	AF305571	**AAL09368.1**		
α-2,3-sialyltransferase (Cst-III)		*Campylobacter jejuni ATCC 43429*	2.4.99.-	AY044156	**AAK73183.1**		
α-2,3-sialyltransferase (Cst-III)		*Campylobacter jejuni ATCC 43430*	2.4.99.-	AF400047	**AAK85419.1**		
α-2,3-sialyltransferase (Cst-II)		*Campylobacter jejuni ATCC 43432*	2.4.99.-	AF215659	**AAG43979.1**	Q9F0M9	
α-2,3/8-sialyltransferase (CstII)		*Campylobacter jejuni ATCC 43438*	n.d.	AF400048	**AAK91725.1**	Q93MQ0	
α-2,3-sialyltransferase cst-II		*Campylobacter jejuni ATCC 43446*	2.4.99.-	AF167344	**AAF34137.1**		
α-2,3-sialyltransferase (Cst-II)		*Campylobacter jejuni ATCC 43456*	2.4.99.-	AF401528	**AAL05990.1**	Q93D05	
α-2,3-/α-2,8-sialyltransferase (CstII)		*Campylobacter jejuni ATCC 43460*	2.4.99.-	AY044868	**AAK96001.1**	Q938X6	
α-2,3/8-sialyltransferase (Cst-II)		*Campylobacter jejuni ATCC 700297*	n.d.	AF216647	**AAL36462.1**		
ORF		*Campylobacter jejuni GB11*	n.d.	AY422197	**AAR82875.1**		
α-2,3-sialyltransferase cstIII		*Campylobacter jejuni MSC57360*	2.4.99.-	AF195055	**AAG29922.1**		
α-2,3-sialyltransferase cstIII Cj1140		*Campylobacter jejuni NCTC 11168*	2.4.99.-	AL139077 NC_002163	**CAB73395.1** NP_282288.1	Q9PNF4	
α-2,3/α-2,8-sialyltransferase II (cstII)		*Campylobacter jejuni O:10*	n.d.	- AX934427	AAO96669.1 **CAF04167.1**		
α-2,3/α-2,8-sialyltransferase II (CstII)		*Campylobacter jejuni O:19*	n.d.	AX934431	**CAF04169.1**		
α-2,3/α-2,8-sialyltransferase II (CstII)		*Campylobacter jejuni O:36*	n.d.	AX934436	**CAF04171.1**		
α-2,3/α-2,8-		*Campylobacter*	n.d.	AX934434	**CAF04170.1**		

FIGURE 7N

Protein	Organism		EC#	GenBank / GenPept		SwissProt	PDB / 3D
sialyltransferase II (CstII)		*jejuni O:4*					
α-2,3/α-2,8-sialyltransferase II (CstII)		*Campylobacter jejuni O:41*	n.d.	- - AX934429	AAO96670.1 AAT17967.1 **CAF04168.1**		
α-2,3-sialyltransferase cst-I		*Campylobacter jejuni OH4384*	2.4.99.-	AF130466 -	**AAF13495.1** AAS36261.1	Q9RGF1	
bifunctional α-2,3/-2,8-sialyltransferase (Cst-II)		*Campylobacter jejuni OH4384*	2.4.99.-	AF130984 AX934425	**AAF31771.1** CAF04166.1	1RO7 1RO8	C A
HI0352 (fragment)		*Haemophilus influenzae Rd*	n.d.	U32720 X57315 NC_000907	AAC22013.1 CAA40567.1 NP_438516.1	**P24324**	
PM1174		*Pasteurella multocida PM70*	n.d.	AE006157 NC_002663	**AAK03258.1** NP_246111.1	Q9CLP3	
Sequence 10 from patent US 6503744		*Unknown.*	n.d.	-	**AAO96672.1**		
Sequence 10 from patent US 6699705		*Unknown.*	n.d.	-	**AAT17969.1**		
Sequence 12 from patent US 6699705		*Unknown.*	n.d.	-	**AAT17970.1**		
Sequence 2 from patent US 6709834		*Unknown.*	n.d.	-	**AAT23232.1**		
Sequence 3 from patent US 6503744		*Unknown.*	n.d.	-	**AAO96668.1**		
Sequence 3 from patent US 6699705		*Unknown.*	n.d.	-	**AAT17965.1**		
Sequence 34 from patent US 6503744		*Unknown.*	n.d.	-	**AAO96684.1**		
Sequence 35 from patent US 6503744 (fragment)		*Unknown.*	n.d.	- -	**AAO96685.1** AAS36262.1		
Sequence 48 from patent US 6699705		*Unknown.*	n.d.	-	**AAT17988.1**		
Sequence 5 from patent US 6699705		*Unknown.*	n.d.	-	**AAT17966.1**		
Sequence 9 from patent US 6503744		*Unknown.*	n.d.	-	**AAO96671.1**		

NUCLEOTIDE SUGAR PURIFICATION USING MEMBRANES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application is a U.S. national phase application of PCT/US2006/043048 filed Nov. 3, 2006, which claims priority under 35 U.S.C. §119(e) to U.S. Provisional Patent Application No. 60/829,242, filed Oct. 12, 2006, U.S. Provisional Application No. 60/823,538, filed Aug. 25, 2006, U.S. Provisional Application No. 60/746,754, filed May 8, 2006, U.S. Provisional Application No. 60/796,281, filed Apr. 28, 2006, and U.S. Provisional Application No. 60/733,975, filed Nov. 3, 2005, the disclosures of which are incorporated herein by reference for all purposes.

BACKGROUND OF THE INVENTION

[0002] Increased understanding of the role of carbohydrates as recognition elements on the surface of cells has led to increased interest in the production of carbohydrate molecules of defined structure. For instance, compounds comprising the oligosaccharide moiety, sialyl lactose, have been of interest as neutralizers for enterotoxins from bacteria such as *Vibrio cholerae, Escherichia coli*, and *Salmonella* (see, e.g., U.S. Pat. No. 5,330,975). Sialyl lactose has also been investigated for the treatment of arthritis and related autoimmune diseases. In particular, sialyl lactose is thought to inhibit or disrupt the degree of occupancy of the Fc carbohydrate binding site on IgG, and thus prevent the formation of immune complexes (see, U.S. Pat. No. 5,164,374). Recently, sialyl-α(2,3)galactosides, sialyl lactose and sialyl lactosamine have been proposed for the treatment of ulcers, and Phase I clinical trials have begun for the use of the former compound in this capacity. See, Balkonen et al., *FEMS Immunology and Medical Microbiology* 7:29 (1993) and BioWorld Today, p. 5, Apr. 4, 1995. As another example, compounds comprising the sialyl Lewis ligands, sialyl Lewis^{x} and sialyl Lewis^{a} are present in leukocyte and non-leukocyte cell lines that bind to receptors such as the ELAM-1 and GMP 140 receptors. Polley et al., *Proc. Natl. Acad. Sci., USA,* 88:6224 (1991) and Phillips et al., *Science,* 250:1130 (1990), see, also, U.S. Ser. No. 08/063,181.

[0003] Because of interest in making desired carbohydrate structures, glycosyltransferases and their role in enzyme-catalyzed synthesis of carbohydrates are presently being extensively studied. The use of glycosyltransferases for enzymatic synthesis of carbohydrate offers advantages over chemical methods due to the virtually complete stereoselectivity and linkage specificity offered by the enzymes (Ito et al., *Pure Appl. Chem.,* 65:753 (1993) U.S. Pat. Nos. 5,352,670, and 5,374,541). Consequently, glycosyltransferases are increasingly used as enzymatic catalysts in synthesis of a number of carbohydrates used for therapeutic and other purposes.

[0004] Carbohydrate compounds produced by enzymatic synthesis or by other methods are often obtained in the form of complex mixtures that include not only the desired compound but also contaminants such as unreacted sugars, salts, pyruvate, phosphate, PEP, nucleosides, nucleotides, and proteins, among others. The presence of these contaminants is undesirable for many applications for which the carbohydrate compounds are useful. Previously used methods for purifying oligosaccharides, such as chromatography, i.e., ion exchange and size exclusion chromatography, have several disadvantages. For example, chromatographic purification methods are not amenable to large-scale purifications, thus precluding their use for commercial production of saccharides. Moreover, chromatographic purification methods are expensive. Therefore, a need exists for purification methods that are faster, more efficient, and less expensive than previously used methods. The present invention fulfills this and other needs.

SUMMARY OF THE INVENTION

[0005] The present invention provides methods of purifying a carbohydrate compound from a feed solution containing a contaminant. The methods involve contacting the feed solution with a nanofiltration or reverse osmosis membrane under conditions such that the membrane retains the desired carbohydrate compound while a majority of the contaminant passes through the membrane. The invention provides methods for purifying carbohydrate compounds such as sialyl lactosides, sialic acid, lacto-N-neotetraose (LNnT) and GlcNAcβ1,3Galβ1,4Glc (LNT-2), NeuAcα(2→3)Galβ(1→4)(Fucα1→3)Glc(R^{1})β1-OR^{2}, wherein R^{1} is OH or NAc; R^{2} is a hydrogen, an alkoxy, a saccharide, an oligosaccharide or an aglycon group having at least one carbon atom; and Galα(1→3)Galβ(1→4)Glc(R^{1})β-O—R^{3}, wherein R^{1} is OH or NAc; R^{3} is —$(CH_2)_n$—COX, with X=OH, OR^{4}, —$NHNH_2$, R^{4} being a hydrogen, a saccharide, an oligosaccharide or an aglycon group having at least one carbon atom, and n=an integer from 2 to 18.

[0006] Also provided are methods for purifying carbohydrate compounds having a formula NeuAcα(2→3)Galβ(1→4)GlcN(R^{1})β-OR^{2}, NeuAcα(2→3)Galβ(1→4)GlcN(R^{1})β(1→3)Galβ-OR^{2}, NeuAcα(2→3)Galβ(1→4)(Fucα1→3)GlcN(R^{1})β-OR^{2}, or NeuAcα(2→3)Galβ(1→4)(Fucα1→3)GlcN(R^{1})β(1→3)Galβ-OR^{2}, wherein R^{1} is alkyl or acyl from 1-18 carbons, 5,6,7,8-tetrahydro-2-naphthamido; benzamido; 2-naphthamido; 4-aminobenzamido; or 4-nitrobenzamido, and R^{2} is a hydrogen, a saccharide, an oligosaccharide or an aglycon group having at least one carbon atom.

[0007] In another embodiment, the invention provides methods of purifying a carbohydrate compound from a feed solution comprising a reaction mixture used to synthesize the carbohydrate compound. The synthesis can be enzymatic or chemical, or a combination thereof. The methods involve removing any proteins present in the feed solution by contacting the feed solution with an ultrafiltration membrane so that proteins are retained the membrane while the carbohydrate compound passes through the membrane as a permeate. The permeate from the ultrafiltration step is then contacted with a nanofiltration or reverse osmosis membrane under conditions such that the nanofiltration or reverse osmosis membrane retains the carbohydrate compound while a majority of an undesired contaminant passes through the membrane.

[0008] Another embodiment of the invention provides methods for purifying nucleotides, nucleosides, and nucleotide sugars by contacting a feed solution containing the nucleotide or related compound with a nanofiltration or reverse osmosis membrane under conditions such that the membrane retains the nucleotide or related compound while a majority of the contaminant passes through the membrane.

[0009] The present invention also provides methods for removing one or more contaminants from a solution that contains a carbohydrate of interest. The methods involve contacting the solution with a first side of a semipermeable membrane having rejection coefficients so as to retain the carbohydrate while allowing the contaminant to pass through the membrane. The membrane is selected from the group consisting of an ultrafiltration membrane, a nanofiltration membrane, and a reverse osmosis membrane, depending on the size and charge of the carbohydrate of interest relative to those of the contaminants. The membrane separates a feed solution containing a carbohydrate into a retentate portion and a permeate portion. If the rejection coefficient of the membrane is greater for the carbohydrate than for the contaminant, the retentate portion will have a lower concentration of the contaminant relative to the contaminant concentration in the feed solution, and generally also a higher ratio of the carbohydrate to the undesired contaminant. Conversely, a membrane having a rejection coefficient for the carbohydrate that is lesser than that for the contaminant will effect a separation wherein the concentration of the contaminant is lower in the permeate than in the feed solution, and the permeate will have a higher ratio of carbohydrate to contaminant than the feed solution. If desired, the fraction containing the carbohydrate can be recycled through the membrane system for further purification.

[0010] Examples of contaminants that can be removed from solutions containing the compound of interest using the methods of the invention include, but are not limited to, unreacted sugars, inorganic ions, pyruvate, phosphate, phosphoenolpyruvate, and proteins.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] FIG. **1** is a diagram of an exemplary purification of a nucleotide sugar.

[0012] FIG. **2** is a chromatogram recorded for an exemplary purification of CMP-SA-PEG-30 kDa using Q Sepharose chromatography as described in Example 6. CMP-SA-PEG-30 kDa was collected in two fractions. Fraction 1 contained pure product and Fraction 2 contained residual CMP-SA-Gly reagent and was reprocessed.

[0013] FIG. **3** is a chromatogram recorded for an exemplary separation of CMP-SA-PEG 30 kDa from CMP-SA-Glycine using Q-Sepharose chromatography, showing a typical baseline separation.

[0014] FIG. **4** is a diagram outlining an exemplary process for the preparation of an exemplary GSC (CMP-5'-Glycyl-Sialic Acid).

[0015] FIG. **5** is a diagram outlining an exemplary process for the preparation of an exemplary PSC (CMP-SA-PEG).

[0016] FIG. **6** is a diagram outlining an exemplary process for the purification of a glycosyltransferase.

[0017] FIG. **7** is a table of exemplary sialyltransferases.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

[0018] The term “sialic acid” refers to any member of a family of nine-carbon carboxylated sugars. The most common member of the sialic acid family is N-acetyl-neuraminic acid (2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galactononulopyranos-1-onic acid (often abbreviated as Neu5Ac, NeuAc, or NANA). A second member of the family is N-glycolyl-neuraminic acid (Neu5Gc or NeuGc), in which the N-acetyl group of NeuAc is hydroxylated. A third sialic acid family member is 2-keto-3-deoxy-nonulosonic acid (KDN) (Nadano et al. (1986) *J. Biol. Chem.* 261: 11550-11557; Kanamori et al., *J. Biol. Chem.* 265: 21811-21819 (1990)). Also included are 9-substituted sialic acids such as a 9-O—C_1-C_6 acyl-Neu5Ac like 9-O-lactyl-Neu5Ac or 9-O-acetyl-Neu5Ac, 9-deoxy-9-fluoro-Neu5Ac and 9-azido-9-deoxy-Neu5Ac. For review of the sialic acid family, see, e.g., Varki, *Glycobiology* 2: 25-40 (1992); *Sialic Acids: Chemistry, Metabolism and Function*, R. Schauer, Ed. (Springer-Verlag, New York (1992)). The synthesis and use of sialic acid compounds in a sialylation procedure is disclosed in international application WO 92/16640, published Oct. 1, 1992.

[0019] As used herein, the term “modified sugar,” refers to a naturally- or non-naturally-occurring carbohydrate. The modified sugar is preferably selected from a number of enzyme substrates including, but not limited to sugar nucleotides (mono-, di-, and tri-phosphates), activated sugars (e.g., glycosyl halides, glycosyl mesylates) and sugars that are neither activated nor nucleotides. The “modified sugar” is covalently functionalized with a “modifying group.” Useful modifying groups include, but are not limited to, water-soluble polymers, targeting moieties therapeutic moieties, diagnostic moieties, radioactive moieties, cytotoxic moieties, biomolecules and the like. The modifying group is preferably not a naturally occurring, or an unmodified carbohydrate. The locus of functionalization with the modifying group is preferably selected such that it does not prevent the “modified sugar” from being added enzymatically to a peptide.

[0020] The term “water-soluble” refers to moieties that have some detectable degree of solubility in water. Methods to detect and/or quantify water solubility are well known in the art. Exemplary water-soluble polymers include peptides, saccharides, poly(ethers), poly(amines), poly(carboxylic acids) and the like. Peptides can have mixed sequences of be composed of a single amino acid, e.g., poly(lysine). An exemplary polysaccharide is poly(sialic acid). An exemplary poly (ether) is poly(ethylene glycol), e.g., m-PEG. Poly(ethylene imine) is an exemplary polyamine, and poly(acrylic) acid is a representative poly(carboxylic acid).

[0021] The polymer backbone of the water-soluble polymer can be poly(ethylene glycol) (i.e. PEG). However, it should be understood that other related polymers are also suitable for use in the practice of this invention and that the use of the term PEG or poly(ethylene glycol) is intended to be inclusive and not exclusive in this respect. The term PEG includes poly(ethylene glycol) in any of its forms, including alkoxy PEG, difunctional PEG, multiarmed PEG, forked PEG, branched PEG, pendent PEG (i.e. PEG or related polymers having one or more functional groups pendent to the polymer backbone), or PEG with degradable linkages therein.

[0022] The polymer backbone can be linear or branched. Branched polymer backbones are generally known in the art. Typically, a branched polymer has a central branch core moiety and a plurality of linear polymer chains linked to the central branch core. PEG is commonly used in branched forms that can be prepared by addition of ethylene oxide to various polyols, such as glycerol, pentaerythritol and sorbitol. The central branch moiety can also be derived from several amino acids, such as lysine. The branched poly(ethylene glycol) can be represented in general form as $R(\text{-PEG-OH})_m$ in which R represents the core moiety, such as glycerol or pentaerythritol, and m represents the number of arms. Multi-armed PEG molecules, such as those described in U.S. Pat.

No. 5,932,462, which is incorporated by reference herein in its entirety, can also be used as the polymer backbone.

[0023] Many other polymers are also suitable for the invention. Polymer backbones that are non-peptidic and water-soluble, with from 2 to about 300 termini, are particularly useful in the invention. Examples of suitable polymers include, but are not limited to, other poly(alkylene glycols), such as poly(propylene glycol) ("PPG"), copolymers of ethylene glycol and propylene glycol and the like, poly(oxyethylated polyol), poly(olefinic alcohol), poly(vinylpyrrolidone), poly(hydroxypropylmethacrylamide), poly(α-hydroxy acid), poly(vinyl alcohol), polyphosphazene, polyoxazoline, poly(N-acryloylmorpholine), such as described in U.S. Pat. No. 5,629,384, which is incorporated by reference herein in its entirety, and copolymers, terpolymers, and mixtures thereof. Although the molecular weight of each chain of the polymer backbone can vary, it is typically in the range of from about 100 Da to about 100,000 Da, often from about 6,000 Da to about 80,000 Da.

[0024] The term "targeting moiety," as used herein, refers to species that will selectively localize in a particular tissue or region of the body. The localization is mediated by specific recognition of molecular determinants, molecular size of the targeting agent or conjugate, ionic interactions, hydrophobic interactions and the like. Other mechanisms of targeting an agent to a particular tissue or region are known to those of skill in the art. Exemplary targeting moieties include antibodies, antibody fragments, transferrin, HS-glycoprotein, coagulation factors, serum proteins, β-glycoprotein, G-CSF, GM-CSF, M-CSF, EPO and the like.

[0025] As used herein, "therapeutic moiety" means any agent useful for therapy including, but not limited to, antibiotics, anti-inflammatory agents, anti-tumor drugs, cytotoxins, and radioactive agents. "Therapeutic moiety" includes prodrugs of bioactive agents, constructs in which more than one therapeutic moiety is bound to a carrier, e.g, multivalent agents. Therapeutic moiety also includes proteins and constructs that include proteins. Exemplary proteins include, but are not limited to, Erythropoietin (EPO), Granulocyte Colony Stimulating Factor (GCSF), Granulocyte Macrophage Colony Stimulating Factor (GMCSF), Interferon (e.g., Interferon-α, -β, -γ), Interleukin (e.g., Interleukin II), serum proteins (e.g., Factors VII, VIa, VIII, IX, and X), Human Chorionic Gonadotropin (HCG), Follicle Stimulating Hormone (FSH) and Lutenizing Hormone (LH) and antibody fusion proteins (e.g. Tumor Necrosis Factor Receptor ((TNFR)/Fc domain fusion protein)).

[0026] As used herein, "anti-tumor drug" means any agent useful to combat cancer including, but not limited to, cytotoxins and agents such as antimetabolites, alkylating agents, anthracyclines, antibiotics, antimitotic agents, procarbazine, hydroxyurea, asparaginase, corticosteroids, interferons and radioactive agents. Also encompassed within the scope of the term "anti-tumor drug," are conjugates of peptides with anti-tumor activity, e.g. TNF-α. Conjugates include, but are not limited to those formed between a therapeutic protein and a glycoprotein of the invention. A representative conjugate is that formed between PSGL-1 and TNF-α.

[0027] As used herein, "a cytotoxin or cytotoxic agent" means any agent that is detrimental to cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracinedione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Other toxins include, for example, ricin, CC-1065 and analogues, the duocarmycins. Still other toxins include diptheria toxin, and snake venom (e.g., cobra venom).

[0028] As used herein, "a radioactive agent" includes any radioisotope that is effective in diagnosing or destroying a tumor. Examples include, but are not limited to, indium-111, cobalt-60. Additionally, naturally occurring radioactive elements such as uranium, radium, and thorium, which typically represent mixtures of radioisotopes, are suitable examples of a radioactive agent. The metal ions are typically chelated with an organic chelating moiety.

[0029] Many useful chelating groups, crown ethers, cryptands and the like are known in the art and can be incorporated into the compounds of the invention (e.g., EDTA, DTPA, DOTA, NTA, HDTA, etc. and their phosphonate analogs such as DTPP, EDTP, HDTP, NTP, etc). See, for example, Pitt et al., "The Design of Chelating Agents for the Treatment of Iron Overload," In, INORGANIC CHEMISTRY IN BIOLOGY AND MEDICINE; Martell, Ed.; American Chemical Society, Washington, D.C., 1980, pp. 279-312; Lindoy, THE CHEMISTRY OF MACROCYCLIC LIGAND COMPLEXES; Cambridge University Press, Cambridge, 1989; Dugas, BIOORGANIC CHEMISTRY; Springer-Verlag, New York, 1989, and references contained therein.

[0030] Additionally, a manifold of routes allowing the attachment of chelating agents, crown ethers and cyclodextrins to other molecules is available to those of skill in the art. See, for example, Meares et al., "Properties of In Vivo Chelate-Tagged Proteins and Polypeptides." In, MODIFICATION OF PROTEINS: FOOD, NUTRITIONAL, AND PHARMACOLOGICAL ASPECTS;" Feeney, et al., Eds., American Chemical Society, Washington, D.C., 1982, pp. 370-387; Kasina et al., *Bioconjugate Chem.,* 9: 108-117 (1998); Song et al., *Bioconjugate Chem.,* 8: 249-255 (1997).

[0031] A compound is "substantially purified" from an undesired component in a solution if the concentration of the undesired component after purification is no greater than about 40% of the concentration of the component prior to purification. Preferably, the post-purification concentration of the undesired component will be less than about 20% by weight, and more preferably less than about 10%, and still more preferably less than about 5% of the pre-purification concentration.

[0032] The term "pharmaceutically pure," as used herein, refers to a compound that is sufficiently purified from undesired contaminants that the compound is suitable for administration as a pharmaceutical agent. Preferably, the compound is purified such that the undesired contaminant is present after purification in an amount that is about 5% by weight or less of the pre-purification concentration of the contaminant in the feed solution. More preferably, the post-purification concentration of the contaminant is about 1% or less of the pre-purification contaminant concentration, and most preferably about 0.5% or less of the pre-purification concentration of contaminant.

[0033] A "feed solution" refers to any solution that contains a compound to be purified. For example, a reaction mixture used to synthesize an oligosaccharide can be used as a feed solution from which the desired reaction product is purified using the methods of the invention.

[0034] Where substituent groups are specified by their conventional chemical formulae, written from left to right, they equally encompass the chemically identical substituents, which would result from writing the structure from right to left, e.g., $—CH_2O—$ is intended to also recite $—OCH_2—$.

[0035] The term "alkyl," by itself or as part of another substituent means, unless otherwise stated, a straight or branched chain, or cyclic hydrocarbon radical, or combination thereof, which may be fully saturated, mono- or polyunsaturated and can include di- and multivalent radicals, having the number of carbon atoms designated (i.e. C_1-C_{10} means one to ten carbons). Examples of saturated hydrocarbon radicals include, but are not limited to, groups such as methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl, isobutyl, sec-butyl, cyclohexyl, (cyclohexyl)methyl, cyclopropylmethyl, homologs and isomers of, for example, n-pentyl, n-hexyl, n-heptyl, n-octyl, and the like. An unsaturated alkyl group is one having one or more double bonds or triple bonds. Examples of unsaturated alkyl groups include, but are not limited to, vinyl, 2-propenyl, crotyl, 2-isopentenyl, 2-(butadienyl), 2,4-pentadienyl, 3-(1,4-pentadienyl), ethynyl, 1- and 3-propynyl, 3-butynyl, and the higher homologs and isomers. The term "alkyl," unless otherwise noted, is also meant to include those derivatives of alkyl defined in more detail below, such as "heteroalkyl." Alkyl groups that are limited to hydrocarbon groups are termed "homoalkyl".

[0036] The term "alkylene" by itself or as part of another substituent means a divalent radical derived from an alkane, as exemplified, but not limited, by $—CH_2CH_2CH_2CH_2—$, and further includes those groups described below as "heteroalkylene." Typically, an alkyl (or alkylene) group will have from 1 to 24 carbon atoms, with those groups having 10 or fewer carbon atoms being preferred in the present invention. A "lower alkyl" or "lower alkylene" is a shorter chain alkyl or alkylene group, generally having eight or fewer carbon atoms.

[0037] The terms "alkoxy," "alkylamino" and "alkylthio" (or thioalkoxy) are used in their conventional sense, and refer to those alkyl groups attached to the remainder of the molecule via an oxygen atom, an amino group, or a sulfur atom, respectively.

[0038] The term "heteroalkyl," by itself or in combination with another term, means, unless otherwise stated, a stable straight or branched chain, or cyclic hydrocarbon radical, or combinations thereof, consisting of the stated number of carbon atoms and at least one heteroatom selected from the group consisting of O, N, Si and S, and wherein the nitrogen and sulfur atoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. The heteroatom (s) O, N and S and Si may be placed at any interior position of the heteroalkyl group or at the position at which the alkyl group is attached to the remainder of the molecule. Examples include, but are not limited to, $—CH_2—CH_2—O—CH_3$, $—CH_2—CH_2—NH—CH_3$, $—CH_2—CH_2—N(CH_3)—CH_3$, $—CH_2—S—CH_2—CH_3$, $—CH_2—CH_2$, $—S(O)—CH_3$, $—CH_2—CH_2—S(O)_2—CH_3$, $—CH{=}CH—O—CH_3$, $—Si(CH_3)_3$, $—CH_2—CH{=}N—OCH_3$, and $—CH{=}CH—N(CH_3)—CH_3$. Up to two heteroatoms may be consecutive, such as, for example, $—CH_2—NH—OCH_3$ and $—CH_2—O—Si(CH_3)_3$. Similarly, the term "heteroalkylene" by itself or as part of another substituent means a divalent radical derived from heteroalkyl, as exemplified, but not limited by, $—CH_2—CH_2—S—CH_2—CH_2—$ and $—CH_2—S—CH_2—CH_2—NH—CH_2—$. For heteroalkylene groups, heteroatoms can also occupy either or both of the chain termini (e.g., alkyleneoxy, alkylenedioxy, alkyleneamino, alkylenediamino, and the like). Still further, for alkylene and heteroalkylene linking groups, no orientation of the linking group is implied by the direction in which the formula of the linking group is written. For example, the formula $—C(O)_2R'—$ represents both $—C(O)_2R'—$ and $—R'C(O)_2—$.

[0039] The terms "cycloalkyl" and "heterocycloalkyl", by themselves or in combination with other terms, represent, unless otherwise stated, cyclic versions of "alkyl" and "heteroalkyl", respectively. Additionally, for heterocycloalkyl, a heteroatom can occupy the position at which the heterocycle is attached to the remainder of the molecule. Examples of cycloalkyl include, but are not limited to, cyclopentyl, cyclohexyl, 1-cyclohexenyl, 3-cyclohexenyl, cycloheptyl, and the like. Examples of heterocycloalkyl include, but are not limited to, 1-(1,2,5,6-tetrahydropyridyl), 1-piperidinyl, 2-piperidinyl, 3-piperidinyl, 4-morpholinyl, 3-morpholinyl, tetrahydrofuran-2-yl, tetrahydrofuran-3-yl, tetrahydrothien-2-yl, tetrahydrothien-3-yl, 1-piperazinyl, 2-piperazinyl, and the like.

[0040] The terms "halo" or "halogen," by themselves or as part of another substituent, mean, unless otherwise stated, a fluorine, chlorine, bromine, or iodine atom. Additionally, terms such as "haloalkyl," are meant to include monohaloalkyl and polyhaloalkyl. For example, the term "halo(C_1-C_4)alkyl" is mean to include, but not be limited to, trifluoromethyl, 2,2,2-trifluoroethyl, 4-chlorobutyl, 3-bromopropyl, and the like.

[0041] The term "aryl" means, unless otherwise stated, a polyunsaturated, aromatic, substituent that can be a single ring or multiple rings (preferably from 1 to 3 rings), which are fused together or linked covalently. The term "heteroaryl" refers to aryl groups (or rings) that contain from one to four heteroatoms selected from N, O, and S, wherein the nitrogen and sulfur atoms are optionally oxidized, and the nitrogen atom(s) are optionally quaternized. A heteroaryl group can be attached to the remainder of the molecule through a heteroatom. Non-limiting examples of aryl and heteroaryl groups include phenyl, 1-naphthyl, 2-naphthyl, 4-biphenyl, 1-pyrrolyl, 2-pyrrolyl, 3-pyrrolyl, 3-pyrazolyl, 2-imidazolyl, 4-imidazolyl, pyrazinyl, 2-oxazolyl, 4-oxazolyl, 2-phenyl-4-oxazolyl, 5-oxazolyl, 3-isoxazolyl, 4-isoxazolyl, 5-isoxazolyl, 2-thiazolyl, 4-thiazolyl, 5-thiazolyl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrimidyl, 4-pyrimidyl, 5-benzothiazolyl, purinyl, 2-benzimidazolyl, 5-indolyl, 1-isoquinolyl, 5-isoquinolyl, 2-quinoxalinyl, 5-quinoxalinyl, 3-quinolyl, tetrazolyl, benzo[b]furanyl, benzo[b]thienyl, 2,3-dihydrobenzo[1,4]dioxin-6-yl, benzo [1,3]dioxol-5-yl and 6-quinolyl. Substituents for each of the above noted aryl and heteroaryl ring systems are selected from the group of acceptable substituents described below.

[0042] For brevity, the term "aryl" when used in combination with other terms (e.g., aryloxy, arylthioxy, arylalkyl) includes both aryl and heteroaryl rings as defined above. Thus, the term "arylalkyl" is meant to include those radicals in which an aryl group is attached to an alkyl group (e.g., benzyl, phenethyl, pyridylmethyl and the like) including those alkyl groups in which a carbon atom (e.g., a methylene group) has been replaced by, for example, an oxygen atom (e.g., phenoxymethyl, 2-pyridyloxymethyl, 3-(1-naphthyloxy)propyl, and the like).

[0043] Each of the above terms (e.g., "alkyl," "heteroalkyl," "aryl" and "heteroaryl") is meant to include both substituted and unsubstituted forms of the indicated radical. Preferred substituents for each type of radical are provided below.

[0044] Substituents for the alkyl and heteroalkyl radicals (including those groups often referred to as alkylene, alkenyl, heteroalkylene, heteroalkenyl, alkynyl, cycloalkyl, heterocycloalkyl, cycloalkenyl, and heterocycloalkenyl) are generically referred to as "alkyl group substituents," and they can be one or more of a variety of groups selected from, but not limited to: —OR', ═O, ═NR', ═N—OR', —NR'R", —SR', -halogen, —SiR'R"R'", —OC(O)R', —C(O)R', —CO$_2$R', —CONR'R", —OC(O)NR'R", —NR"C(O)R', —NR'—C(O)NR"R'", —NR"C(O)$_2$R', —NR—C(NR'R"R'")═NR"", —NR—C(NR'R")═NR'", —S(O)R', —S(O)$_2$R', —S(O)$_2$NR'R", —NRSO$_2$R', —CN and —NO$_2$ in a number ranging from zero to (2m'+1), where m' is the total number of carbon atoms in such radical. R', R", R'" and R"" each preferably independently refer to hydrogen, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, e.g., aryl substituted with 1-3 halogens, substituted or unsubstituted alkyl, alkoxy or thioalkoxy groups, or arylalkyl groups. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R", R'" and R"" groups when more than one of these groups is present. When R' and R" are attached to the same nitrogen atom, they can be combined with the nitrogen atom to form a 5-, 6-, or 7-membered ring. For example, —NR'R" is meant to include, but not be limited to, 1-pyrrolidinyl and 4-morpholinyl. From the above discussion of substituents, one of skill in the art will understand that the term "alkyl" is meant to include groups including carbon atoms bound to groups other than hydrogen groups, such as haloalkyl (e.g., —CF$_3$ and —CH$_2$CF$_3$) and acyl (e.g., —C(O)CH$_3$, —C(O)CF$_3$, —C(O)CH$_2$OCH$_3$, and the like).

[0045] Similar to the substituents described for the alkyl radical, substituents for the aryl and heteroaryl groups are generically referred to as "aryl group substituents." The substituents are selected from, for example: halogen, —OR', ═O, ═NR', ═N—OR', —NR'R", —SR', -halogen, —SiR'R"R'", —OC(O)R', —C(O)R', —CO$_2$R', —CONR'R", —OC(O)NR'R", —NR"C(O)R', —NR'—C(O)NR"R'", —NR"C(O)$_2$R', —NR—C(NR'R"R'")═NR"", —NR—C(NR'R")═NR'", —S(O)R', —S(O)$_2$R', —S(O)$_2$NR'R", —NRSO$_2$R', —CN and —NO$_2$, —R', —N$_3$, —CH(Ph)$_2$, fluoro(C$_1$-C$_4$)alkoxy, and fluoro(C$_1$-C$_4$)alkyl, in a number ranging from zero to the total number of open valences on the aromatic ring system; and where R', R", R'" and R"" are preferably independently selected from hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl and substituted or unsubstituted heteroaryl. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R", R'" and R"" groups when more than one of these groups is present. In the schemes that follow, the symbol X represents "R" as described above.

[0046] Two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula -T-C(O)—(CRR')$_q$—U—, wherein T and U are independently —NR—, —O—, —CRR'— or a single bond, and q is an integer of from 0 to 3. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula -A-(CH$_2$)$_r$—B—, wherein A and B are independently —CRR'—, —O—, —NR—, —S—, —S(O)—, —S(O)$_2$—, —S(O)$_2$NR'— or a single bond, and r is an integer of from 1 to 4. One of the single bonds of the new ring so formed may optionally be replaced with a double bond. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula —(CRR')$_s$—X—(CR"R'")$_d$—, where s and d are independently integers of from 0 to 3, and X is —O—, —NR'—, —S—, —S(O)—, —S(O)$_2$—, or —S(O)$_2$NR'—. The substituents R, R', R" and R'" are preferably independently selected from hydrogen or substituted or unsubstituted (C$_1$-C$_6$)alkyl.

[0047] As used herein, the term "heteroatom" is meant to include oxygen (O), nitrogen (N), sulfur (S) and silicon (Si).

EMBODIMENTS OF THE INVENTION

[0048] The present invention provides methods for rapidly and efficiently purifying specific carbohydrate and oligosaccharide structures to a high degree of purity using semipermeable membranes such as reverse osmosis and/or nanofiltration membranes. The methods are particularly useful for separating desired oligosaccharide compounds from reactants and other contaminants that remain in a reaction mixture after synthesis or breakdown of the oligosaccharides. For example, the invention provides methods for separating oligosaccharides from enzymes and/or other components of reaction mixtures used for enzymatic synthesis or enzymatic degradation of oligosaccharides, nucleotide sugars, glycolipids, liposaccharides, nucleotides, nucleosides, and other saccharide-containing compounds. Also provided are methods for removing salts, sugars and other components from feed solutions using ultrafiltration, nanofiltration or reverse osmosis. Using these techniques, the saccharides (e.g., sialyl lactose, SLe^x, and many others) can be produced at up to essentially 100% purity. Moreover, the purification methods of the invention are more efficient, rapid, and amenable to large-scale purifications than previously known carbohydrate purification methods.

[0049] Often, a desired purification can be effected in a single step; additional purification steps such as crystallization and the like are generally not required. Accordingly, the invention provides single-step methods for purifying saccharide-containing compounds.

[0050] To purify saccharides according to the methods of the invention, a membrane is selected that is appropriate for separating the desired carbohydrate from the undesired components (contaminants) of the solution from which the carbohydrate is to be purified. The goal in selecting a membrane is to optimize for a particular application the molecular weight cutoff (MWCO), membrane composition, permeability, and rejection characteristics, that is, the membrane's total capacity to retain specific molecules while allowing other species, e.g., salts and other, generally smaller or opposite charged molecules, to pass through. The percent retention of a component i (R_i) is given by the formula $R_i=(1-C_{ip}/C_{ir})\times 100\%$, wherein C_{ip} is the concentration of component i in the permeate and C_{ir} is the concentration of component i in the retentate, both expressed in weight percent. The percent retention of a component is also called the retention characteristic or the membrane rejection coefficient.

[0051] In and exemplary embodiment, a membrane is chosen that has a high rejection ratio for the saccharide of interest relative to the rejection ratio for compounds from which separation is desired. If a membrane has a high rejection ratio for a first compound relative to a second compound, the concentration of the first compound in the permeate solution which passes through the membrane is decreased relative to that of the second compound. Conversely, the concentration of the first compound increases relative to the concentration of the second compound in the retentate. If a membrane does not reject a compound, the concentration of the compound in both the permeate and the reject portions will remain essentially the same as in the feed solution. It is also possible for a membrane to have a negative rejection rate for a compound if the compound's concentration in the permeate becomes greater than the compound's concentration in the feed solution. A general review of membrane technology is found in "Membranes and Membrane Separation Processes," in Ullmann's *Encyclopedia of Industrial Chemistry* (VCH, 1990); see also, Noble and Stem, *Membrane Separations Technology: Principles and Applications* (Elsevier, 1995).

[0052] As a starting point, one will generally choose a membrane having a molecular weight cut-off (MWCO, which is often related to membrane pore size) that is expected to retain the desired compounds while allowing an undesired compound present in the feed stream to pass through the membrane. The desired MWCO is generally less than the molecular weight of the compound being purified, and is typically greater than the molecular weight of the undesired contaminant that is to be removed from the solution containing the compound being purified. For example, to purify a compound having a molecular weight of 200 Da, one would choose a membrane that has a MWCO of less than about 200 Da. A membrane with a MWCO of 100 Da, for example, would also be a suitable candidate. The membranes that find use in the present invention are classified in part on the basis of their MWCO as ultrafiltration (UF) membranes, nanofiltration (NF) membranes, or reverse osmosis (RO) membranes, depending on the desired separation. For purposes of this invention, UF, NF, and RO membranes are classified as defined in the *Pure Water Handbook*, Osmonics, Inc. (Minnetonka Minn.). RO membranes typically have a nominal MWCO of less than about 200 Da and reject most ions, NF membranes generally have a nominal MWCO of between about 150 Da and about 5 kDa, and UF membranes generally have a nominal MWCO of between about 1 kDa and about 300 kDa (these MWCO ranges assume a saccharide-like molecule).

[0053] A second parameter that is considered in choosing an appropriate membrane for a particular separation is the polymer type of the membrane. Exemplary membranes of use in the invention are made of conventional membrane material whether inorganic, organic, or mixed inorganic and organic. Typical inorganic materials include glasses, ceramics, cermets, metals and the like. Ceramic membranes, which are preferred for the UF zone, may be made, for example, as described in U.S. Pat. No. 4,692,354 to Asaeda et al, U.S. Pat. No. 4,562,021 to Alary et al., and others. The organic materials which are preferred for the NF and RO applications, are typically polymers, whether isotropic, or anisotropic with a thin layer or "skin" on either the bore side or the shell side of the fibers. Preferred materials for fibers are polyamides, polybenzamides, polysulfones (including sulfonated polysulfone and sulfonated polyether sulfone, among others), polystyrenes, including styrene-containing copolymers such as acrylo-nitrile-styrene, butadiene-styrene and styrene-vinylbenzylhalide copolymers, polycarbonates, cellulosic polymers including cellulose acetate, polypropylene, poly(vinyl chloride), poly(ethylene terephthalate), polyvinyl alcohol, fluorocarbons, and the like, such as those disclosed in U.S. Pat. Nos. 4,230,463, 4,806,244, and 4,259,183. The NF and RO membranes often consist of a porous support substrate in addition to the polymeric discrimination layer.

[0054] Of particular importance in selecting a suitable membrane composition is the membrane surface charge. Within the required MWCO range, a membrane is selected that has a surface charge that is appropriate for the ionic charge of the carbohydrate and that of the contaminants. While MWCO for a particular membrane is generally invariable, changing the pH of the feed solution can affect separation properties of a membrane by altering the membrane surface charge. For example, a membrane that has a net negative surface charge at neutral pH can be adjusted to have a net neutral charge simply by lowering the pH of the solution. An additional effect of adjusting solution pH is to modulate the ionic charge on the contaminants and on the carbohydrate of interest. Therefore, by choosing a suitable membrane polymer type and pH, one can obtain a system in which both the contaminant and the membrane are neutral, facilitating pass-through of the contaminant. If, for instance, a contaminant is negatively charged at neutral pH, it is often desirable to lower the pH of the feed solution to protonate the contaminant. For example, removal of phosphate is facilitated by lowering the pH of the solution to about 3, which protonates the phosphate anion, allowing passage through a membrane. For purification of an anionic carbohydrate, the pH will generally between about pH 1 and about pH 7. Conversely, if contaminant has a positive surface charge, the pH of the feed solution can be adjusted to between about pH 7 and about pH 14. For example, increasing the pH of a solution containing a contaminant having an amino group ($-NH_3^+$) will make the amino group neutral, thus facilitating its passage through the membrane. Thus, one aspect of the invention involves modulating a separation by adjusting the pH of a solution in contact with the membrane; this can change the ionic charge of a contaminant and can also affect the surface charge of the membrane, thus facilitating purification if the desired carbohydrate. Of course, the manufacturer's instructions must be followed as to acceptable pH range for a particular membrane to avoid damage to the membrane.

[0055] For some applications, a mixture is first subjected to nanofiltration or reverse osmosis at one pH, after which the retentate containing the saccharide of interest is adjusted to a different pH and subjected to an additional round of membrane purification. For example, filtration of a reaction mixture used to synthesize sialyl lactose through an Osmonics MX07 membrane (a nanofiltration membrane having a MWCO of about 500 Da) at pH 3.0 will retain the sialyl lactose and remove most phosphate, pyruvate, salt and manganese from the solution, while also removing some of the GlcNAc, lactose, and sialic acid. Further recirculation through the MXO7 membrane after adjusting the pH of the retentate to 7.4 will remove most of the remaining phosphate, all of the pyruvate, all of the lactose, some of the sialic acid, and substantial amounts of the remaining manganese.

[0056] If a saccharide is to be purified from a mixture that contains proteins, such as enzymes used to synthesize a desired oligosaccharide or nucleotide sugar, it is often desirable to remove the proteins as a first step of the purification procedure. For a saccharide that is smaller than the proteins, this separation is accomplished by choosing a membrane that has an MWCO which is less than the molecular mass of the protein or other macromolecule to be removed from the solution, but is greater than the molecular mass of the oligosaccharide being purified (i.e., the rejection ratio in this case is higher for the protein than for the desired saccharide). Proteins and other macromolecules that have a molecular mass greater than the MWCO will thus be rejected by the membrane, while the saccharide will pass through the membrane. Conversely, if an oligosaccharide or nucleotide sugar is to be purified from proteins that are smaller than the oligosaccharide or nucleotide sugar, a membrane is used that has a MWCO that is larger than the molecular mass of the protein but smaller than that of the oligosaccharide or nucleotide sugar. Generally, separation of proteins from carbohydrates will employ membranes that are commonly referred to as ultrafiltration (UF) membranes. UF membranes that are suitable for use in the methods of the invention are available from several commercial manufacturers, including Millipore Corp. (Bedford, Mass.), Osmonics, Inc. (Minnetonka, Minn.), Filmtec (Minneapolis, Minn.), UOP, Desalination Systems, Advanced Membrane Technologies, and Nitto.

[0057] The invention also provides methods for removing salts and other low molecular weight components from a mixture containing a saccharide of interest by using a nanofiltration (NF) or a reverse osmosis (RO) membrane. Nanofiltration membranes are a class of membranes for which separation is based both on molecular weight and ionic charge. These membranes typically fall between reverse osmosis and ultrafiltration membranes in terms of the size of species that will pass through the membrane. Nanofiltration membranes typically have micropores or openings between chains in a swollen polymer network. Molecular weight cut-offs for non-ionized molecules are typically in the range from 100-20,000 Daltons. For ions of the same molecular weight, membrane rejections (retentions) will increase progressively for ionic charges of 0, 1, 2, 3 etc. for a particular membrane because of increasing charge density (see, e.g., Eriksson, P., "Nanofiltration Extends the Range of Membrane Filtration," *Environmental Progress,* 7: 58-59 (1988)). Nanofiltration is also described in *Chemical Engineering Progress*, pp. 68-74 (March 1994), Rautenbach et al., *Desalination* 77: 73 (1990), and U.S. Pat. No. 4,806,244). In a typical application, saccharides of interest will be retained by the nanofiltration membrane and contaminating salts and other undesired components will pass through. A nanofiltration membrane useful in the methods of the invention will typically have a retention characteristic for the saccharide of interest of from about 40% to about 100%, preferably from about 70% to about 100%, more preferably from about 90% to about 100%. The nanofilter membranes used in the invention can be any one of the conventional nanofilter membranes, with polyamide membranes being particularly suitable. Several commercial manufacturers, including Millipore Corp. (Bedford, Mass.), Osmonics, Inc. (Minnetonka, Minn.), Filmtec, UOP, Advanced Membrane Technologies, Desalination Systems, and Nitto, among others, distribute nanofiltration membranes that are suitable for use in the methods of the invention. For example, suitable membranes include the Osmonics MX07, YK, GH (G-10), GE (G-5), and HL membranes, among others.

[0058] Reverse osmosis (RO) membranes also allow a variety of aqueous solutes to pass through them while retaining selected molecules. Generally, osmosis refers to a process whereby a pure liquid (usually water) passes through a semipermeable membrane into a solution (usually sugar or salt and water) to dilute the solution and achieve osmotic equilibrium between the two liquids. In contrast, reverse osmosis is a pressure driven membrane process wherein the application of external pressure to the membrane system results in a reverse flux with the water molecules passing from a saline or sugar solution compartment into the pure water compartment of the membrane system. A RO membrane, which is semipermeable and non-porous, requires an aqueous feed to be pumped to it at a pressure above the osmotic pressure of the substances dissolved in the water. An RO membrane can effectively remove low molecular weight molecules (<200 Daltons) and also ions from water. Preferably, the reverse osmosis membrane will have a retention characteristic for the saccharide of interest of from about 40% to about 100%, preferably from about 70% to about 100%, and more preferably from about 90% to about 100%. Suitable RO membranes include, but are not limited to, the Filmtec BW-30, Filmtec SW-30, Filmtec SW-30HR, UOP RO membranes, Desal RO membranes, Osmonics RO membranes, Advanced Membrane Technologies RO membranes, and the Nitto RO membranes, among others. One example of a suitable RO membrane is Millipore Cat. No. CDRN500 60 (Millipore Corp., Bedford Mass.).

[0059] The membranes used in the invention may be employed in any of the known membrane constructions. For example, the membranes can be flat, plate and frame, tubular, spiral wound, hollow fiber, and the like. In a preferred embodiment, the membrane is spiral wound. The membranes can be employed in any suitable configuration, including either a cross-flow or a depth configuration. In "cross-flow" filtration, which is preferred for ultrafiltration, nanofiltration and reverse osmosis purifications according to the invention, the "feed" or solution from which the carbohydrate of interest is to be purified flows through membrane channels, either parallel or tangential to the membrane surface, and is separated into a retentate (also called recycle or concentrate) stream and a permeate stream. To maintain an efficient membrane, the feed stream should flow, at a sufficiently high velocity, parallel to the membrane surface to create shear forces and/or turbulence to sweep away accumulating particles rejected by the membrane. Cross-flow filtration thus entails the flow of three streams—feed, permeate and retentate. In contrast, a "dead end" or "depth" filter has only two streams—feed and filtrate (or permeate). The recycle or retentate stream, which retains all the particles and large molecules rejected by the membrane, can be entirely recycled to the membrane module in which the recycle stream is generated, or can be partially removed from the system. When the methods of the invention are used to purify saccharides from lower molecular weight components, for example, the desired saccharides are contained in the retentate stream (or feed stream, for a depth filter), while the permeate stream contains the removed contaminants.

[0060] The purification methods of the invention can be further optimized by adjusting the pressure, flow rate, and temperature at which the filtration is carried out. UF, NF, and RO generally require increasing pressures above ambient to overcome the osmotic pressure of the solution being passed through the membrane. The membrane manufacturers' instructions as to maximum and recommended operating pressures can be followed, with further optimization possible by making incremental adjustments. For example, the recommended pressure for UF will generally be between about 25 and about 100 psi, for NF between about 50 psi and about 1500 psi, and for RO between about 100 and about 1500 psi. Flow rates of both the concentrate (feed solution) and the permeate can also be adjusted to optimize the desired purification. Again, the manufacturers' recommendations for a particular membrane serve as a starting point from which to begin the optimization process by making incremental adjustments. Typical flow rates for the concentrate (P_c) will be between about 1 and about 15 gallons per minute (GPM), and more preferably between about 3 and about 7 GPM. For the permeate, flow rates (Pf) of between about 0.05 GPM and about 10 GPM are typical, with flow rates between about 0.2 and about 1 GPM being preferred. The temperature at which the purification is carried out can also influence the efficiency and speed of the purification. Temperatures of between about 0 and about 100° C. are typical, with temperatures between about 20 and 40° C. being preferred for most applications. Higher temperatures can, for some membranes, result in an increase in membrane pore size, thus providing an additional parameter that one can adjust to optimize a purification.

[0061] In a preferred embodiment, the filtration is performed in a membrane purification machine which provides a means for automating control of flow rate, pressure, temperature, and other parameters that can affect purification. For example, the Osmonics 213T membrane purification machine is suitable for use in the methods of the invention, as are machines manufactured by other companies listed above.

[0062] The membranes can be readily cleaned either after use or after the permeability of the membrane diminishes. Cleaning can be effected at a slightly elevated temperature if so desired, by rinsing with water or a caustic solution. If the streams contain small amounts of enzyme, rinsing in the presence of small amounts of surfactant, for instance ULTRASIL, is useful. Also, one can use prefilters (100-200 μm) to protect the more expensive nanofiltration membranes. Other cleaning agents can, if desired, be used. The choice of cleaning method will depend on the membrane being cleaned, and the membrane manufacturer's instructions should be consulted. The cleaning can be accomplished with a forward flushing or a backward flushing.

[0063] The purification methods of the invention can be used alone or in combination with other methods for purifying carbohydrates. For example, an ion exchange resin can be used to remove particular ions from a mixture containing a saccharide of interest, either before or after nanofiltration/reverse osmosis, or both before and after filtration. Ion exchange is particularly desirable if it is desired to remove ions such as phosphate and nucleotides that remain after a first round of nanofiltration or reverse osmosis. In the case of sialyl lactose synthesis as discussed above, this can be accomplished, for example, by adding an anion exchange resin such as AG1X-8 (acetate form, BioRad; see, e.g., BioRad catalog for other ion exchange resins) to a retentate that is at about pH 3.0 or lower until the phosphate concentration is reduced as desired. In this process, acetic acid is released, so one may wish to follow the ion exchange with an additional purification through the nanofiltration or reverse osmosis system. For example, one can circulate the pH 3.0 or lower solution through an Osmonics MX07 or similar membrane until the conductivity of the permeate is low and stabilized. The pH of the solution can then be raised to 7.4 with NaOH and the solution recirculated through the same membrane to remove remaining sodium acetate and salt. Cations can be removed in a similar manner; for example, to remove Mn^{2+}, an acidic ion exchange resin can be used, such as AG50WX8 (He) (BioRad).

[0064] The purification methods of the invention are particularly useful for purifying oligosaccharides, modified saccharides, nucleotide sugars and modified nucleotide sugars prepared using enzymatic synthesis. Enzymatic synthesis using glycosyltransferases provides a powerful method for preparing oligosaccharides; for some applications it is desirable to purify the oligosaccharide from the enzymes and other reactants in the enzymatic synthesis reaction mixture. Preferred methods for producing many oligosaccharides involve glycosyl transferase cycles, which produce at least one mole of inorganic pyrophosphate for each mole of product formed and are typically carried out in the presence of a divalent metal ion. Examples of glycosyltransferase cycles are the sialyltransferase cycles, which use one or more enzymes as well as other reactants. See, e.g., U.S. Pat. No. 5,374,541 WO 9425615 A, PCT/US96/04790, and PCT/US96/04824. For example, a reaction used for synthesis of sialylated oligosaccharides can contain a sialyltransferase (FIG. **7**), a CMP-sialic acid synthetase, a sialic acid, an acceptor for the sialyltransferase, CTP, and a soluble divalent metal cation. An exemplary α(2,3)sialyltransferase referred to as α(2,3)sialtransferase (EC 2.4.99.6) transfers sialic acid to the non-reducing terminal Gal of a Galβ163Glc disaccharide or glycoside. See, Van den Eijnden et al., *J. Biol. Chem.*, 256:3159 (1981), Weinstein et al., *J. Biol. Chem.*, 257:13845 (1982) and Wen et al, *J. Biol. Chem.*, 267:21011 (1992). Another exemplary α2,3-sialyltransferase (EC 2.4.99.4) transfers sialic acid to the non-reducing terminal Gal of the disaccharide or glycoside. See, Rearick et al., *J. Biol. Chem.*, 254:4444 (1979) and Gillespie et al., *J. Biol. Chem.*, 267:21004 (1992). Further exemplary enzymes include Gal-β-1,4-GlcNAc α-2,6 sialyltransferase (See, Kurosawa et al. *Eur. J. Biochem.* 219: 375-381 (1994)). The reaction mixture will also contain an acceptor for the sialyltransferase, preferably having a galactosyl unit. Suitable acceptors, include, for example, Galβ1→3 GalNAc, lacto-N-tetraose, Galβ1→3 GlcNAc, Galβ1→3Ara, Galβ1→6GlcNAc, Galβ1→4Glc (lactose), Galβ1→4Glcβ1-OCH_2CH_3, Galβ1→4Glcβ1-$OCH_2CH_2CH_3$, Galβ1→4Glcβ1-$OCH_2C_6H_5$, Galβ1→4GlcNAc, Galβ1-OCH_3, melibiose, raffinose, stachyose, and lacto-N-neotetraose (LNnT). The sialic acid present in the reaction mixture can include not only sialic acid itself (5-N-acetylneuraminic acid; 5-N-acetylamino-3,5-dideoxy-D-glycero-D-galacto-2-nonulosonic acid; NeuAc, and sometimes also abbreviated AcNeu or NANA), but also 9-substituted sialic acids such as a 9-O—C_1-C_6 acyl-NeuAc like 9-O-lactyl-NeuAc or 9-O-acetyl-NeuAc, 9-deoxy-9-fluoro-NeuAc and 9-azido-9-deoxy-NeuAc. The synthesis and use of these compounds in a sialylation procedure is described in international application WO 92/16640, published Oct. 1, 1992.

[0065] In preferred embodiments the reaction medium can further comprise a CMP-sialic acid recycling system comprising at least 2 moles of phosphate donor per each mole of sialic acid, and catalytic amounts of an adenine nucleotide, a kinase capable of transferring phosphate from the phosphate donor to nucleoside diphosphates, and a nucleoside monophosphate kinase capable of transferring the terminal phosphate from a nucleoside triphosphate to CMP. For example, a suitable CMP-sialic acid regenerating system comprises cytidine monophosphate (CMP), a nucleoside triphosphate (for example adenosine triphosphate (ATP), a phosphate donor (for example, phosphoenolpyruvate or acetyl phosphate), a kinase (for example, pyruvate kinase or acetate kinase) capable of transferring phosphate from the phosphate donor to nucleoside diphosphates and a nucleoside monophosphate kinase (for example, myokinase) capable of transferring the terminal phosphate from a nucleoside triphosphate to CMP. The previously discussed α(2,3)sialyltransferase and CMP-sialic acid synthetase can also be formally viewed as part of the CMP-sialic acid regenerating system. For those embodiments in which a CMP-sialic acid recycling system is not used, the reaction medium will preferably further comprise a phosphatase.

[0066] Pyruvate is a byproduct of the sialyltransferase cycle and can be made use of in another reaction in which N-acetylmannosamine (ManNAc) and pyruvate are reacted in the presence of NeuAc aldolase (EC 4.1.3.3) to form sialic acid. Alternatively, advantage can be taken of the isomerization of GlcNAc to ManNAc, and the less expensive GlcNAc can be used as the starting material for sialic acid generation. Thus, the sialic acid can be replaced by ManNAc (or GlcNAc) and a catalytic amount of NeuAc aldolase. Although NeuAc aldolase also catalyzes the reverse reaction (NeuAc to ManNAc and pyruvate), the produced NeuAc is irreversibly incorporated into the reaction cycle via CMP-NeuAc catalyzed by CMP-sialic acid synthetase. In addition, the starting material, ManNAc, can also be made by the chemical conversion of GlcNAc using methods known in the art (see, e.g., Simon et al., *J. Am. Chem. Soc.* 110:7159 (1988). The enzymatic synthesis of sialic acid and its 9-substituted derivatives and the use of a resulting sialic acid in a different sialylating reaction scheme is disclosed in International application WO 92/16640, published on Oct. 1, 1992, and incorporated herein by reference.

[0067] When a galactosyltransferase is used for enzymatic synthesis of an oligosaccharide, the reaction medium will preferably contain, in addition to a galactosyltransferase, donor substrate, acceptor sugar and divalent metal cation, a donor substrate recycling system comprising at least 1 mole of glucose-1-phosphate per each mole of acceptor sugar, a phosphate donor, a kinase capable of transferring phosphate from the phosphate donor to nucleoside diphosphates, and a pyrophosphorylase capable of forming UDP-glucose from UTP and glucose-1-phosphate and catalytic amounts of UDP and a UDP-galactose-4-epimerase. Exemplary galactosyltransferases include α(1,3) galactosyltransferase (E.C. No. 2.4.1.151, see, e.g., Dabkowski et al., *Transplant Proc.* 25: 2921 (1993) and Yamamoto et al., *Nature* 345:229-233 (1990)) and β(1,4) galactosyltransferase (E.C. No. 2.4.1.38).

[0068] Oligosaccharides synthesized by other enzymatic methods can also be purified by the methods of the invention. For example, the methods are useful for purifying oligosaccharides produced in non-cyclic or partially cyclic reactions such as simple incubation of an activated saccharide and an appropriate acceptor molecule with a glycosyltransferase under conditions effective to transfer and covalently bond the saccharide to the acceptor molecule. Glycosyltransferases, which include those described in, e.g., U.S. Pat. No. 5,180,674, and International Patent Publication Nos. WO 93/13198 and WO 95/02683, as well the glycosyltransferases encoded by the los locus of *Neisseria* (see, U.S. Pat. No. 5,545,553), can be bound to a cell surface or unbound. Oligosaccharides that can be obtained using these glycosyltransferases include, for example,

Galα(1→4)Galβ(1→4)Glc, GlcNAcβ(1,3)Galβ(1,4)Glc,

Galβ(1→4)GlcNAcβ(1→3)Galβ(1→4)Glc, and

[0069] GalNAcβ(1→3)Galβ(1→4)GlcNAcβ(1→3) Galβ(1→4)Glc, among many others.

[0070] Among the compounds that one can purify using the described methods are sialic acid and any sugar having a sialic acid moiety. Exemplary species include sialic acid species modified with a linker (e.g., glycyl sialic acid) and with a polymer (e.g., poly(ethylene glycol). Other compounds include sialyl galactosides, including the sialyl lactosides, as well as compounds having the formula:

NeuAcα(2→3)Galβ(1→4)GlcN(R')β-OR or

NeuAcα(2→3)Galβ(3→4)GlcN(R')β(1→3)Galβ-OR.

[0071] In these formulae, R' is alkyl or acyl from 1-18 carbons, 5,6,7,8-tetrahydro-2-naphthamido; benzamido; 2-naphthamido; 4-aminobenzamido; or 4-nitrobenzamido. R is a hydrogen, a alkyl C_1-C_6, a saccharide, an oligosaccharide or an aglycon group having at least one carbon atom. The term "aglycon group having at least one carbon atom" refers to a group -A-Z, in which A represents an alkylene group of from 1 to 18 carbon atoms optionally substituted with halogen, thiol, hydroxy, oxygen, sulfur, amino, imino, or alkoxy; and Z is hydrogen, —OH, —SH, —NH_2, —NHR^1, —$N(R^1)_2$, —CO_2H, —CO_2R^1, —$CONH_2$, —$CONHR^1$, —$CON(R^1)_2$, —$CONHNH_2$, or —OR^1 wherein each R^1 is independently alkyl of from 1 to 5 carbon atoms. In addition, R can be:

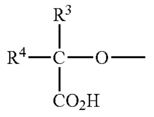
$$(CH_2)_nCH(CH_2)_mCH_3$$

where n, m, and o are independently selected from the integers 1-18; $(CH_2)_n$—R^2 (in which n=0-18), wherein R^2 is a variously substituted aromatic ring, preferably, a phenyl group, being substituted with one or more alkoxy groups, preferably methoxy or $O(CH_2)_mCH_3$, (in which m=0-18), or a combination thereof. R can also be 3-(3,4,5-trimethoxyphenyl)propyl.

[0072] The present invention is also useful for purifying a variety of compounds that comprise selectin-binding carbohydrate moieties. These selectin-binding moieties have the general formula:

$$R^1Gal\beta1,m(Fuc\alpha1,n)GlcNR^0(R^2)_p—$$

in which R^0 is (C_1-C_8 alkyl)carbonyl, (C_1-C_8 alkoxy)carbonyl, or (C_2-C_9 alkenyloxy)carbonyl, R^1 is an oligosaccharide or a group having the formula:



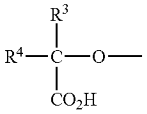
$$R^4-\underset{\displaystyle CO_2H}{\overset{\displaystyle R^3}{C}}-O-$$

[0073] R^3 and R^4 may be the same or different and may be H, C_1-C_8 alkyl, hydroxy-(C_1-C_8 alkyl), aryl-(C_1-C_8 alkyl), or (C_1-C_8 alkoxy)-(C_1-C_8 alkyl), substituted or unsubstituted. R^2 may be H, C_1-C_8 alkyl, hydroxy-(C_1-C_8 alkyl), aryl-(C_1-C_8-alkyl), (C_1-C_8 alkyl)-aryl, alkylthio, α1,2Man, α1,6GalNAc, β1,3Galβ1,4Glc, α1,2Man-R^8, α1,6GalNAc-R^8, and β1,3Gal-R^8. R^8 may be H, C_1-C_8 alkyl, C_1-C_8 alkoxy, hydroxy-(C_1-C_8 alkyl), aryl-(C_1-C_8 alkyl), (C_1-C_8 alkyl)-aryl, or alkylthio. In the formula, m and n are integers and may be either 3 or 4; p may be zero or 1.

[0074] The substituted groups mentioned above may be substituted by hydroxy, hydroxy(C_1-C_4 alkyl), polyhydroxy (C_1-C_4 alkyl), alkanoamido, or hydroxyalknoamido substituents. Preferred substituents include hydroxy, polyhydroxy (C_3 alkyl), acetamido and hydroxyacetamido. A substituted radical may have more than one substitution, which may be the same or different.

[0075] For embodiments in which R^1 is an oligosaccharide, the oligosaccharide is preferably a trisaccharide. Preferred trisaccharides include NeuAcα2,3Galβ1,4GlcNAcβ1,3 or NeuGcα2,3Galβ1,4GlcNAcβ1,3.

[0076] For embodiments in which R^1 is the group having the formula


$$R^4-\overset{\overset{\large R^3}{|}}{\underset{\underset{\large CO_2H}{|}}{C}}-O-$$

[0077] R^3 and R^4 preferably form a single radical having the formula

$—R^5—$ or $—(R^6)_q—O—(R^7)_r—$

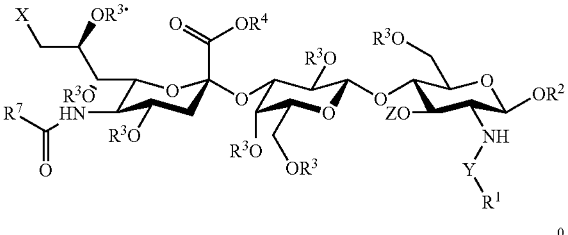
in which R^5 is C_3-C_7 divalent alkyl, substituted or unsubstituted, R^6 and R^7 are the same or different and are C_1-C_6 divalent alkyl, substituted or unsubstituted. In the formula, q and r are integers which may be the same or different and are either zero or 1. The sum of q and r is always at least 1.

[0078] A more preferred structure for a single radical formed by R^3 and R^4 is one having the formula

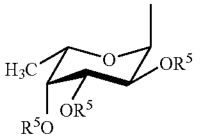
$—(R^6)—O—$

in which R^6 is C_3-C_4 divalent alkyl, substituted or unsubstituted. For instance, R^6 may have the formula $—CH_2—CH_2—CH_2—CH_2—$, preferably substituted. The radical can be substituted with hydroxy, polyhydroxy(C_3 alkyl), and substituted or unsubstituted alkanoamido groups, such as acetamido or hydroxyacetamido. The substituted structure will typically form a monosaccharide, preferably a sialic acid such as NeuAc or NeuGc linked α2,3 to the Gal residue.

[0079] In the general formula, above, both m and n are integers and can be either 3 or 4. Thus, in one set of structures Gal is linked β1,4 and Fuc is linked α1,3 to GlcNAc. This formula includes the SLe^x tetrasaccharide. SLe^x has the formula NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAcβ1-. This structure is selectively recognized by LECCAM-bearing cells. SLe^x compounds that can be purified using the methods of the invention include NeuAcα2,3 Galβ1,4(Fucα1,3)GlcNAcβ1-Gal-OEt, NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAcβ1,4Galβ1-OEt, and others that are described in international application WO 91/19502. Other compounds that one can purify using the methods include those described in U.S. Pat. No. 5,604,207 having the formula:



wherein Z is hydrogen, C_1-C_6 acyl or

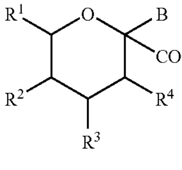


[0080] Y is selected from the group consisting of C(O), SO_2, HNC(O), OC(O) and SC(O). R^1 is selected from the group consisting of an aryl, a substituted aryl and a phenyl C_1-C_3 alkylene group, wherein said aryl substitutent is selected from the group consisting of a halo, trifluoromethyl, nitro, C_1-C_{18} alkyl, C_1-C_{18} alkoxy, amino, mono-C_1-C_{18} alkylamino, di-C_1-C_{18} alkylamino, benzylamino, C_1-C_{18} alkylbenzylamino, C_1-C_{18} thioaklyl and C_1-C_{18} alkyl carboxamido groups, or R^1Y is allyloxycarbonyl or chloroacetyl. R^2 is selected from the group consisting of monosaccharide (including β1,3Gal-OR, where R=H, alkyl, aryl or acyl), disaccharide, hydrogen, C_1-C_{18} straight chain, branched chain or cyclic hydrocarbyl, C_1-C_6 alkyl, 3-(3,4,5-trimethoxyphenyl)propyl, C_1-C_5 alkylene-carboxylate, trisubstituted silyl C_2-C_4 alkylene wherein said trisubstituted silyl is a silyl group having three substituents independently selected from the group consisting of C_1-C_4 alkyl, phenyl, or OR^2 together form a C_1-C_{18} straight chain, branched chain or cyclic hydrocarbyl carbamate; R^3 is hydrogen or C_1-C_6 acyl; R^4 is hydrogen, C_1-C_6 alkyl or benzyl. R^5 is selected from the group consisting of hydrogen, benzyl, methoxybenzyl, dimethoxybenzyl and C_1-C_6 acyl. R^7 is methyl or hydroxymethyl. X is selected from the group consisting of C_1-C_6 acyloxy, C_2-C_6 hydroxylacyloxy, hydroxy, halo and azido.

[0081] A related set of structures included in the general formula are those in which Gal is linked β1,3 and Fuc is linked α1,4. For instance, the tetrasaccharide, NeuAcα2,3Galβ1,3(Fucα1,4)GlcNAcβ1-, termed here SLe^a, is recognized by selectin receptors. See, Berg et al., *J. Biol. Chem.,* 266:14869-14872 (1991). In particular, Berg et al. showed that cells transformed with E-selectin cDNA selectively bound neoglycoproteins comprising SLe^a.

[0082] The methods of the invention are also useful for purifying oligosaccharide compounds having the general formula Galα1,3Gal-, including Galα1,3Galβ1,4Glc(R)β-O—R^1, wherein R^1 is $—(CH_2)_n—COX$, with X=OH, OR^2, $—NHNH_2$, R=OH or NAc, and R^2 is a hydrogen, a saccharide, an oligosaccharide or an aglycon group having at least one carbon atom, and n=an integer from 2 to 18, more preferably from 2 to 10. For example, one can purify a compound having the formula Galα1,3Galβ1,4GlcNAcβ-O—$(CH_2)_5$—COOH using procedures such as those described in Examples 7-8. Also among the compounds that can be purified according to the invention are lacto-N-neotetraose (LNnT), GlcNAcβ1,3Galβ1,4Glc (LNT-2), sialyl(α2,3)-lactose, and sialyl(α2,6)-lactose.

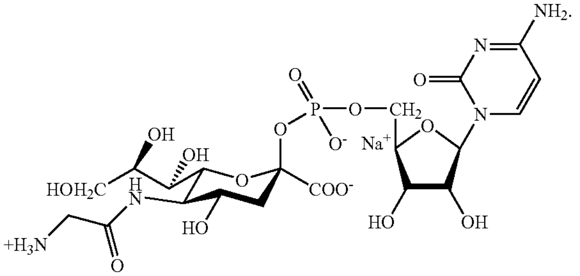
[0083] In one embodiment, a modified sialic acid has the following structure:



wherein, R^1, R^2, R^3 and R^4 are each independently selected from H, OR^5, NR^6R^7, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl and unsubstituted heteroalkyl. R^5 is H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl or unsubstituted heteroalkyl. The symbols R^6 and R^7 independently represent H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl and unsubstituted heteroalkyl. B is a nucleoside. Exemplary nucleosides include AMP, UMP, GMP, CMP, TMP, ADP, UDP, GDP, CDP, TDP, ATP, UTP, GTP, CTP, TTP, cAMP and cGMP.

[0084] In a preferred embodiment, the sialic acid is modified with a linker group. Preferred sites for such modification are R^1 or R^2. Thus, in a preferred embodiment, at least one of R^1 and R^2 includes a linker. An exemplary linker is a glycyl linker.

[0085] In a preferred embodiment, the modified sialic acid has the following structure:



[0086] In yet another preferred embodiment, a modifying group is attached to the sialic acid through the linker. An exemplary species according to this description includes a modifying group attached through the free amine moiety of the linker in the figure above. A presently preferred modifying group is a water-soluble polymer. Poly(ethylene glycol) is a preferred water-soluble polymer.

[0087] The methods of the invention are useful not only for purifying carbohydrates (and modified carbohydrates and nucleotide sugars) that that are newly synthesized, but also those that are the products of degradation, e.g., enzymatic degradation. See, e.g., Sinnott, M. L., *Chem. Rev.* 90: 1171-1202 (1990) for examples of enzymes that catalyze degradation of oligosaccharides.

[0088] The invention also provides methods for purifying nucleotides, nucleotide sugars, and related compounds. For example, a nucleotide sugar such as GDP-fucose, GDP-mannose, CMP-NeuAc, UDP-glucose, UDP-galactose, UDP-N-acetylgalactosamine, and the like, can be purified by the methods described herein. The methods are also useful for purifying nucleotides and nucleotides in various states of phosphorylation (e.g., CMP, CDP, CTP, GMP, GDP, GTP, TMP, TDP, TTP, AMP, ADP, ATP, UMP, UDP, UTP), as well as the deoxy forms of these and other nucleotides, including modified nucleotides. The method of the invention can be used to prepare and purify nucleotide sugars to a high degree of purity on a multi-kilogram scale (e.g., at least about 1 kg, preferably at least about 1.5 kg, more preferably at least about 2 kg, and even more preferably, at least about 3 kg of purified sugar nucleotide per synthesis/purification run). An exemplary process flow chart is set forth in FIG. **1**.

[0089] In the discussion that follows, focus is placed on the purification of nucleotide sugars. The methods set forth hereinbelow are equally applicable to the purification of sugars, modified sugars and modified nucleotides sugars (e.g., those bearing a linker arm (e.g., a glycl linker arm), a modifying group (e.g., a water-soluble polymer (e.g., PEG)), or a modifying group attached to the linker arm (e.g., PEG attached to the sugar through a glycyl linker).

[0090] The process of the invention routinely provides nucleotide sugars, e.g., CMP-NAN, in recovered yields of purified materials in greater that 40%, e.g., of from about 40% to about 80%. In a preferred embodiment, the yield of isolated CMP-NAN is from about 50% to about 70% of the theoretical synthesis yield.

[0091] In general, the process of the invention provides nucleotide sugars that are at least 80% pure, preferably at least 85% pure, more preferably, at least 90% pure and still more preferably, at least 95% pure.

[0092] In a representative embodiment, the nucleotide sugar is a CMP-sialic acid, e.g., CMP-NAN (N-acetylneuraminic acid). The generic process for purification of nucleotide sugars is exemplified in the context of CMP-sialic acid, however, this focus is for clarity of illustration and should not be construed as limiting the process to practice with CMP-sialic acids.

[0093] In a preferred embodiment, a membrane-based methodology is utilized to purify the nucleotide sugar from reaction components. In the case of CMP-sialic acid, exemplary reaction components include cytidine monophosphate and its active analogues, and cytidine diphosphate, unreacted sialic acid, salts (e.g., PO_4^{3-}, Mn^{2+}).

[0094] In a preferred embodiment, the amount of CMP, CDP and/or CTP of the product is less than about 20%, preferably, less than about 15%, more preferably, less than about 10% and still more preferably less than 5%.

[0095] In another preferred embodiment, the content of unreacted sialic acid, e.g., NAN, in the final product is less than about than about 20%, preferably, less than about 15%, more preferably, less than about 10%, still more preferably, less than about 5% and even more preferably, less than about 2%.

[0096] In yet another preferred embodiment, the phosphate content of the final product is less than about 5%, preferably, less than about 2%, and more preferably, about 0%.

[0097] The invention also provides methods for synthesizing and purifying nucleotide sugars. The nucleotide sugar is enzymatically synthesized from a nucleotide and a sugar in the presence of an enzyme. After the nucleotide sugar is synthesized, the nucleotide sugar is purified according to a method of the invention.

[0098] In one embodiment, following synthesis, a nucleotide sugar solution is optionally clarified by filtration. The nucleotide sugar solution passes through a membrane bag filter in which contaminating salts and other undesired contaminants are filtered out of the nucleotide sugar solution. The clarification step can be incorporated at any step of the process. In a preferred embodiment, the nucleotide sugar solution is clarified after synthesis of the nucleotide sugar. The nucleotide sugar solution may be clarified one or more times.

[0099] In another embodiment, the nucleotide sugar solution is purified using hollow fiber filtration. Hollow fiber filtration removes proteins introduced by the enzyme preparation of the nucleotide sugar. The hollow fiber membrane retains proteins from the enzyme preparation while allowing for passage of the nucleotide sugar solution through the membrane. In an exemplary embodiment, the hollow fiber membrane comprises a hollow fiber membrane with a tangential filtration skid. The hollow fiber filtration step can be incorporated at any step of the process. In one embodiment, the nucleotide sugar solution goes through hollow fiber filtration after clarification. In another embodiment, the nucleotide sugar solution goes through hollow fiber filtration after synthesis of the nucleotide sugar. The nucleotide sugar solution may be filtered one or more times using hollow fiber filtration.

[0100] In another embodiment, the nucleotide sugar solution is purified using nanofiltration. Nanofiltration removes salts and other low molecular weight components from a mixture. Nanofiltration membranes separate molecules based on molecular weight and ionic charge. Molecular weight-cutoffs for non-ionized molecules are typically in the range from 100-20,000 daltons. In an exemplary application, saccharides of interest will be retained by the nanofiltration membrane and contaminating salts and other undesired components will pass through. The nanofiltration step can be incorporated at any step of the process. In one embodiment, the nucleotide sugar solution goes through hollow-fiber filtration first and then nanofiltration. In another embodiment, the nucleotide sugar solution goes through nanofiltration first and then hollow fiber filtration. In the alternative, the nucleotide sugar solution may be purified using either hollow-fiber filtration or nanofiltration. In another embodiment, the nucleotide sugar solution goes through nanofiltration after clarification. In yet another embodiment, the nucleotide sugar solution goes through nanofiltration after synthesis of the nucleotide sugar. The nucleotide sugar solution may be filtered one or more times using nanofiltration. After nanofiltration, the purified nucleotide sugar solution may generally be stored or may undergo further purification.

[0101] In another embodiment, the nucleotide sugar solution may optionally be decolorized (e.g., by passing the solution over activate carbon). In a preferred embodiment, decolorization involves passing the nucleotide sugar solution over a pre-packed column of activated carbon attached to a chromatography system. Decolorization can be incorporated at any step of the process. In one embodiment, the nucleotide sugar solution is decolorized after nanofiltration. In another embodiment, the nucleotide sugar solution is decolorized after hollow-fiber filtration. In yet another embodiment, the nucleotide sugar solution is decolorized after clarification. The nucleotide sugar solution may be decolorized one or more times.

[0102] In another embodiment, the nucleotide sugar solution is purified using a charged depth media filter. The charged depth media filter removes endotoxins from the nucleotide sugar solution. Endotoxins are toxic, natural compounds such as lipopolysaccharides found inside pathogens on the outer cell wall of bacteria. Purification by a charged depth media filter can be incorporated at any step of the process. In one embodiment, the nucleotide sugar solution is filtered after decolorization.

[0103] In another embodiment, the nucleotide sugar solution is purified by a charged depth media filter after nanofiltration. In yet another embodiment, the nucleotide sugar solution is purified by a charged depth media filter after hollow-fiber filtration. In another embodiment, the nucleotide sugar solution is purified by a charged depth media filter after clarification. In another embodiment, the nucleotide sugar solution is purified by a charged depth media filter after synthesis of the nucleotide sugar. The nucleotide sugar solution may be filtered one or more times using a charged depth media filter.

[0104] In another embodiment, the nucleotide sugar solution is purified using a sterile filter. The sterile filter removes contaminating salts and other undesired contaminants from the nucleotide sugar solution. In a more preferred embodiment, the sterile filter is pre-packaged and sterilized with a bag manifold system for final filtration and storage. Purification by a sterile filter can be incorporated at any step of the process. In one embodiment, the nucleotide sugar solution is filtered by a sterile filter after purification by a charged depth media filter. In another embodiment, the nucleotide sugar solution is purified by a sterile filter after decolorization. In yet another embodiment, the nucleotide sugar solution is purified by a sterile filter after nanofiltration. In another embodiment, the nucleotide sugar solution is purified by a sterile filter after hollow fiber filtration. In another embodiment, the nucleotide sugar solution is purified by a sterile filter after clarification. In another embodiment, the nucleotide sugar solution is purified by a sterile filter after synthesis of the nucleotide sugar. The nucleotide sugar solution may be filtered one or more times using a sterile filter.

[0105] An exemplary process of nucleotide sugar purification is described in FIG. **1**. The nucleotide sugar is first synthesized (1) from a nucleotide and a sugar in the presence of an enzyme.

[0106] After the nucleotide sugar is synthesized, the nucleotide sugar is clarified by filtration (2) and transferred into a mobile tank (3). The clarified nucleotide sugar solution is concentrated using a hollow fiber filtration unit with a tangential flow filtration skid and diafiltered with purified water (4) and (5). The nucleotide sugar solution is transferred to a mobile tank (6) and the pH is adjusted.

[0107] The purified solution is concentrated again and diafiltered with purified water using a nanofiltration system (7) and (8). The nucleotide sugar solution is then decolorized in which the color is removed from the nucleotide sugar solution (9).

[0108] The nucleotide sugar solution subsequently undergoes two filtrations. First, the nucleotide sugar solution is filtered using a charged depth media filter in which endotoxins are removed (10). In an exemplary embodiment, the charged depth media filter is a CUNO Zeta Plus 60 ZA filter or an equivalent. After the nucleotide sugar solution is filtered through the charged depth media filter, the nucleotide sugar solution is optionally filtered using a 0.2 μm sterile filter (11). In an exemplary embodiment, the sterile filter is a CUNO LifeASSURE 0.2 μm sterile filter or an equivalent. The purified nucleotide sugar solution is appropriate for storage.

[0109] Exemplary nucleotide sugars that can be purified by the method described above include, but are not limited to, CMP-NAN, GDP-fucose, GDP-mannose, CMP-NeuAc, UDP-Glucose, UDP-galactose and UDP-N-acetylgalactosamine, and modified analogues thereof. In a preferred embodiment, the nucleotide sugar is CMP-NAN or a modified CMP-NAN.

[0110] The invention also provides methods for synthesizing and purification of a nucleotide-Glycyl Sialic Acid ("nucleotide-GSC").

[0111] In one embodiment, the synthesis of the nucleotide-GSC begins with the synthesis of the protected Fmoc-glycyl-mannosamine ("FGM"). In an exemplary embodiment, mannosamine and Fmoc-Glycyl-OSU are reacted in an aqueous solution under basic conditions. The aqueous solution may contain a base, e.g., sodium methoxide and an organic cosolvent, e.g., methanol, to facilitate the reaction.

[0112] The FGM is optionally purified. In an exemplary embodiment, FGM is purified by chromatography, e.g., silica gel chromatography. FGM may be chromatographed one or more times.

[0113] In another embodiment, FGM converted to the corresponding sialic acid analogue by reaction with pyruvate to form Fmoc-glycyl-sialic acid ("FSC"). This reaction is efficiently catalyzed by a sialic acid aldolase. Appropriate sialic acid aldolases are commercially available. In an exemplary embodiment, the reaction mixture includes at least the nucleotide, the aldolase, $MnCl_2$ and water. The resulting FSC is then coupled to a nucleotide in the presence of an enzyme to form the resulting product, nucleotide-FSC.

[0114] The nucleotide-FSC is optionally purified. In an exemplary embodiment, the nucleotide-FSC is purified by chromatography, e.g., reverse phase chromatography. In an exemplary embodiment, the reverse phase chromatography is C18 reverse phase chromatography. The nucleotide FSC can be filtered (e.g., 0.22 filter) prior to and/or after chromatography.

[0115] The nucleotide-FSC is preferably deprotected to produce the free amine analogue of the linker-nucleotide sugar construct. In an exemplary embodiment, deprotection is effected with methanol:water:dimethylamine. Deprotection results in a modified sialic acid, nucleotide-Glycyl-Sialic Acid ("GSC").

[0116] The GSC is optionally purified, filtered and/or lyophilized.

[0117] An exemplary process for nucleotide-GSC synthesis and purification is described in FIG. **4**.

[0118] The synthesis of nucleotide-GSC begins with the synthesis and concentration of FGM (12). FGM is then purified using silica flash column chromatography (13). FGM is reacted with pyruvate to form FSC (14). The resulting FSC is then coupled to a nucleotide in the presence of an enzyme to form the resulting product, nucleotide-FSC (14). The nucleotide-FSC is then purified (15). The nucleotide-FSC is deprotected (16) thus cleaving off the Fmoc group from the nucleotide-FSC. The resultant product is a nucleotide-GSC. The nucleotide-GSC is further purified and concentrated (16). The nucleotide-GSC is then lyophilized (17) and subsequently released for testing (18).

[0119] An exemplary nucleotide that can be used for the synthesis and purification of a nucleotide-GSC includes, but is not limited to, CMP, CDP, CTP, GMP, GDP, GTP, TMP, TDP, TTP, AMP, ADP, ATP, UMP, UDP, UTP, as well as the deoxy forms of these and other nucleotides. In a preferred embodiment, the nucleotide is CMP.

[0120] The invention provides also methods for synthesizing and purification of a nucleotide-Sialic Acid ("SA")-PEG. In one embodiment, the synthesis of the nucleotide-SA-PEG begins with Fmoc-glycyl-mannosamine ("FGM"). Synthesis and purification of this starting material is discussed above. GSC and methoxy-paranitrophenyl-carbomate-polyethylene glycol ("mPEG-pNP") are combined in under conditions suitable to allow formation of a conjugate between the PEG and the free amine of the glycyl linker. In a preferred embodiment, the synthesis reaction is performed in a 80% THF: 20% H_2O solution.

[0121] In another embodiment, the nucleotide-SA-PEG is purified. In a preferred embodiment, the nucleotide-SA-PEG is purified by reverse phase chromatography.

[0122] An exemplary process of nucleotide-SA-PEG ("PSC") synthesis and purification is described in FIG. **5**.

[0123] The synthesis of nucleotide-SA-PEG begins with the synthesis of FGM (19). FGM is rotovapped (20). FGM is then purified using silica column chromatography (21). FGM is detected by a UV light (22) and rotovapped (23). FGM is reacted with pyruvate to form FSC (24). The resulting FSC is then coupled to a nucleotide in the presence of an enzyme to form the resulting product, nucleotide-FSC (24). The nucleotide-FSC is then purified using a 0.2 μm filter (25) and a C18 reverse phase chromatography (26). The solution is then detected by a UV ray (27). The nucleotide-FSC is deprotected (28) thus cleaving off the Fmoc group from the nucleotide-FSC. The resultant product is a nucleotide-GSC. The nucleotide-GSC is rotovapped (29). The nucleotide-GSC is further purified (30) by a 0.2 μm filter. The nucleotide-GSC is then lyophilized (31). The nucleotide-GSC is reacted with a mPEG-pNP to form nucleotide-SA-PEG ("PSC") (32). The PSC is purified by reverse phase chromatography (33). The PSC is detected by UV light (34) and rotovapped (35). The PSC is then lyophilized (36).

[0124] An exemplary nucleotide that can be used for the synthesis and purification of a nucleotide-SA-PEG includes, but is not limited to, CMP, CDP, CTP, GMP, GDP, GTP, TMP, TDP, TTP, AMP, ADP, ATP, UMP, UDP, UTP, as well as the deoxy forms of these and other nucleotides. In a preferred embodiment, the nucleotide is CMP.

[0125] The invention further provides methods for purifying a glycosyltransferase. In one embodiment, a glycosyltransferase solution is harvested, isolating the enzyme from cell culture and other debris to produce a suitable feed material for subsequent purification steps. In an exemplary embodiment, the harvesting reaction occurs at about pH 6. The harvesting step can be incorporated at any step of the process.

[0126] The glycosyltransferase is optionally precipitated from the solution. In an exemplary embodiment, the glycosyltransferase is precipitated by addition of calcium chloride ("$CaCl_2$") to the solution. The pH of the solution can be adjusted as appropriate. In an exemplary embodiment, the pH of the precipitation reaction is adjusted to about 7.5. The mixture can be incubated for any suitable time under appropriate conditions. In an exemplary embodiment, the precipitation step lasts for about 30 minutes at about 4° C. Other exemplary additives include EDTA.

[0127] The recovery of glycosyltransferase after precipitation is about 80% to about 100%, preferably from about 90% to about 100%, even more preferably about 100%.

[0128] In another embodiment, the glycosyltransferase solution is subjected to membrane filtration. Exemplary membrane filters have a pore size of about 0.1 μm to about 0.5 μm, preferably about 0.1 μm to about 0.3 μm, and more preferably about 0.20 μm to about 0.25 μm. The filtration step can be incorporated at any step of the process.

[0129] In another embodiment, the glycosyltransferase solution is ultrafiltrated. In ultrafiltration, products of high molecular weight are retained on the membrane, while low molecular weight solutes pass through the membrane. In an exemplary embodiment, the ultrafiltration membrane has a molecular weight cut-off (MWCO) between about 5 kDa and about 200 kDa.

[0130] In an exemplary embodiment, the glycosyltransferase solution is equilibrated with 15 mM sodium phosphate buffer, pH 7.5 and conductivity is 2 ms/cm. In another embodiment, the glycosyltransferase solution is equilibrated with 15 mM sodium phosphate, pH 7.5. In another embodiment, the glycosyltransferase solution is equilibrated with 15 mM sodium phosphate, 0.01M NaCl, pH 7.5. In another embodiment, the glycosyltransferase is equilibrated with 15 mM sodium phosphate, 0.05M NaCl, pH 7.5. In another embodiment, the glycosyltransferase solution is equilibrated with 15 mM sodium phosphate, 0.10M NaCl, pH 7.5. In another embodiment, the pH range of the sodium phosphate may range from about 5.5 to about 8.5. In a preferred embodiment, the pH range of the glycosyltransferase solution may range from about 6.5 to about 7.5. In another embodiment, the test excipient includes, but is not limited to, glycerol, mannitol, sorbitol, sucrose and Tween-20. In one embodiment, the percent recovery of glycosyltransferase after ultrafiltration is about 45% to about 85%. In a preferred embodiment, the percent recovery of glycosyltransferase after ultrafiltration is about 55% to about 75%. In a more preferred embodiment, the percent recovery of glycosyltransferase after ultrafiltration is about 65%. The ultrafiltration step can be incorporated at any step of the process. In a preferred embodiment, the glycosyltransferase solution is ultrafiltrated after the filtration step. In another preferred embodiment, the glycosyltransferase solution is ultrafiltrated after the precipitation step.

[0131] In another preferred embodiment, the glycosyltransferase solution is ultrafiltrated after the harvesting step. The glycosyltransferase solution may be ultrafiltrated one or more times.

[0132] In another embodiment, the glycosyltransferase is purified by chromatography, e.g., ion exchange chromatography. In an exemplary embodiment, the glycosyltransferase is purified using an anion exchange column. In a further exemplary embodiment, the anion exchange column is a Mustang Q or an equivalent. The recovery of glycosyltransferase after anion exchange chromatography is preferably from about 80% to about 100%. In a preferred embodiment, the recovery of glycosyltransferase after chromatography is about 90% to about 100%. In a more preferred embodiment, the recovery of glycosyltransferase after chromatography is about 100%. The chromatography step can be incorporated at any step of the process. In a preferred embodiment, the glycosyltransferase is purified by chromatography after an ultrafiltration step. In a preferred embodiment, the glycosyltransferase is purified by chromatography after the filtration step. In another preferred embodiment, the glycosyltransferase is purified by chromatography after the precipitation step. In another preferred embodiment, the glycosyltransferase is purified by chromatography after the harvesting step.

[0133] In another exemplary embodiment, the glycosyltransferase solution purified using a cation exchange column. An exemplary cation exchange chromatography protocol utilizes a Unosphere S resin or an equivalent. In a preferred embodiment, the cation exchange column is a 30S or an equivalent.

[0134] The cation exchange column is eluted with at least one buffer. In one embodiment, the first buffer comprises a 15 mM sodium phosphate, pH 7.5. In another embodiment, the column is further eluted with a second buffer. An exemplary second buffer includes 1M NaCl, 15 mM sodium phosphate, pH 7.5.

[0135] Elution of the column is optionally performed while developing a eluant gradient. In an exemplary embodiment, the added elution step has a rate of about 5 ms/cm to about 10 ms/cm. The recovery of glycosyltransferase after cation exchange chromatography is about 35% to about 75%. In a preferred embodiment, the recovery of glycosyltransferase after chromatography is about 45% to about 65%. In a more preferred embodiment, the percent recovery of glycosyltransferase after chromatography is about 55%. The chromatography step can be incorporated at any step of the process. In a preferred embodiment, the glycosyltransferase is purified by cation exchange chromatography after the anion exchange chromatography step described in the immediately preceding paragraph. In a preferred embodiment, the glycosyltransferase is purified by anion or cation exchange chromatography after the ultrafiltration step. In another preferred embodiment, the glycosyltransferase is purified by ion exchange chromatography after the filtration step. In yet another preferred embodiment, the glycosyltransferase is purified by chromatography after the precipitation step. In another preferred embodiment, the glycosyltransferase solution is purified by chromatography after the harvesting step. The glycosyltransferase solution may be purified by chromatography one or more times.

[0136] In an exemplary embodiment, the glycosyltransferase is purified by hydroxyapatite (HA) chromatography. Exemplary hydroxyapatite sorbents are selected from ceramic and crystalline hydroxyapatite materials. In an exemplary embodiment the particle size of the ceramic hydroxyapatite sorbent is between about 20 μm and about 180 μm, preferably about 60 to about 100 μm, and, more preferably about 80 μm. In a preferred embodiment, the recovery of glycosyltransferase after HA chromatography is about 50% to about 90%, preferably from about 60% to about 90%. In a more preferred embodiment, the percent recovery of glycosyltransferase after chromatography is about 72%. HA chromatography can be incorporated at any step of the process. In a preferred embodiment, the glycosyltransferase is purified by HA chromatography after the cation chromatography step described above in the immediately preceding paragraph. In a preferred embodiment, the glycosyltransferase is purified by HA chromatography after the anion exchange chromatography step described above. In another preferred embodiment, the glycosyltransferase is purified by HA chromatography after the ultrafiltration step. In still another preferred embodiment, the glycosyltransferase is purified by HA chromatography after the filtration step. In another preferred embodiment, the glycosyltransferase is purified by HA chromatography after the precipitation step. In another preferred embodiment, the glycosyltransferase is purified by HA chromatography after the harvesting step. The glycosyltransferase solution may be purified by HA chromatography one or more times.

[0137] In another exemplary embodiment, the glycosyltransferase is purified by hydrophobic interaction chromatography ("HIC"). In an exemplary embodiment, the hydrophobic moieties of the column matrix are selected from, but are not limited to, alkyl groups, aromatic groups and ethers. In an exemplary embodiment, the HIC column is packed with a phenyl 650M resin, butyl 650M resin, phenyl HP resin.

[0138] In an exemplary embodiment, the glycosyltransferase solution is equilibrated with 0.5M ammonium sulfate, 20 mM sodium phosphate, pH 7.

[0139] HIC chromatography can be incorporated at any step of the process. In a preferred embodiment, the glycosyltransferase is purified by HIC chromatography after purification by the HA chromatography step described above in the immediately preceding paragraph. In a preferred embodiment, the glycosyltransferase is purified by chromatography after the cation exchange chromatography step described above. In another preferred embodiment, the glycosyltransferase is purified by chromatography after the anion exchange chromatography step described above. In a preferred embodiment, the glycosyltransferase solution is purified by chromatography after the ultrafiltration step. In a preferred embodiment, the glycosyltransferase solution is purified by chromatography after the filtration step. In another preferred embodiment, the glycosyltransferase solution is purified by chromatography after the precipitation step. In another preferred embodiment, the glycosyltransferase solution is purified by chromatography after the harvesting step. The glycosyltransferase solution may be purified by chromatography one or more times.

[0140] The glycosyltransferase can be purified by ultrafiltration. In an exemplary embodiment, the glycosyltransferase solution is equilibrated with 5 mM Bis-Tris, 0.1M NaCl, 5% sorbitol, pH 6.5. It is generally preferred that the recovery of glycosyltransferase after ultrafiltration is about 80% to about 100%. In a preferred embodiment, the percent recovery of glycosyltransferase after chromatography is about 90% to about 100%.

[0141] Ultrafiltration can be incorporated at any step of the process. In a preferred embodiment, the glycosyltransferase is purified by ultrafiltration after the HIC chromatography step in the immediately preceding paragraph described above. In a preferred embodiment, the glycosyltransferase is purified by ultrafiltration after purification by the HA chromatography step described in the preceding paragraph above. In another preferred embodiment, the glycosyltransferase is purified by ultrafiltration after the cation exchange chromatography step described above. In still a further preferred embodiment, the glycosyltransferase is purified by ultrafiltration after the anion exchange chromatography step described above. In another embodiment, the glycosyltransferase is purified by a second ultrafiltration step after the first ultrafiltration step described above. The glycosyltransferase can be purified by ultrafiltration after the filtration step or precipitation steps. In still another embodiment, the glycosyltransferase is purified by ultrafiltration after the harvesting step. The glycosyltransferase may be purified by ultrafiltration one or more times.

[0142] In another embodiment, the total percent recovery of glycosyltransferase from the purification process is about 5% to about 45%. In a preferred embodiment, the percent recovery of glycosyltransferase from the purification process is about 15% to about 35%.

[0143] An exemplary process of a glycosyltransferase purification scheme is described in FIG. **6**. The glycosyltransferase is first harvested (37). In an exemplary embodiment, the harvesting reaction takes place at pH 6. After harvesting, the glycosyltransferase is precipitated using calcium chloride (38). The glycosyltransferase is then filtered (39). After filtration, the glycosyltransferase undergoes ultrafiltration (40). The glycosyltransferase passes through an anion exchange column such as Mustang Q (41) or an equivalent. After passing through the anion exchange column, the glycosyltransferase passes through a cation exchange column, such as UNOsphere S (42) or an equivalent. The glycosyltransferase is then passed through a HA Type I chromatography column (43). The glycosyltransferase then undergoes ultrafiltration (44).

[0144] In one embodiment, the glycosyltransferase is a sialyltransferase. In a more preferred embodiment, the sialyltranferase includes, but is not limited to, ST6GalNac, ST3Gal3, α(2,3)-sialyltransferase, α(2,6)-sialyltransferase and α(2,8)-sialyltransferase. In another embodiment, the sialyltransferase includes, but is not limited to, a sialyltranferase listed in the table in FIG. **7**. In a more preferred embodiment, the sialyltransferase is ST6GalNac.

[0145] The following examples are offered solely for the purposes of illustration, and are intended neither to limit nor to define the invention.

EXAMPLES

Example 1

Fmoc-Glycyl-Mannosamine Synthesis and Purification

[0146] The synthesis of Fmoc-glycyl-mannosamine (FGM) occurred in a non-aqueous solution involving two main compounds: D-Mannosamine HCl and Fmoc-Glycyl-OSu. Both materials were dry powders that were introduced into a system comprised of anhydrous methanol and sodium methoxide. The reaction was agitated at 25° C. for 1 hThe reaction was complete when the FGM concentration was greater than 15 mg/mL, determined by HPLC. The FGM synthesis was then rotovapped (20° C.) to about 8% of the initial volume. The chromatographic purification was performed using a Biotage pre-packed silica column. The FGM solution was loaded onto the column in a 50:50 $CHCl_3$:CH_3OH solution. The silica column was then washed with 18 column volumes (CV) of 3% $CHCl_3$/97% CH_3OH. Following the wash, FGM was eluted from the column using 14 CV of 15% $CHCl_3$/85% CH_3OH. Fractions containing material were pooled and then rotovapped (20° C.) to dryness and stored at 4° C. The average recovery for this step from the consistency batches was 73.8%.

[0147] Raw Materials: The table below lists the materials used during the synthesis and purification of Fmoc-Glycyl-Mannosamine (FGM). The first four reagents in the table were used during the synthesis of FGM. The last two reagents were used during the purification of FGM.

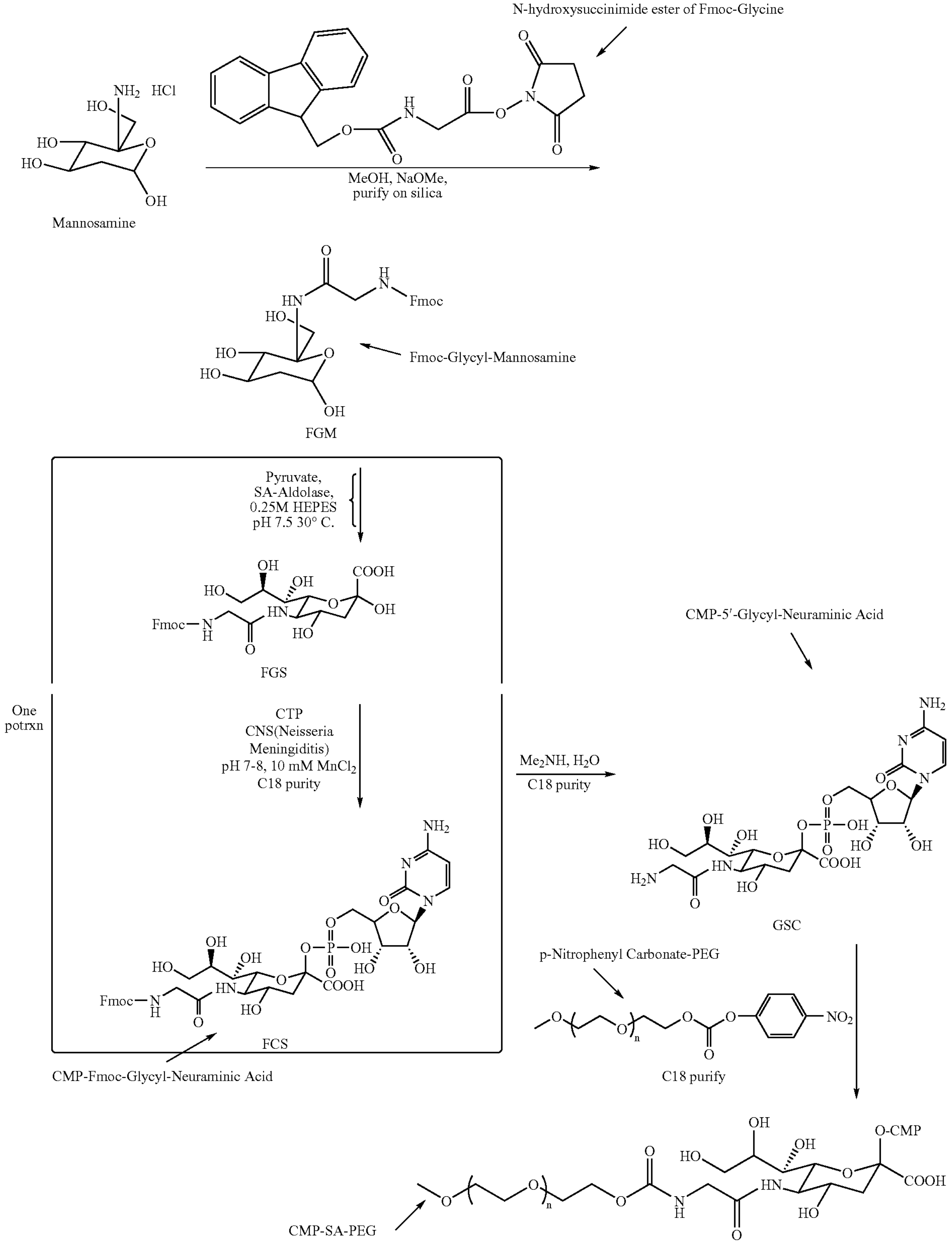
D-Mannosamine HCl	9.0	g
Fmoc-Glycyl-OSu	36.2	g
Sodium Methoxide, 0.5 M	184	mL
Anhydrous Methanol	726	mL
Methanol, ACS	1.4	L
Chloroform, ACS	16.2	L

[0148] The silica column was a Biotage 75M Silica column. The column volume for a 75M column was 0.5 L.

Example 2

I. Description of CMP-Glycyl-Sialic Acid and CMP-Sialic Acid-PEG Synthesis and Purification

[0149] The production of CMP-Sialic Acid-PEG (PSC) was performed in two segments. First, a key intermediate, CMP-Glycyl-Sialic Acid (GSC), was synthesized, purified, and dried, and second, this intermediate was PEGylated, purified, and dried. A synthetic pathway for CMP-SA-PEG is shown below.


N-hydroxysuccinimide ester of Fmoc-Glycine
Mannosamine
MeOH, NaOMe,
purify on silica
Fmoc-Glycyl-Mannosamine
FGM
Pyruvate,
SA-Aldolase,
0.25M HEPES
pH 7.5 30° C.
FGS
One
potrxn
CTP
CNS(Neisseria
Meningiditis)
pH 7-8, 10 mM MnCl₂
C18 purity
FCS
CMP-Fmoc-Glycyl-Neuraminic Acid
Me₂NH, H₂O
C18 purity
CMP-5′-Glycyl-Neuraminic Acid
GSC
p-Nitrophenyl Carbonate-PEG
C18 purify
CMP-SA-PEG

Synthetic Pathway for CMP-SA-PEG

[0150] The first step in the synthesis of GSC was the reaction of mannosamine with Fmoc-Gly-OSu in methanol under basic conditions. The resulting Fmoc-glycyl-mannosamine was purified on a silica flash chromatography column. The purified Fmoc-glycyl-mannosamine then entered a two step enzymatic reaction. Fmoc-glycyl-mannosamine (FGM) was reacted with pyruvate to convert to Fmoc-glycyl-sialic acid. This reaction was catalyzed by a commercially available sialic acid aldolase. Fmoc-glycyl-sialic acid was then coupled to cytidine-5'-monophosphate through a CMP-NAN synthetase catalyzed reaction with cytidine-5'-triphosphate. The resulting product, CMP-Fmoc-glycyl-sialic acid, was purified on a C18 reverse phase column.

[0151] Finally, the Fmoc-group was removed from the CMP-Fmoc-glycyl-sialic acid by deprotection with dimethylamine, forming a precipitate. The resulting precipitate was filtered out of solution using a 0.2 μm membrane and discarded. The resulting solution was dried in a freeze dryer, yielding a white powder of CMP-glycyl-sialic acid (GSC).

[0152] Both CMP-SA-10K PEG and CMP-SA-20K PEG were synthesized in a single step reaction of GSC with the appropriately sized mPEG-pNP. This reaction was performed in an 80% THF:20% H_2O solution. Purification of the final product, CMP-SA-PEG (PSC), was performed by reverse phase chromatography. CMP-SA-10K PEG was purified using a C8 reverse phase chromatography resin, and CMP-SA-10K PEG was purified using a C4 reverse phase chromatography resin. The eluent from the reverse phase column was dried by lyophilization, yielding a white powder of purified CMP-SA-PEG.

[0153] The process was scaled to produce approximately 10 g of the GSC intermediate. Approximately 5.5 g of CMP-SA-20K was produced from 0.5 g of GSC, while approximately 5.0 g of CMP-SA-10K PEG was produced from 1.0 g of GSC. The final CMP-SA-PEG products were approximately 90% pure with the major impurities being CMP and sialic acid-PEG, the primary breakdown products of CMP-SA-PEG.

II. CMP-Fmoc-Glycyl-Sialic Acid Synthesis

Process Description

[0154] The synthesis of CMP-Fmoc-glycyl-sialic acid (FSC) was a two-step enzymatic process performed simultaneously in one pot. Reacting with pyruvate, Fmoc-glycyl-mannosamine (FGM) was converted to Fmoc-glycyl-sialic acid, catalyzed by sialic acid-aldolase. Fmoc-glycyl-sialic acid was then coupled to cytidine-5'-monophosphate through a CMP-NAN synthetase catalyzed reaction with cytidine-5'-triphosphate.

[0155] The pH of the reaction was controlled at 7.5±0.5. Temperature was controlled at 30±2° C., and the reaction was continuously agitated. The reaction reached completion in 6-24 hrs. and the extent-of-reaction was determined as a function of the FSC concentration (≧14.0 g/L, 95% of theoretical conversion). The resulting product solution can be stored at 4° C. until the purification is executed or for up to 72 h. The average recovery for the step during consistency batches was 91.2%.

[0156] The quantities of materials required for the synthesis of CMP-Fmoc-glycyl-sialic acid varied with the quantity of Fmoc-glycyl-mannosamine generated from the purification step of the process. Exemplary quantities are as follows:

Fmoc-glycyl-mannosamine	11	g
Cytidine-5'-triphosphate	15.8	g
Manganese Chloride	9.5	g
Pyruvate	26.4	g
Sodium Hydroxide, 50% (w/w)	17	mL
CMP-NAN-Synthetase (*N. meningitidis*)	50,600	U
N-Acetyneuraminic acid Aldolase	1.19	g

III. CMP-Fmoc-Glycyl-Sialic Acid Purification

Process Description

[0157] The purification of CMP-Fmoc-glycyl-sialic acid (FSC) consists of two steps: the clarification of the reaction solution by 0.2 μm filtration and the reverse phase chromatographic purification of the FSC. To clarify the reaction, the solution was simply pumped through a Millipore Millipak 0.2 μm filter cartridge. Little backpressure was generated during this filtration. After the filtration was complete, the filter cake and filter were rinsed with 500 mL of purified water.

[0158] The chromatographic purification was performed using a Biotage pre-packed C18 reverse phase column. The FSC was loaded in an aqueous solution. The FSC binds to the column, and the column was washed with six column volumes of purified water. The FSC was then eluted in 10% methanol in purified water.

[0159] The purification step was performed using the Biotage chromatography system (Z-1405).

IV. CMP-Fmoc-Glycyl-Sialic Acid (FSC) Deprotection and CMP-5'-Glycyl-Sialic Acid (GSC) Filtration

Process Description

[0160] The deprotection of CMP-Fmoc-Glycyl-Sialic Acid (FSC) occurred in a 10% methanol:water solution in a reaction with dimethylamine. Dimethylamine (40 wt % in water) is a solution that was added to the FSC C18 fraction pool. The Fmoc group was cleaved off, resulting in key intermediate, CMP-5'-Glycyl-Sialic Acid (GSC). The reaction was agitated at 25° C. for 75 minutes.

[0161] The reaction was complete when the peak area ratio of GSC:FSC was greater than 20, as measured by HPLC. The resulting GSC solution was then rotovapped (30° C.) to about 35% of the original volume. The free Fmoc-derivative formed a white precipitate that needed to be removed from the GSC solution. This material was filtered through a Millipore Millipak-200 Filter Unit (0.22 μm). The filter was then rinsed with RO water. This GSC solution was stored at 4° C. or frozen at −20° C. until lyophilization. The average recovery for this step from the consistency batches was 86.1%.

[0162] The table below lists the materials used during the deprotection of CMP-Fmoc-Glycyl-Sialic Acid (FSC) and filtration of CMP-5'-Glycyl-Sialic Acid (GSC).

Raw Materials for CMP-Glycyl-Sialic Acid (GSC) Deprotection and Filtration

CMP-Fmoc-Glycyl-Sialic Acid (FSC) C18 Fraction Pool	Volume determined from P30 batch record
Dimethylamine (40 wt %)	Volume calculated from P40 batch record See Section A, step 1

-continued

Raw Materials for CMP-Glycyl-Sialic Acid (GSC) Deprotection and Filtration	
RO H_2O	Volume calculated from P40 batch record See Section B, step 6

V. CMP-SA-Glycine Production

[0163] Another method of producing and purifying CMP-SA-Glycine is provided below:

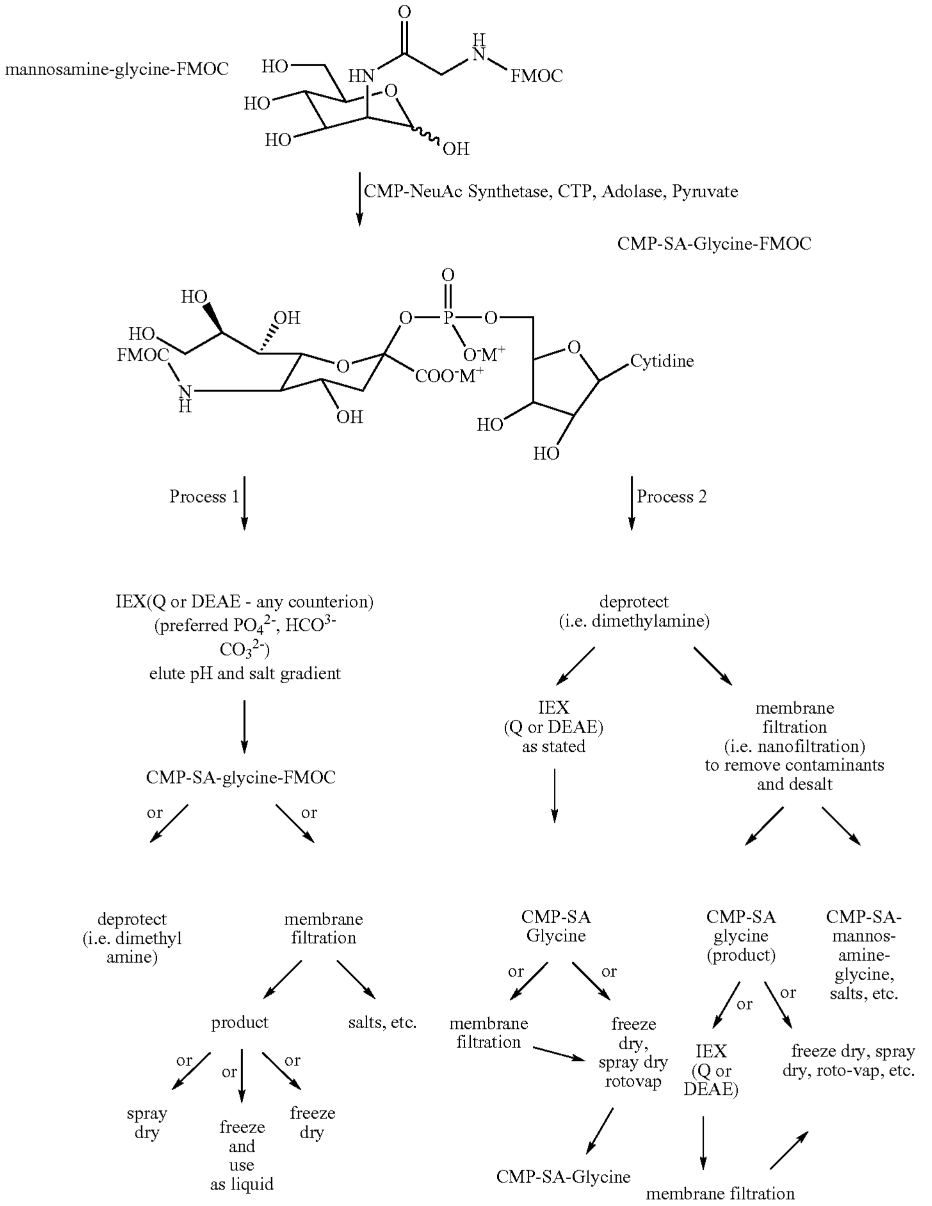
Example 3

I. Summary of Consistency Batches of 10k and 20k Cmp-SA-PEG After Process Development

[0164] Consistency batches were performed for 10K and 20K CMP-Sialic Acid-PEG after development of the synthesis and purification operations. These batches demonstrated that a reproducible process had been developed to produce high-purity CMP-SA-PEG with very low contaminant levels, suitable for the glycopegylation projects.

[0165] 10K CMP-SA-PEG was produced at greater than 80% purity at overall process yields of approximately 60%, and 20K CMP-SA-PEG was produced at greater than 70% purity at overall process yields of approximately 50%. The

CMP-SA-Glycine Production



products were low in endotoxin, bioburden, and protein, and NMR has shown that the balance of the material was nearly all mPEG-OH, a by-product of the synthesis process.

Materials and Methods

[0166] CMP-SA-PEG (PSC) was produced in a reaction of CMP-Glycyl-Sialic Acid (GSC) with paranitrophenyl-carbomate-polyethylene glycol (pNP-PEG). The reaction conditions for the consistency batches of each size CMP-SA-PEG are summarized below.

CMP-SA-PEG Synthesis Conditions

	10K PSC	20K PSC
Raw Materials		
CMP-5'-Glycyl-Sialic Acid	1.0 g	0.5 g
(GSC)	10K	20K
	10K	20K
pNP-PEG	24 g 10K pNP-PEG	24 g 20K pNP-PEG
	10K	20K
	10K	20K
RO H_2O	200 mL	100 mL
Tetrahydrofuran (THF)	800 mL	400 mL
unstabilized	10K	20K
	10K	20K
Reaction Conditions		
Length of Reaction	24 ± 1 hrs.	20 ± 1 hrs.
pH adjustment to 8.9 ± 0.1	17 ± 1 hrs.	17 ± 1 hrs.
Additions of PEG	3 additions (90 min. between) each of 8 g of pNP-PEG	3 additions (90 min. between) each of 8 g of pNP-PEG

[0167] At the end of the reaction, a molar ratio of PSC to GSC (product to reactant) of greater than 7:1 is found for 10K PSC and greater than 5:1 for 20K PSC.

[0168] The reaction solution were then rotovapped at <30° C. to less than 80% of its original volume to remove the THF. The remaining aqueous solution was then diluted to five times the original reaction volume (diluted to 5 L for 10K PSC and 2.5 L for 20K PSC) using RO water. The diluted solution was then adjusted to pH 9.5±0.1 with 1M NaOH and allowed to stir for at least 1 hr. This elevated pH caused breakdown of residual pNP-PEG to free paranitrophenol, carbon dioxide, and methoxyPEG-OH (mPEG-OH). After at least 1 hr. at the elevated pH, the solution is adjusted to pH 8.0±0.1 using 1M HCl.

[0169] Both 10K and 20K PSC were then purified on a C4 reverse-phase chromatography column. The table below summarizes the purification conditions.

CMP-SA-PEG Purification Conditions

	10K PSC	20K PSC
Column	Biotage C4 75 L (1 L bed volume, 7.5 cm diameter)	Biotage C4 75 L (1 L bed volume, 7.5 cm diameter)
Flowrate	100 mL/min (136 cm/hr)	100 mL/min (136 cm/hr)
Wash Conditions	10 column volumes (CVs) 10% MeOH, 1 mL 1M NaOH/L water	10 CVs 10% MeOH, 1 mL 1M NaOH/L water
Elution Conditions	4 CVs 40% MeOH, 1 mL 1M NaOH/L water	4CVs 43% MeOH, 1 mL 1M NaOH/L water
Regeneration Conditions	3CVs 100% MeOH 2CVs 50% MeOH, 1 mL 1M NaOH/L water 2CVs 25% MeOH, 1 mL 1M NaOH/L water 3CVs 100% RO water, 1 mL 1M NaOH/L water	3CVs 100% MeOH 2CVs 50% MeOH, 1 mL 1M NaOH/L water 2CVs 25% MeOH, 1 mL 1M NaOH/L water 3CVs 100% RO water, 1 mL 1M NaOH/L water

[0170] From the column, fractions during the elution that contain UV (27 nm) absorbance of at least 10% of the main peak absorbance were pooled, and the methanol from the pool was removed by rotovap.

[0171] This concentrated fraction pool was then freeze dried on a Labconco flask-style freeze-drier. Final testing was performed on the resulting powder from this drying.

Equipment

[0172] The syntheses was performed in 2 L and 5 L sealed bottles, temperature controlled in a shaker incubator, SI-0017, B. Braun Certomat BS1.

[0173] Chromatographic purification was performed on the Biotage Flash Chromatography System, Z-1405. Drying was performed on a lab-scale Labconco freeze dryer.

Results

[0174] Using the methods described above, two batches each of 10K and 20K PSC were synthesized, purified, and dried. The chart below summarizes the yield and recoveries from the synthesis and purification steps of the process. Note that the extent-of-reaction is noted as a ratio of peak areas of PSC to GSC from the HPLC assay. Quantitative yields from the syntheses are not available because the use of THF in the reaction disrupts the quantitative ability of the HPLC method.

CMP-SA-PEG Consistency Batch Yields and Recovery

	PSC Synthesis			
	10K PSC A	10K PSC B	20K PSC A	20K PSC B
GSC content (wet weight)	60.66%	67.38%	67.38%	61.15%
EOR	13.8:1	8.0:1	6.3:1	5.4:1

-continued

CMP-SA-PEG Consistency Batch Yields and Recovery

	PSC Purification					
	10K PSC A	10K PSC B	Average	20K PSC A	20K PSC B	Average
Quantity (g)	6.7	7.9		5.5	7.3	
Yield PSC (g)	5.5	6.6	6.1	4.2	5.5	4.8
% Recovery	59.8%	64.0%	61.9%	42.4%	60.5%	51.4%

Results for the four batches are summarized below.

PSC Consistency Batch Testing Results

	10K PSC A	10K PSC B	20K PSC A	20K PSC B
Identification (RP-LC)	Match with standard	Match with standard	Match with standard	Match with standard
10K PSC purity (g/100 g of dry wt)	82.62	83.01	76.30	74.56
CMP (g/100 g of dry wt)	0.02	0.02	0.02	0.02
Moisture content (g/100 g of wet wt)	0.60	0.49	0.52	0.48
Sodium % Na$^+$ (g/100 g of wet wt)	0.45	0.50	0.22	0.14
Aldolase/CNS2 impurity	None visible	None visible	None visible	None visible
Endotoxin (EU/mg of wet wt)	0.011	0.007	0.0113	0.006
Microbial limits testing	***	***	***	***
Total aerobic counts (CFU/g of wet wt.)	<10	<10	<10	<10
Total yeast and mold (CFU/g of wet wt.)	<10	<10	<10	<10

[0175] The HPLC traces of the 4 batches showed that the CMP-SA-PEG was >98% by peak area. CMP levels were also very low and much lower than seen in previous lots of PSC.

[0176] A silver stain SDS-PAGE gel was run to analyze for residual CNS and aldolase. None was detectable in either the 10K or 20K PSC batches.

[0177] Also, as requested in the materials specifications, ^{1}H-NMR was performed on the batches.

[0178] The 10K PSC samples from Consistency Batch#2 and Consistency Batch#3, were submitted for ^{1}H NMR analysis. The NMR spectra indicated that the PSC is reasonably clean, agreeing with the HPLC analysis. There are no observable resonances for sialic acid-PEG (expected at 2.22 & 1.83 ppm) or CMP (distinct resonance expected ~8.11 ppm). A very small amount of PEG-carbamate-DMA (the product of the reaction of dimethylamine with pNP-PEG, 2.93 ppm) is seen in Batch #2, while none is seen in Batch #3. Although non-quantitative, the ratio of the methyl singlet (3.39 ppm) to the unique PSC resonances (~8.00, 2.50, & 1.55 ppm) is ~⅓, which would indicate relatively low levels of mPEG-OH impurities.

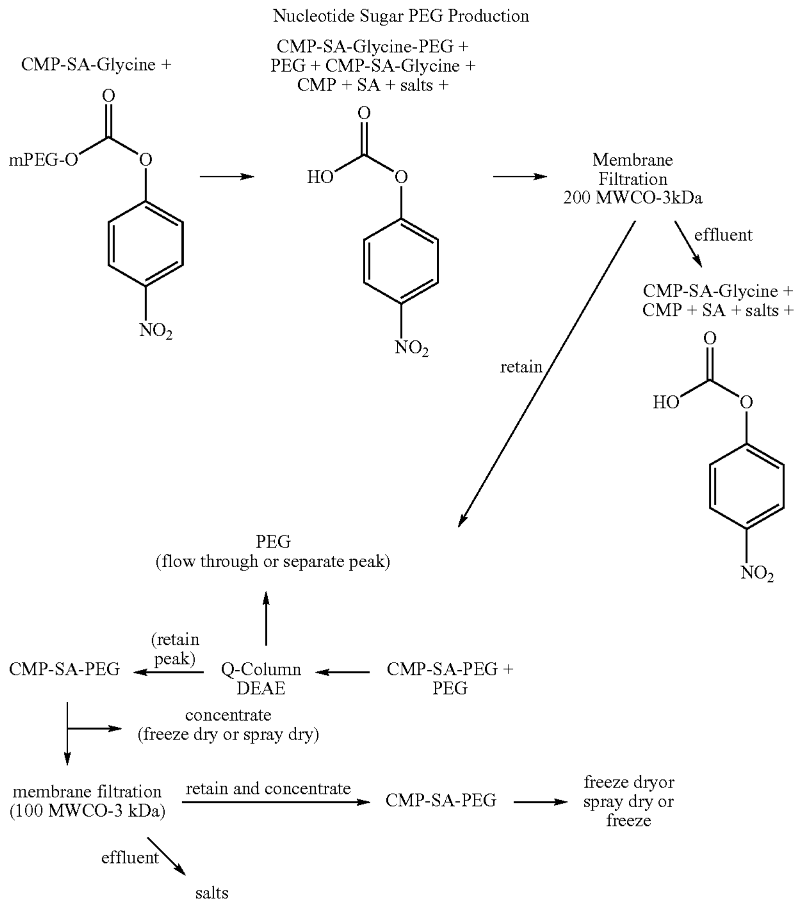
[0179] The 20K PSC samples from Consistency Batch#2 and Consistency Batch#3, submitted for ^{1}H NMR analysis showed that the PSC is also reasonably clean. There are no observable resonances for sialic acid-PEG (2.22 & 1.83 ppm), CMP (distinct resonance at ~8.11 ppm), or PEG-carbamate-DMA (~2.93 ppm). Although non-quantitative, the ratio of the methyl singlet (3.39 ppm) to the unique PSC resonances (~8.00, 2.50, & 1.55 ppm) is ~⅓, which would indicate relatively low levels of mPEG-OH impurities.

Example 4

Nucleotide Sugar-PEG Purification

General Overview

[0180] The current method discusses PEG coupling with a nucleotide sugar, such as CMP-SA-glycine. The nucleotide sugar-PEG product can be separated from the reaction mixture by first desalting the reaction mixture using dialysis. Membrane filtration (reverse osmosis, nanofiltration, etc.) or size exclusion techniques (i.e. polyacrylamide) resin, Sephadex resin, Sepharose resin) can be used next to further purify the mixture. After purification, the reaction mixture containing the nucleotide sugar-PEG product undergoes further purification involving ion exchange chromatography. For most reaction mixtures, a DEAE or Q-resin can be used to remove the unreacted PEG from the product. Q-Sepharose resin is currently preferred, although any polymer with a quaternary amine will also work. The Q-Sepharose resin can include ions such as —OH, Cl$^-$, HCO$_3^-$, CO$_3^{2-}$, PO$_4^{2-}$, SO$_3^-$, Br$^-$, BO$_3^{2-}$ or the like. The product can be loaded and eluted using known methods in the art such as changes in pH or ion strength (NaCl, KCl, etc.). The product may be eluted by a step-wise process or a gradient process. The nucleotide sugar-PEG product eluted from the column can be collected and desalted again using dialysis, membrane filtration, or size-exclusion techniques. The process would look similar to the figure below but can be varied dependent on processing results and desires.



Specific Example

[0181] CMP-SA-glycine and the p-nitrophenyl-carbamate-mPEG are mixed together to obtain a reaction mixture comprising the product CMP-SA-glycine-PEG, as well as PEG, p-nitrophenyl-carboxylic acid, salts, CMP-SA-glycine, CMP and sialic acid (SA). The reaction mixture underwent membrane filtration wherein the membrane has MWCO of 200 (units)-300 kDa. CMP-SA-PEG and PEG were retained while p-nitrophenyl-carboxylic acid, salts, CMP-SA-glycine, CMP and SA passed through the membrane. The retained products underwent further purification with a Q-column or DEAE column in which PEG flows through the membrane and CMP-SA-PEG is retained and concentrated. At this step, the CMP-SA-PEG may be freeze-dried or spray dried. The CMP-SA-PEG underwent membrane filtration in which salts pass through and while CMP-SA-PEG was retained and concentrated. The membrane has a MWCO of 100 MW-3 kDa. This sample may be freeze dried, spray dried, or frozen.

[0182] Any membrane size can be used from reverse osmosis (RO) pore sizes (molecular weight cut-offs) to microfiltration MWCOs depending on the separation desired. During membrane filtration, the pH can be varied between 2 and 12, more specifically between 5-10 and more specifically between 7-9 for CMP-SA-PEG.

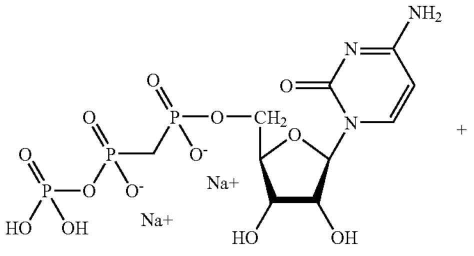
[0183] The membrane filtration step can be used to remove impurities by such techniques as diafiltration and can be used to concentrate the process streams.

Example 5

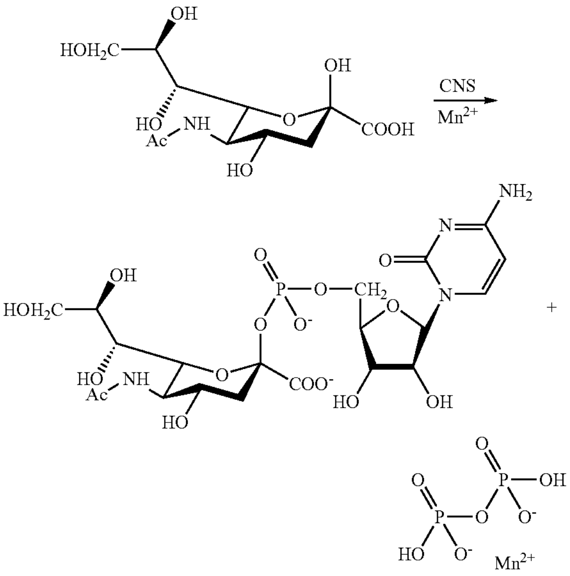
[0184] Cytidine 5'-monophospho N-acetylneuraminic acid (CMP-NAN) is enzymatically synthesized from cytidine 5'-triphosphate (CTP) and N-acetylneuraminic acid (NAN) in the presence of *E. coli-expressed* CMP-NAN Synthetase (CNS). FIG. **1** illustrates the reaction.

CMP-NAN Synthesis Reaction

[0185]



-continued



[0186] CTP and NAN were incubated in a reactor with CNS and $MnCl_2$. Using a small excess of CTP, the reaction proceeded to almost 100% conversion of NAN to CMP-NAN in under two hours.

[0187] The primary by-product of the synthesis reaction was the formation of a manganese phosphate precipitate. Approximate 30% of the final volume of the reaction consisted of this heavy, brown precipitate. In order to remove this precipitate, the solution was filtered. The cake that formed on the filter was washed with water, and the final solution was then filtered to remove any fine particulates that were not held back by the first filtration.

[0188] In order to remove proteins introduced from the CNS, the CMP-NAN solution was permeated through a hollow fiber filter. The membrane successfully retains proteins from the enzyme preparation while allowing the passage of CMP-NAN.

[0189] Residual salts were then removed from the solution by concentration and diafiltration of the CMP-NAN using a nanofiltration membrane. This membrane retains CMP-NAN during concentration and diafiltration while allowing passage of salts that remain in solution, primarily Tris HCl (the buffer from the enzyme preparation) and NaCl (from the synthesis reaction).

[0190] The concentrated CMP-NAN was subsequently decolorized by passing the solution over a pre-packed column of activated carbon. The color is likely from manganese oxide formed during the addition of sodium hydroxide to control the pH during synthesis.

[0191] Finally, endotoxin was removed using a charged media depth filter, and then the CMP-NAN solution was filtered through a 0.2 μm filter and aliquotted into sterile containers.

[0192] An aliquot was spray dried and tested. The CMP-NAN purity was 83.3%. The remainder of the material was stored as a frozen liquid at −20° C. A portion of the frozen bulk was lyophilized. This material was analyzed and released per specification RM0300 with a purity of 82%.

Process Summary

[0193] Cytidine 5'-monophospho N-Acetylneuraminic acid (CMP-NAN) was synthesized in a temperature and pH controlled vessel from Cytidine 5'-triphosphate (CTP) and sialic acid (NAN) using CMP-NAN Synthetase (CNS) in the presence of $MnCl_2$. The CNS is a recombinant protein cloned from *N. meningitidis* and was expressed in JM109 *E. coli* cells. The reaction proceeded for approximately 1.5 hours with sufficient agitation to keep the precipitate from settling. The resultant CMP-NAN solution was chilled to <20° C., clarified by filtration, and transferred into a mobile tank.

[0194] The clarified CMP-NAN solution was concentrated 10 times using a hollow fiber filtration unit and diafiltered with 5 volumes of USP purified water. The CMP-NAN was transferred to a mobile tank and the pH was adjusted. This solution was concentrated 10 times and diafiltered with 6 volumes of USP purified water using a nanofiltration system.

[0195] Decolorization was achieved by running the CMP-NAN through an activated carbon column and endotoxin was removed using a CUNO Zeta Plus 60ZA filter.

[0196] The purified CMP-NAN solution was then aseptically filtered (CUNO LifeASSURE 0.2 μm) and aliquoted into MITOS Sugar Nucleotide Bags and stored at −20° C.

Consistency Runs

[0197] Three consistency runs were performed at the 20 L scale before the Engineering Trial took place. The tables below summarize the yields and recoveries and some of the analysis results.

Consistency Runs Summary Data

Batch	Synthesis Yield	Theoretical Yield	Overall Process Yield	Purification Recovery
1	1339 g	98.1%	607.8 g	45.4%
2	1264 g	92.6%	793.6 g	62.7%
3	1373 g	100.6%	952.0 g	69.3%

Analysis Results from Consistency Runs

Chemical Component	Batch #1	Batch #2	Batch #3
CMP-NAN	75.0%	77.5%	82.5%
CMP	5.6%	5.0%	5.0%
CDP	0%	0.5%	0%
NAN	9.4%	8.5%	4.6%
Moisture	7.5%	7.3%	7.7%
Mass Balance	97.5%	98.8%	99.8%
Other Impurities	0.2%	0.9%	0.4%
PO_4			

Property	Requirement	Result
Appearance	White to off-white powder	White powder
Identification	Compares to reference spectra	Compares to reference spectra
Moisture Content	≦5% moisture	5%
Purity	≧80% CMP-NAN	82%

-continued

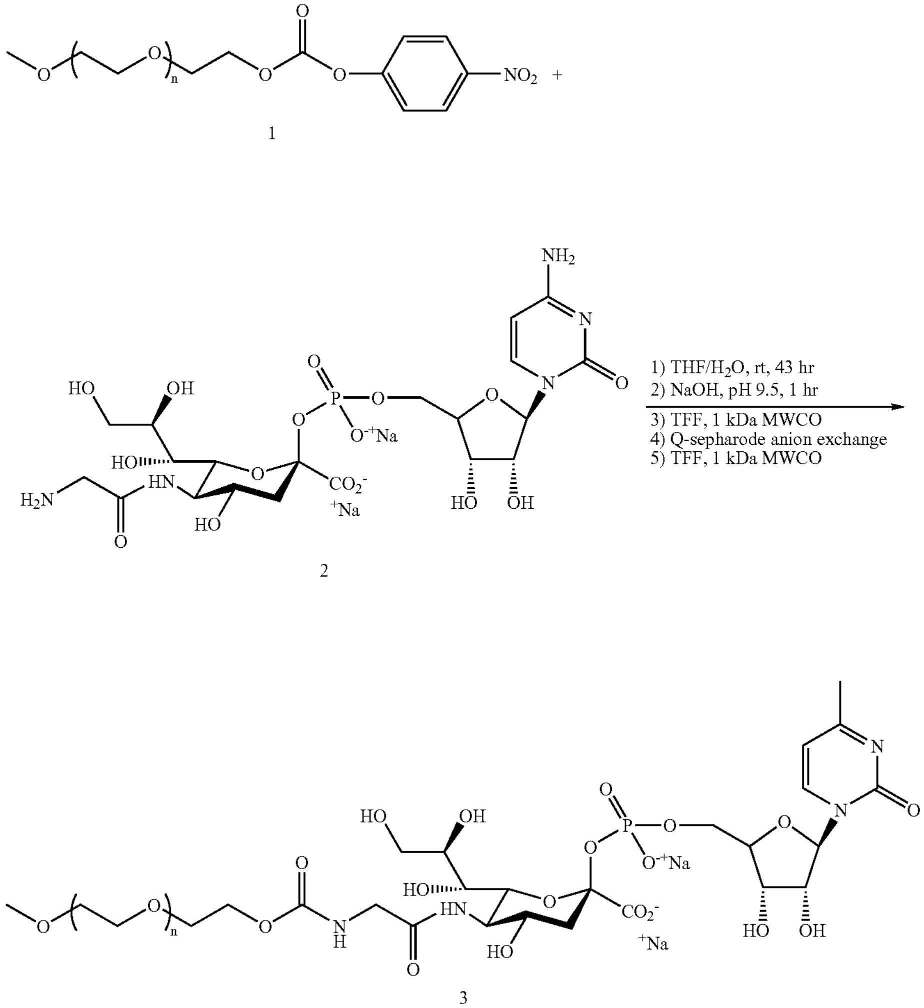
Property	Requirement	Result
Percent CMP, CDP and CTP	<10% CMP, CDP, and CTP	5%
Manganese by ICP	Report Results	120 ppm
Sodium % by ICP	Report Results	7.5%
Heavy Metals (as Pb)	Report Results	<20 ppm
Host Cell Protein	Report Results	Non detected
Total Aerobic Count	≦10 cfu/mg	<1 cfu/mg
Yeast and Mold	≦10 cfu/mg	<1 cfu/mg
Endotoxin	≦0.5 EU/mg	.00165 EU/mg

Example 6

Synthesis and Purification of CMP-Sialic Acid-PEG 30 kDa

[0198] This example describes the preparation and purification of CMP-SA-PEG 30 kDa (compound 3, below). The mPEG-p-nitrophenyl carbonate-30 kDa (compound 1, below) was reacted with sodium CMP-SA-Glycine (compound 2, below) in a mixture of THF/Water. The crude product was desalted by Tangential Flow Filtration (TFF), purified by Q Sepharose chromatography (IEX), and again desalted by TFF to provide 13.5 g of CMP-SA-PEG-30 kDa product (compound 3, below). Reprocessing of mixed IEX fractions afforded an additional 8.2 grams of 3 (overall yield 72.3%).

Scheme 1



I. Materials and Methods

[0199]

Materials

Name	Unit
Q Sepharose	Big Beads
Double 1K Pellicon-2 "MINI" filters	PBLC 1K Regen. Cellulose Membrane; Screen Type: V; 0.1 m^2
CMP-SA-Glycine (2) Dimethylamine salt	Powder
30K mPEG-p-nitrophenyl carbonate (1) (obtained from NOF America)	Powder
NaCl	Powder
NaOH	1.0 N
Tris-Glycine gel, 4-20%	1.0 mm, 10 wells
SeeBlue-plus2 Standard	12 proteins
Tris-Glycine Running Buffer	10× concentrated
Tris-Glycine Sample Buffer	50 mL solution
$BaCl_2$	Powder
Iodine Solution	N/10
Sodium Bicarbonate	Powder

II. Reaction Conditions.

[0200] CMP-SA-Glycine (dimethyl amine salt form, 1.35 g, 2.0 mmole) was dissolved in 20 mL H_2O, and the pH was adjusted to 10.5 with 0.1 N NaOH (~20 mL). The basic solution was degassed under reduced pressure (vacuum 30 min), frozen and lyophilized to dryness. The resulting sodium CMP-SA-Glycine was redissolved in water (80 mL). The pH was measured and found to be 8.5. The pH was adjusted to 7.8 by addition of 0.2 N NaH_2PO4 (<1.0 mL) and the resulting solution was diluted with THF (200 mL). The mPEG-p-nitrophenyl carbonate-30 kDa (30.0 g, 1 mmol) was added to the CMP-SA-Glycine solution in small portions over 3 hr at room temperature. The reaction mixture was stirred at room temperature for 43 hrs. The THF was then removed by rotary evaporation at reduced pressure without heating (water bath temperature at or below 30° C.). The aqueous residue (80 mL) was diluted with water to 600 mL, and adjusted to pH 9.5 with 1.0 N NaOH (about 1.0 mL). The basic solution was stirred at room temperature for 1 hour to hydrolyze any unreacted mPEG-p-nitrophenyl carbonate-30 kDa, and then purified by Tangential Flow Filtration (TFF), Q-Sepharose chromatography (IEX), and then TFF as described below. The final purified fractions were freeze-dried to afford 21.7 g (72.3%) of a white solid (3).

III. Tangential Flow Filtration (TFF) Purification

[0201] A Watson-Marlow peristaltic pump (505S) was connected through Tygon tubing (¼" ID) to a Millipore Pellicon-2 Mini Holder equipped with two Millipore 1K Pellicon 2 "MINI" filter (PLAC-V 1K Regenerated Cellulose Membrane; Screen Type: V; 0.1 m2) (FIG. **1**). The crude aqueous product solution (500 mL, pH 9.5) from above was transferred to a 1000 mL bottle immersed in an ice bath, equipped with a conductivity meter and a pH meter. The product solution was fed onto the Pellicon Mini filter through Tygon tubing (¼" ID) for diafiltration with a pump speed of 90 rpm (Cross flow rate: 430 mL/min; Flux rate: 20 mL/min; Pressure 13 psi). The retentate solution was returned to the bottle containing the bulk chilled product solution (PharMed tubing, ¼" ID) which was maintained at a constant volume (600 mL) by addition of cold DI water (4° C.). The permeate solution was collected in 2 L fractions. The pH and conductivity values of the retentate/product solution were measured and recorded over time as shown in the table below. The pH of the retentate/product solution was maintained above pH 7.5 by the dropwise addition of 1.0 N NaOH, as needed. The retentate/product solution was diafiltered until the conductivity dropped below 0.8 mS, and then the retentate/product solution was allowed to concentrate to a volume of 500 mL. The concentrated retentate was then purified by anion exchange chromatography as described below. The permeate fractions were checked for product break-through by SDS-PAGE as described below:

Time (min.)	Conductivity (mS)	pH Value	Total Vol (mL)
0	1.93	9.58	600
30	1.59	9.50	600
60	1.10	9.07	600
90	0.899	8.69	600
120	0.818	8.47	600
150	0.743	8.35	600
180	0.755	8.56	500

IV. SDS-PAGE Analysis.

[0202] Samples of the TFF permeate fractions (0.5 mL) were concentrated to dryness under a stream of N_2 gas, resuspended in 10 μL water and mixed with 10 μL Tris-Gly SDS-PAGE sample buffer and loaded onto 4-20% polyacrylamide Tris-Gly SDS-PAGE gels. See Blue Plus2 protein standard was also loaded as a marker. Gels were run at a constant voltage of 125 V for 1 hr 50 min. After electrophoresis, the gels were washed with water (100 mL) for 10 min, and then incubated with a 5% barium chloride aqueous solution for 10 min. Iodine solution (0.1 N, 4.0 mL) was added to visualize any mPEG present. The staining process was stopped by washing the gels with water. The proteins used as a standard were a mix of myosin (250 kDa), phosphorylase (148 kDa), BSA (98 kDa, glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), lysozyme (22 kDa), aprotinin (6 kDa), and insulin B-chain (4 kDa). The gels were visualized and scanned with an HP Scanjet 7400C, and the image of the gel was optimized with the HP Precision Scan Program.

V. Anion Exchange Column Chromatography (IEX) Purification.

[0203] The Q Sepharose Big Beads (4.0 L) were treated with 1.0 M NaOH (8.0 L), and then with saturated aqueous sodium bicarbonate (8.0 L) to generate the bicarbonate form of the resin. The newly generated resin was packed in a 17×18 cm (ID) column which was connected to an HPLC system equipped with a UV (274 nm) and an ELS detector (Evaporation temp: 120° C.; Nebulizer temp: 90° C.; Gas flow rate: 1.85 SLM). The TFF retentate from above was slowly loaded on the Q column (60 mL/min.). The column was then washed with solvent A (cold DI water, 8.0 L) at a flow rate of 125 mL/min until ELS detection indicated that all non-binding impurities (mPEG-OH) had been washed from the column. The product was then eluted with a gradient of 90% Solvent A/10% Solvent B (1.0 N NaCl) to 20% Solvent A/80% Solvent B over 100 min, and then to 100% B for 5 minutes at a flow rate of 125 ml/min. Product-containing fractions were collected (detected by UV, 274 nM) as shown in FIG. **2**. Fraction 1 was desalted by TFF as described below. Fraction 2 was reprocessed (data not shown).

VI. Tangential Flow Filtration (TFF) for Desalting.

[0204] The first fraction (Fraction 1) of the ion-exchange-purified product was desalted using the TFF procedure described above. Upon reaching a constant conductivity, the retentate/product solution was allowed to concentrate to 400 mL. The final retentate (400 mL) was freeze-dried to yield 13.5 g of white solid.

Time (min.)	Conductivity (mS)	pH Value	Total Vol (mL)
0	23.9	7.79	600
30	23.1	7.81	600
120	10.8	7.91	600
150	8.46	7.94	600
180	5.59	8.14	600
210	2.68	8.30	600
240	1.14	8.46	600
270	0.706	8.68	600
300	0.489	8.81	600
330	0.403	8.90	500
360	0.389	8.53	400

Example 7

Process Description for the Synthesis and Downstream Processing of CMP-NAN

[0205] Cytidine-monophospho 5'-N-acetylneuraminic acid (CMP-NAN) is enzymatically synthesized from cytidine 5'-triphosphate (CTP) and N-acetylneuraminic acid (NAN) in the presence of *E. coli-expressed* CMP-NAN synthetase (CNS).

[0206] The reactants (CTP and NAN) are incubated (30° C., pH 8.5-9.5) in a reactor (20 or 40 L Synthesis Reactor, jacketed temperature control is <10° C. to 30° C., manual or automatic pH control is 8.5-9.5, and agitated) with CNS and manganese chloride ($MnCl_2$). Using a small excess of CTP, the reaction proceeds to almost 100% conversion of NAN to the CMP-NAN product in under two hours. When the reaction is complete, the solution is chilled to <10.0° C. Once chilled the process stream is filtered through a 0.5 μm membrane bag filter (CUNO Polynet bag filter with CUNO bag filter housing and pressure-fed (nitrogen) filtration).

[0207] In order to remove proteins introduced by the enzyme preparation, the process stream is next permeated through a 10K hollow fiber membrane with a tangential flow filtration (TFF) skid (Amersham Bioscience (formerly AG Technology) 10K MWCO hollow fiber cartridge, temperature control is <10° C., and inlet, outlet and permeate pressure indication is 0-60 psig). The 10K membrane successfully retains proteins from the enzyme preparation while allowing for passage of the CMP-NAN. The result is a protein-free CMP-NAN solution.

[0208] Residual salts are then removed from solution by concentration and diafiltration of the CMP-NAN using a nanofiltration membrane with a nanofiltration TFF skid (Millipore Nanomax-50 Helicon RO4 spiral wound membrane cartridge installed on a Millipore Prolab II skid, temperature control is <10° C. and pressure indication is 0-600 psig). This membrane retains CMP-NAN during concentration and diafiltration while allowing passage of salts that remain in solution from the synthesis reaction and hollow fiber filtration.

[0209] This concentrated CMP-NAN stream is subsequently de-colored by passing the solution over a pre-packed column of activated carbon attached to a Biotage chromatography system (75M activated chromatography column and Biotage skid). The activated carbon efficiently removes the color from the solution in one or two passes of the CMP-NAN stream.

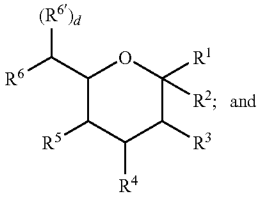
[0210] The process is completed by two filtrations. First, the CMP-NAN solution is filtered using a charged media depth filter (CUNO Zeta Plus 60ZA, 1 ft^2). Second, the solution is sent through a final 0.2 μm filter that is pre-packaged and sterilized with a bag manifold system (provided by Mitos Technologies) for final filtration and storage. The product can be stored as a frozen solution or dried as a white powder for refrigerated storage.

[0211] All publications, patents and patent applications mentioned in this specification are herein incorporated by reference into the specification to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference.

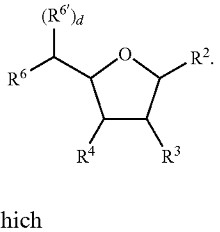
What is claimed is:

1. A method of removing essentially all of a contaminant from a mixture comprising said contaminant and a desired product comprising a moiety with a structure selected from:

I



II



in which

R^1 is selected from H, CH_2OR^7, $COOR^7$ or OR^7 wherein

R^7 is a member selected from H, substituted or unsubstituted alkyl or substituted or unsubstituted heteroalkyl;

R^2 is selected from H, OH, NH and a moiety that includes a nucleotide;

R^3, R^4, R^5, R^6 and $R^{6'}$ are independently selected from H, substituted or unsubstituted alkyl, OR^9, and NHC(O)R^{10};

wherein

R^9 and R^{10} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and sialic acid

the index d is 0 or 1;

with the proviso that at least one of R^3, R^4, R^5, R^6, and $R^{6'}$ includes the linker or linker-modifying group, said method comprising:

contacting said mixture with a first membrane for a length of time sufficient to allow essentially all of said contaminant to separate from said desired product

wherein

said mixture has a pH such that said first membrane and said desired product have a net charge of the same sign and said contaminant has a net charge which is a member selected from neutral and a sign which is opposite of the sign of the net charge for the first membrane; and

said first membrane has a molecular weight cut-off that is greater than the molecular weight of said desired product,

thereby removing essentially all of the contaminant from the mixture.

2. The method according to claim **1**, wherein said desired product and said first membrane each have a net negative charge and said contaminant has a net charge which is a member selected from neutral and a net positive charge.

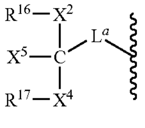
3. The method according to claim **1**, wherein the contaminant is a member selected from phosphate, pyrophosphate, nucleotide monophosphate, nucleotide diphosphate, nucleotide triphosphate, sodium phosphate, manganese chloride, sodium pyruvate, GlcNAc, magnesium sulfate, tetrasodium pyrophosphate, lactose, benzoic acid, LNT-2, LNnT, sialic acid, cytidine, CMP, benzyl alcohol, CyLac, cylexin, cytilene and sodium chloride.

4. The method according to claim **1**, wherein said first membrane is contacted with at least about 500 mg of the desired product.

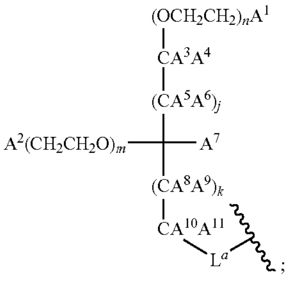
5. The method according to claim **1**, wherein the desired product is a member selected from a nucleotide sugar, glycolipid, sialylated ganglioside, LNnT, sialyl lactose and salts thereof.

6. The method according to claim **5**, wherein said nucleotide sugar is a member selected from CMP-Nan, GDP-Man, GDP-Fuc, UDP-Glc, UDP-Gal, UDP-GlcNAc, UDP-GalNAc, UDP-GlcA, UDP-IdoA, UDP-Xyl.

7. The method according to claim **5**, wherein the desired product includes a sugar moiety comprising a moiety having a structure which is a member selected from:



and



;

wherein

L^a is a linker selected from a bond, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

X^5, R^{16} and R^{17} are independently selected from polymeric moieties and non-reactive groups;

X^2 and X^4 are independently selected linkage fragments joining polymeric moieties R^{16} and R^{17} to C;

m and n are integers independently selected from 0 to 5000;

A^1, A^2, A^3, A^4, A^5, A^6, A^7, A^8, A^9, A^{10} and A^{11} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, $-NA^{12}A^{13}$, $-OA^{12}$ and $-SiA^{12}A^{13}$

wherein

A^{12} and A^{13} are members independently selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, and substituted or unsubstituted heteroaryl.

8. The method according to claim **5**, wherein, prior to said contacting, said method further comprises forming the mixture by contacting a nucleotide with a sugar and a nucleotide sugar synthetase capable of ligating said nucleotide and said sugar.

9. The method according to claim **5**, wherein, prior to said contacting, said method further comprises forming the mixture by subjecting a cell system to conditions whereby said cell system produces a nucleotide sugar.

10. The method according to claim **1**, wherein the mixture is not further purified prior to said contacting.

11. The method according to claim **1**, wherein the desired product is not further purified after said contacting.

12. The method according to claim **1**, wherein the desired product is further purified after said contacting.

13. The method according to claim **1**, wherein, prior to said contacting, said method further comprises contacting said mixture with a second membrane for a length of time sufficient to allow said desired product to pass through said second membrane and to allow molecules with a molecular weight greater than about 500 kDa to be retained in said second membrane.

14. The method according to claim **1**, wherein, prior to said contacting, said method further comprises contacting said mixture with a third membrane for a length of time sufficient to allow said desired product to pass through said third membrane and to allow molecules with a molecular weight greater than about 10 kDa to be retained in said third membrane.

15. A method of purifying a desired product from a mixture comprising a phosphorus-containing contaminant and said desired product, said method comprising:

contacting said mixture with a first membrane for a length of time sufficient to allow essentially all of said phosphorus-containing contaminant to pass through said first membrane

wherein

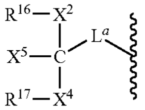
said mixture has a pH such that said first membrane and said desired product have a net negative charge and said contaminant has a net charge which is a member selected from a neutral and a positive charge; and said first membrane has a molecular weight cut-off that is greater than the molecular weight of said desired product, thereby purifying the desired product from the mixture.

16. The method according to claim **15**, wherein said first membrane is contacted with at least about 500 mg of the desired product.

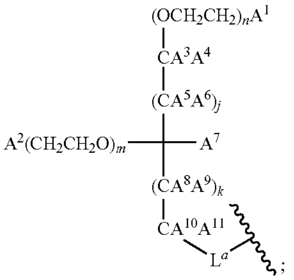
17. The method according to claim **15**, wherein the desired product is a member selected from a nucleotide sugar, glycolipid, sialylated ganglioside, LNnT, sialyl lactose and salts thereof.

18. The method according to claim **17**, wherein said desired product is a nucleotide sugar and said nucleotide sugar is a member selected from CMP-Nan, GDP-Man, GDP-Fuc, UDP-Glc, UDP-Gal, UDP-GlcNAc, UDP-GalNAc, UDP-GlcA, UDP-IdoA and UDP-Xyl.

19. The method according to claim **17**, wherein said desired product includes a sugar moiety comprising a moiety having a structure which is a member selected from:



and



;

wherein

L^a is a linker selected from a bond, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

X^5, R^{16} and R^{17} are independently selected from polymeric moieties and non-reactive groups;

X^2 and X^4 are independently selected linkage fragments joining polymeric moieties R^{16} and R^{17} to C;

m and n are integers independently selected from 0 to 5000;

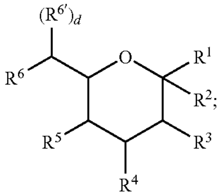
A^1, A^2, A^3, A^4, A^5, A^6, A^7, A^8, A^9, A^{10} and A^{11} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, —$NA^{12}A^{13}$, —OA^{12} and —$SiA^{12}A^{13}$

wherein

A^{12} and A^{13} are members independently selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, and substituted or unsubstituted heteroaryl.

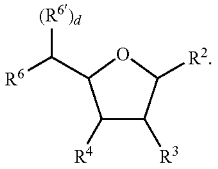
20. The method according to claim **15**, wherein said desired product comprises a moiety with a structure selected from:

I



and

II



in which

R^1 is selected from H, CH_2OR^7, $COOR^7$ or OR^7

wherein

R^7 is a member selected from H, substituted or unsubstituted alkyl or substituted or unsubstituted heteroalkyl;

R^2 is selected from H, OH, NH and a moiety that includes a nucleotide;

R^3, R^4, R^5, R^6 and $R^{6'}$ are independently selected from H, substituted or unsubstituted alkyl, OR^9, and $NHC(O)R^{10}$

wherein

R^9 and R^{10} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and sialic acid

the index d is 0 or 1;

with the proviso that at least one of R^3, R^4, R^5, R^6, and $R^{6'}$ includes the linker or linker-modifying group.

21. A composition of matter comprising a nucleotide sugar, produced by a process comprising:

contacting a mixture with a first membrane for a length of time sufficient to allow essentially all of a contaminant to pass through said first membrane

wherein

said mixture comprises said contaminant and said nucleotide sugar;

said mixture has a pH such that said first membrane and said desired product have a net charge of the same sign and said contaminant has a net charge which is a member selected from a neutral and a positive charge;

said first membrane has a molecular weight cut-off that is greater than the molecular weight of said nucleotide sugar; and

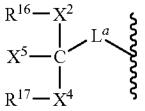
said composition of matter is essentially free of said contaminant.

22. The composition according to claim **21**, wherein said nucleotide sugar and said first membrane each have a net negative charge.

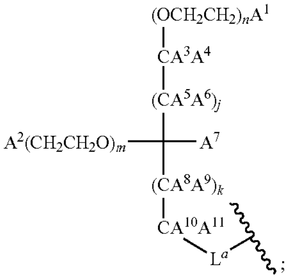
23. The composition according to claim **21**, wherein the contaminant is a member selected from phosphate, pyrophosphate, nucleotide monophosphate, nucleotide diphosphate, nucleotide triphosphate, sodium phosphate, manganese chloride, sodium pyruvate, GlcNAc, magnesium sulfate, tetrasodium pyrophosphate, lactose, benzoic acid, LNT-2, LNnT, sialic acid, cytidine, CMP, benzyl alcohol, CyLac, cylexin, cytilene and sodium chloride.

24. The composition of matter according to claim **21**, wherein said nucleotide sugar is a member selected from CMP-Nan, GDP-Man, GDP-Fuc, UDP-Glc, UDP-Gal, UDP-GlcNAc, UDP-GalNAc, UDP-GlcA, UDP-IdoA and UDP-Xyl.

25. The composition according to claim **21**, wherein the desired product includes a sugar moiety comprising a moiety having a structure which is a member selected from:



and



wherein

L^a is a linker selected from a bond, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

X^5, R^{16} and R^{17} are independently selected from polymeric moieties and non-reactive groups;

X^2 and X^4 are independently selected linkage fragments joining polymeric moieties R^{16} and R^{17} to C;

m and n are integers independently selected from 0 to 5000;

A^1, A^2, A^3, A^4, A^5, A^6, A^7, A^8, A^9, A^{10} and A^{11} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, —$NA^{12}A^{13}$, —OA^2 and —$SiA^{12}A^{13}$

wherein

A^{12} and A^{13} are members independently selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, and substituted or unsubstituted heteroaryl.

26. The composition of matter according to claim **21**, wherein said mixture is formed by contacting a nucleotide with a sugar and a nucleotide sugar synthetase capable of ligating said nucleotide to said sugar.

27. The composition of matter according to claim **21**, wherein said mixture is formed by subjecting a cell system to conditions whereby said cell system produces a nucleotide sugar.

28. A method of removing essentially all of a contaminant from a mixture comprising said contaminant and a desired product, said method comprising:

contacting said mixture with a first membrane for a length of time sufficient to allow essentially all of said contaminant to pass through said first membrane

wherein

said mixture has a pH such that said first membrane and said desired product have a net charge of the same sign and said contaminant is at its isoelectric point;

said first membrane has a molecular weight cut-off that is greater than the molecular weight of said desired product;

thereby removing essentially all of the contaminant from the mixture.

29. A method of purifying a nucleotide sugar from reactive components used to prepare said nucleotide sugar, said method comprising:

a) contacting a reactive solution comprising said nucleotide sugar with a nanofiltration membrane, thereby removing a nucleotide monophosphate and a sugar from said reactive solution while retaining said nucleotide sugar in said reactive solution,

thereby forming a desalted nucleotide sugar solution.

30. The method of claim **28**, further comprising:

b) passing said desalted nucleotide sugar solution over a charged media depth filter.

* * * * *