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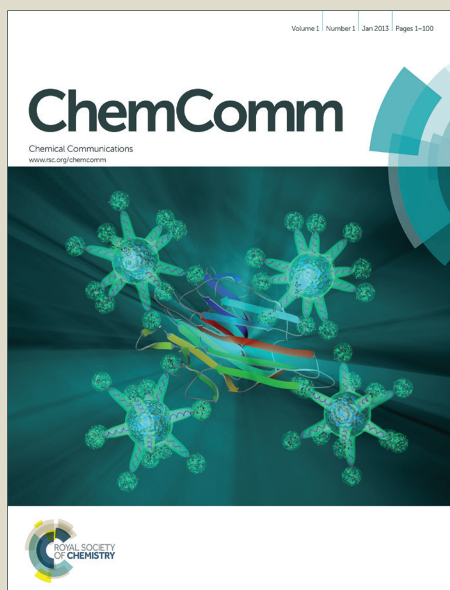

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ARTICLE TYPE

Highly efficient one-pot multienzyme (OPME) synthesis of glycans with fluorous-tag assisted purification

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Oligo(ethylene glycol)-linked light fluorous tags have been found to be optimal for conjugating to glycans for both high-yield enzymatic glycosylation reactions using one-pot multienzyme (OPME) systems and quick product purification by fluorous solid-phase extraction (FSPE) cartridges. The combination of OPME glycosylation systems and the FSPE cartridge purification scheme provides a highly effective strategy for facile synthesis and purification of glycans.

Tagging organic compounds with a light fluorous tail such as a perfluorooctyl (C_8F_{17}) or perfluorohexyl (C_6F_{13}) group followed by product purification using fluorous solid-phase extraction (FSPE) has found increasing synthetic uses.[1] For carbohydrate synthesis, non-cleavable single,[2] double,[3] and cleavable[4-6] fluorous tags have been in the acceptor glycans to allow solution-phase synthesis and fast product purification. Light fluorous protecting groups have also been used in solution-phase[7, 8] or solid-phase[9] synthesis. Odorless fluorinated thioglycosyl donors were prepared and shown excellent reactivities in glycosylation reactions.[10] Mono-perfluorooctyl (e.g. C_8F_{17}- and C_6F_{13}) tags[11-13] and a di-C_6F_{13}-tag[14] allowed non-covalent immobilization of fluorous-tagged monosaccharides and oligosaccharides on fluorocarbon-coated glass slides to generate glycan microarrays that can sustain washing processes in carbohydrate-lectin binding studies.

Except for a limited number of examples (e.g. feeding cultured animal cells with fluorous-tagged lactosides or an *N*-acetylglucosaminide for producing small amounts of elongated fluorous oligosaccharides,[15-17] using a C_3F_7 tag to facility the product purification of enzymatically synthesized heparosan oligosaccharide derivatives,[18] and using non-covalently immobilized light fluorous tagged glycans as substrates for glycosyltransferase[19] and glycosidase[20] activity assays by mass spectrometry), light fluorous tags have not been used broadly to facilitate product purification in preparative-scale enzymatic synthesis . This is most likely due to either the lower tolerance of relatively long fluorous tags (e.g. a C_8F_{17} or longer tag) by enzymatic reactions which often leads to no reaction or low yields, or the lower efficiency in FSPE cartridge-purification inherited by very short fluorous tags (e.g. a C_3F_7 tag). Here we demonstrate that with suitable linkers, light fluorous tags (e.g. C_6F_{13} and C_8F_{17}) attached to simple glycans can be tolerated by a range of glycosyltransferases in one-pot multienzyme (OPME) systems for efficient synthesis. They can facilitate facile purification of neutral and negatively charged oligosaccharide products using FSPE cartridges in less than 10 minutes. Compared to the hydrocarbon [$(CH_2)_8CO_2Me$ and octyl] tags introduced by Hindsgaul et al. which have been used successfully for glycosyltransferase activity assays,[21] the fluorous tags have additional advantages of being silent in ^1H NMR thus reducing the signals from the tag that may interfere with compound characterization.[22]

Carbohydrates are biologically important but synthetically challenging biomolecules. Our group has developed several efficient OPME approaches for synthesizing complex oligosaccharides with high yields, excellent regio- and stereo-selectivities in preparative to large scales.[23, 24] Starting from simple acceptors and free monosaccharides, each OPME reaction can be completed in 10 min – 48 hours by simply mixing multiple enzymes, a monosaccharide, an acceptor, nucleotide triphosphates, and a metal cofactor. Multiple OPME reactions can be carried out in sequential to build up more complex oligosaccharides.[25, 26] While the OPME approaches are extremely effective, the product purification remains tedious and time consuming. Usually, the combination of size-exclusion and silica gel column purifications are required to obtain the desired pure products.

To facilitate product purification of the OPME glycosylation reactions, light fluorous tagged glycosyltransferase acceptors with different lengths of perfluoroalkanes and various linkers were synthesized and tested to identify optimal fluorous tags that are well tolerated by glycosyltransferases in OPME reactions while allow facile product purification by FSPE.

Since lactose is a common core structure in many glycans and a suitable acceptor for numerous glycosyltransferases, lactosides **1–8** containing various linkers and different lengths of perfluoroalkanes including C_8F_{17}, C_6F_{13}, and C_3F_7 were synthesized (Scheme 1).

A simple C_8F_{17}-tagged lactoside **1** synthesized by direct chemical glycosylation (Scheme 1) was initially tested as an acceptor substrate for *Pasteurella multocida* α2–3-sialyltransferase 1 E271F/R313Y mutant (PmST1 E271F/R313Y) with decreased α2–3-sialidase activity.[27] The assay was performed in a one-pot two-enzyme system containing PmST1 E271F/R313Y and *Neisseria meningitidis*

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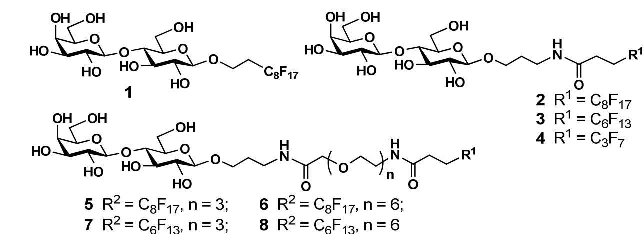
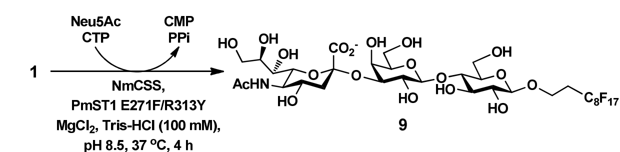
CMP-sialic acid synthetase (NmCSS) (Scheme 2).[28] The reaction was inefficient and only a trace amount (< 10%) of sialylated product was produced as shown by thin-layer chromatography (TLC) and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. The low water solubility of lactoside **1** contributed significantly to the low efficiency of the reaction. Furthermore, the rigid and rod-like perfluorooctyl chain[29] close to the lactoside may also prevent compound **1** from being a good acceptor for PmST1 E271F/R313Y. Adding an organic solvent such as DMF, DMSO, methanol, or acetonitrile for up to 30%, or 0.1% of detergents as Triton X-100, Tween 80, or HFE-7200 did not improve the yield.



Scheme 1. Fluorous-tagged lactosides that were synthesized.



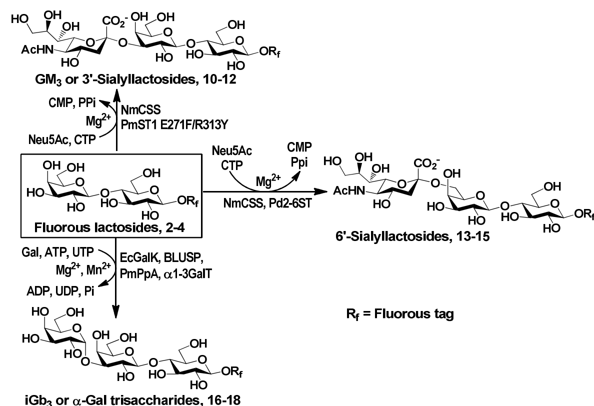
Scheme 2. Test of C_8F_{17}-tagged lactoside **1** as the glycosyltransferase acceptor in a one-pot two-enzyme sialylation system containing NmCSS and PmST1 E271F/R313Y.

To increase the solubility of the fluorous-tagged lactosides and to examine how the length of the fluorous tags affects the efficiency of the enzymatic reactions, fluorous-tagged lactosides (**2**–**4**) (Scheme 1) containing a propylamide linker and different lengths of fluoroalkyl chains were synthesized and used as the acceptors in a one-pot two-enzyme system containing NmCSS and PmST1 E271F/R313Y (Scheme 3). Sialosides (**10**–**12**) were obtained with varied yields of 22%, 92%, and 89% from lactosides tagged with C_8F_{17}, C_6F_{13}, and C_3F_7, respectively (entries **a–c** for products **10–12**) (Table 1). These results indicated that the decrease of the fluorous tag length led to the increase of the enzyme activity. Nevertheless, the short C_3F_7 fluorous tag was not sufficient to retain the sialoside product **12** in FSPE cartridge during the water washing steps (Supporting Information), rendering it an unsuitable tag for FSPE purification. In comparison, C_8F_{17}- and C_6F_{13}-tagged sialosides **10** and **11** were easily purified by a FSPE cartridge. Similar effects of the fluorous tag lengths on the glycosylation yields and FSPE product purification were observed for a one-pot two-enzyme α2–6-sialylation system containing NmCSS and *Photobacterium damselae* α2–6-sialyltransferse (Pd2,6ST)[30, 31] (Table 1, entries **d–f** for products **13–15**) and a one-pot four-enzyme galactosylation system containing *Escherichia coli* galactokinase (EcGalK),[32] *Bifidobacterium longum* UDP-sugar pyrophosphorylase (BLUSP),[33] *Pasteurella multocida* inorganic pyrophosphatase (PmPpA),[24] and bovine α1–3-galactosyltransferase (α1–3GalT)[34] (Table 1, entries **g–i** for products **16–18**).

Table 1. OPME sialylation (pH 8.5) and galactosylation (pH 7.0) of lactosides (**2–4**) containing various lengths of fluorous tags.

Entry	Acceptor	R^1	Enzyme	R^2	R^3	Product	Conversion (%)[a]
a	2	C_8F_{17}	PmST1 E271F/R313Y	Neu5Acα2-3	H	10	22 ± 12[b]
b	3	C_6F_{13}	PmST1 E271F/R313Y	Neu5Acα2-3	H	11	92 ± 5.5[b]
c	4	C_3F_7	PmST1 E271F/R313Y	Neu5Acα2-3	H	12	89 ± 9.8[b]
d	2	C_8F_{17}	Pd2-6ST	H	Neu5Acα2-6	13	31 ± 18[b]
e	3	C_6F_{13}	Pd2-6ST	H	Neu5Acα2-6	14	94 ± 6.0[b]
f	4	C_3F_7	Pd2-6ST	H	Neu5Acα2-6	15	qt.[b]
g	2	C_8F_{17}	α1-3GalT	Galα1-3	H	16	10 ± 0.0[c]
h	3	C_6F_{13}	α1-3GalT	Galα1-3	H	17	60 ± 0.5[c]
i	4	C_3F_7	α1-3GalT	Galα1-3	H	18	qt.[c]

[a]Determined by thin-layer chromatography and ImageQuant 5.2 after staining of the plate. Reaction was assayed at 4 h[b] or 15 h[c].



Scheme 3. OPME glycosylation of fluorous-tagged lactosides.

Table 2. OPME sialylation glycosylation (pH 8.5) and galactosylation (pH 7.0) of fluorous-tagged lactosides (**5–8**) containing a TEG or an HEG linker.

Entry	Acceptor	R^1	n	Enzyme	R^2	R^3	Product	Conversion (%)[a]
j	5	C_8F_{17}	3	PmST1 E271F/R313Y	Neu5Acα2-3	H	19	65 ± 11[b]
k	6	C_8F_{17}	6	PmST1 E271F/R313Y	Neu5Acα2-3	H	20	55 ± 7.8[b]
l	5	C_8F_{17}	3	Pd2-6ST	H	Neu5Acα2-6	21	47 ± 10[b]
m	6	C_8F_{17}	6	Pd2-6ST	H	Neu5Acα2-6	22	52 ± 5.6[b]
n	5	C_8F_{17}	3	α1-3GalT	Galα1-3	H	23	81 ± 8.0[c]
o	6	C_8F_{17}	6	α1-3GalT	Galα1-3	H	24	66 ± 1.0[c]
p	7	C_6F_{13}	3	PmST1 E271F/R313Y	Neu5Acα2-3	H	25	70 ± 4.8[b]
q	8	C_6F_{13}	6	PmST1 E271F/R313Y	Neu5Acα2-3	H	26	80 ± 15[b]
r	7	C_6F_{13}	3	Pd2-6ST	H	Neu5Acα2-6	27	65 ± 7.9[b]
s	8	C_6F_{13}	6	Pd2-6ST	H	Neu5Acα2-6	28	66 ± 25[b]
t	7	C_6F_{13}	3	α1-3GalT	Galα1-3	H	29	71 ± 0.5[c]
u	8	C_6F_{13}	6	α1-3GalT	Galα1-3	H	30	69 ± 1.0[c]

[a]Determined by thin-layer chromatography and ImageQuant 5.2 software after staining. Reaction was assayed at 4 h[b] or 15 h[c].

In order to improve the compatibility of lactosides with longer fluorous tags (e.g. C_8F_{17}) to the OPME glycosylation reactions, a tri- (TEG) or a hexa-ethylene glycol (HEG) linker was introduced to obtain fluorous-tagged lactosides **5–8** (Scheme 1). OPME α2–3/6-sialylation, and α1–3-galactosylation reactions (Scheme 3) showed that the TEG and HEG linkers improved significantly the yields of C_8F_{17} -tagged lactosides (Table 2).

The length of the oligo(ethylene glycol) (OEG) linker with either 3 or 6 ethylene glycol repeats, however, does not seem to affect the efficiency of the OPME reactions significantly (Table 2). While TEG/HEG-C_6F_{13}-tagged lactosides still led to better

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yields, TEG/HEG-C_8F_{17}-tagged lactosides provided sufficiently good yields for practical production of glycosylated products.

Encouraged by these results, preparative-scale synthesis and FSPE cartridge purification of GM3, 6'-sialyllactoside, and iGb_3 glycan analogs (**11**, **14**, **23**, **25**, **27**, and **29**) were carried out (Table 3). After total consumption of the fluorous-tagged lactoside acceptor in each OPME reaction by TLC monitoring, the reaction mixture was centrifuged and the supernatant was purified using an 10 c.c. FSPE cartridge containing 2 g of fluorous silica gel by simply loading the reaction mixture to the cartridge, washing with four-column volumes of water, and eluting the fluorous-tagged product using 100% MeOH. Nuclear magnetic resonance (NMR) spectroscopy and high resolution mass spectrometry (HRMS) analyses demonstrated that one-step FSPE cartridge purification was sufficient to provide desired products with high purity (Supporting information). It is noteworthy that the FSPE cartridge purification is suitable for purifying both neutral (galactosides) and negatively charged (sialosides) trisaccharide products in a simple, quick, and efficient manner. The whole purification process generally takes 5–7 minutes, which is substantially less than conventional silica gel (2–6 hours) and size-exclusion column (>3 hours) purifications for unprotected sugars. The quick purification of fluorous-tagged glycans also allows the yield improvement by subjecting the mixture of fluorous-tagged glycan acceptor and product for the second round of enzymatic synthesis (e.g. synthesizing compound **23** in Table 3).

Table 3. Preparative-scale OPME synthesis of oligosaccharides with FSPE cartridge purification.

Acceptor	Enzyme	Product	Yield (%)[a]
3	PmST1 E271F/R313Y	11	72
3	Pd2-6ST	14	79
5	α1-3GalT	23	82[b]
7	PmST1 E271F/R313Y	25	86
7	Pd2-6ST	27	qt.
7	α1-3GalT	29	89

[a]Isolated yields; [b]The reaction mixture was purified by FSPE and underwent another round of OPME reaction.

In conclusion, a series of fluorous-tagged sialosides and galactosides were synthesized in preparative scales using highly efficient OPME glycosylation approaches and were purified by FSPE cartridges in a simple, quick, and efficient fashion. Introducing a TEG or HEG linker to the C_8F_{17} or C_6F_{13}-tagged lactoside improved the solubility of the substrates and increased the OPME glycosylation yields without compromising the FSPE purification process. The combination of OPME glycosylation systems and the FSPE cartridge purification is a simple and highly effective strategy for facile synthesis and purification of oligosaccharides. The method will be further explored with suitable cleavable linkers for the preparative and large scale synthesis of more complex oligosaccharides and polysaccharides and be adapted to automated enzymatic synthesis of challenging carbohydrate targets.

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Notes and references

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†Electronic Supplementary Information (ESI) available: Experimental details for chemical and enzymatic synthesis, NMR and HRMS data. See DOI: 10.1039/b000000x/

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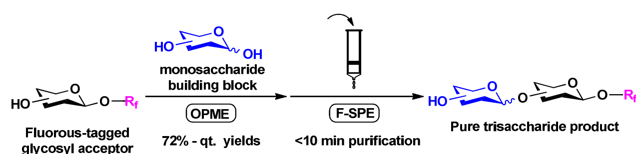
Fluorous-tagged glycans with an oligo(ethylene glycol) linker are well tolerated acceptor substrates for glycosyltransferases which allowed facile synthesis of glycans by efficient one-pot multienzyme (OPME) systems with easy and quick purification by fluorous solid-phase extraction (FSPE) cartridges.



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