Calcium iodide mediated coupling of complex fluorooligosaccharides to phenolic peptide

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Abstract

Besides serving as therapeutic agents and building blocks for glycoprotein synthesis, homogeneous synthetic glycopeptides also benefit understanding function of specific glycoform. However, the selectivity coupling of oligosaccharides to a given peptide remains challenging despite the fact that the synthesis of structure-defined complex oligosaccharides has been greatly facilitated by chemoenzymatic based approaches. Herein, a Ca^{2+}-promoted glycosylation approach was developed to exclusively modify phenolic peptide using a panel of biologically important glycans.

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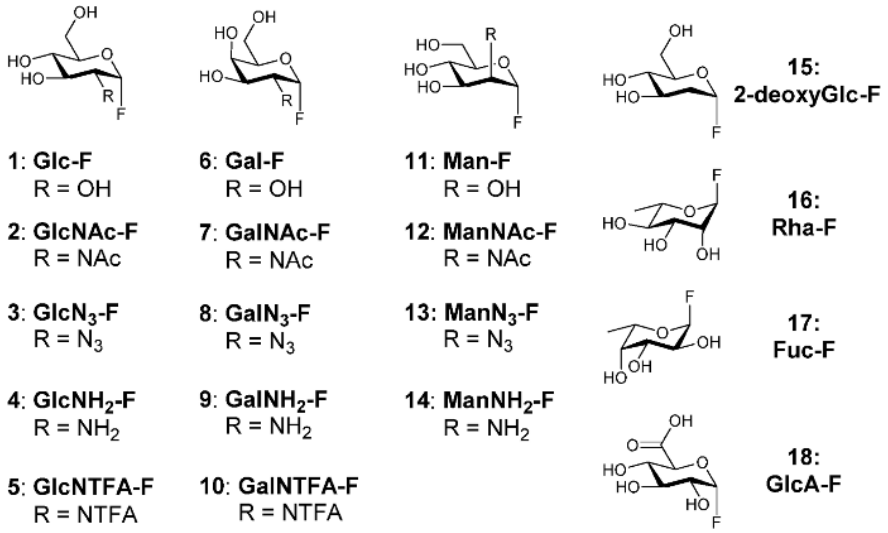
Keywords

Calcium iodide | α-fluoroglycoside | glycosylation | coupling | glycopeptide

Comprehensive Summary

Besides serving as therapeutic agents and building blocks for glycoprotein synthesis, homogeneous synthetic glycopeptides a



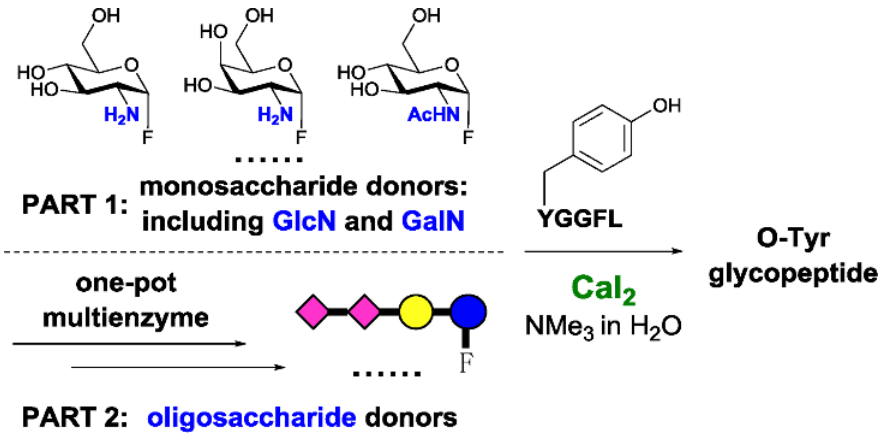
Background and Originality Content

Glycosylation is a ubiquitous post-translational modification and a key mechanism in determining chemical diversity of natural products.[1, 2] Protein glycosylation involving the attachment of complex sugar moieties (glycans) to amino acid side chains plays important roles in protein folding and a diverse range of biological processes.[1, 3] Glycosylation can also modulate the physiological properties of small molecules and peptides, such as improved metabolic stability, subcellular localization, ligand–target interactions and often their bioactivity.[4, 5]

Other than utilizing in the synthesis of large glycopeptides through native chemical ligation (NCL),[6]synthetic glycopeptides are extant as important biochemical probes and therapeutic agents.[5, 7] Typically, glycopeptides are prepared using solid-phase peptide synthesis through incorporating protected-glycosyl residues. The selective glycosylation of unprotected peptides in aqueous solvents, however, has been limited to 'click' S-glycosylation of cysteine,[8, 9] aspartylation of glycol-amide (such as Man9GlcNAc2-NH_2)[10]or enzymatical introduction of glucose to a recognizable N- or O-glycosylation site.[11-13]

More recently, an approach that enable aqueous O-glycosylation of peptide-embedded tyrosine employing glycosyl fluorides has been reported.[14] Originally, the glycosylation reactions have been discovered by Miller between α-glycosyl fluorides and sucrosyl acceptors in the presence of calcium ion and trimethylamine.[15] The formation of O-β-glucosyl products evidenced that both sucrose and Tyr O-glycosylation proceed via a S_N2-type substitution. In nature, GlcNAc and GalNAc are most common hexosamines and initiating sugars of N- and mucin-type O-glycans, respectively. More recently, GalNAc O-glycosylation of Tyr residues were also identified in human and murine proteins.[16-18]It remains to be investigated whether hexosamines can be installed onto Tyr residue in the same manner. Plus, chemo-enzymatic methods, which employ a range of glycosyl transferases to modify a synthetic oligosaccharide precursor, has greatly facilitated the stereo controlled synthesis of complex carbohydrates in two decades.[19-22]Therefore, it is highly intriguing to investigate whether the Ca^{2+}-promoted glycosylation can be extended for coupling structurally well-defined oligosaccharides with peptide-embedded Tyr (Scheme 1).

Scheme 1 Tyr O-glycosylation in water *via*Ca^{2+} promotion of fluoride donors



Results and Discussion

We firstly synthesized α-fluoroGlcNAc and α-fluoroGalNAc, but found that neither of them could give glycosylation product of leu-enkephalin, being an opioid receptor ligand, under the promotion of $Ca(OH)_2$. Next, their fluoro-analogs with amine or azido substitution at C-2 were evaluated for their potentials as glycosyl donors (Figure 1). To our delight, $GlcNH_2$ modified leu-enkephalin formed from α-fluoro$GlcNH_2$ in a low yield (10%) although α-fluoro$GalNH_2$

Figure 1 Eighteen synthesized α-fluoro donors of monosaccharides

Table 1 Optimization of Ca^{2+} promoter: focusing on anions

Entry	Ca^{2+} promoter	Solvent	HPLC Yield (%)[a]
1	$Ca(OH)_2$	H_2O	10
2	Ca-EDTA		4
3	Ca-Levulinate		8
4	Ca-Ascorbate		N.D.[d]
5	Ca-Glycerophosphate	45 wt% NMe_3 in H_2O	6
6	$Ca(ClO_4)_2$		11
7	$Ca(OTf)_2$		11
8	$Ca(H_2PO_4)_2$		4
9	$Ca(OAc)_2$		13
10	$CaCl_2$		13
11	$CaBr_2$		19
12	**CaI_2**		**37**

[a] Yields were calculated based on $\lambda_{214\ nm}$-peak areas of peptide**19** and glyco-peptide **4P** in HPLC spectra.[d] N.D. refers to not detected.

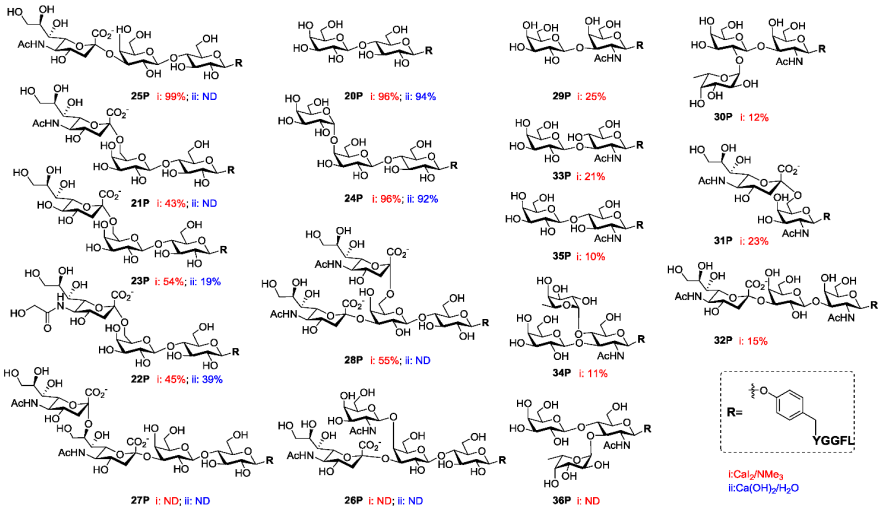
led to no product. Previous studies suggested that the glucosylation of sucrose requires a special array of substrate hydroxyl groups (such as OH-2, OH-1' and OH-3') that presumably engage both Ca^{2+} and sucrose with multiple contacts.[15] OH-2 on glycosyl donors could also involve an analogous interaction and in consequence affected the glycosylation efficiency.

In order to improve the product yield, a series of metal basic or combinations of Ca^{2+} ion and aqueous amine bases were screened (Table 1 and Table S1). Finally, a significant improved conversion of $GlcNH_2$ modified leu-enkephalin (37%) was achieved under the optimized condition in the present of CaI_2 and trimethylamine.

O-GlcNacyl-leu-enkephalin was then quantitatively obtained by one step acylation. Meanwhile, CaI_2 exhibited good compatibility to other fluoromonosaccharides lacking either OH-2 or OH-6, such as GlcNAc-F, $GalNH_2$-F and GlcA-F (Table 2). All monosaccharide coupled leu-enkephalin were characterized by high resolution mass spectroscopy (Supplementary pages 13-18). Only β-anomer products were observed when fluoromonosaccharides including α-fluoro Glc, Gal, derivatives of Glc and Gal, and Fuc were afforded. In contrast, α-fluoro Man and Rha involved reaction produced α-anomer products that is consistent with a proposed double inversion process involving the axial C2-OH group.[14]

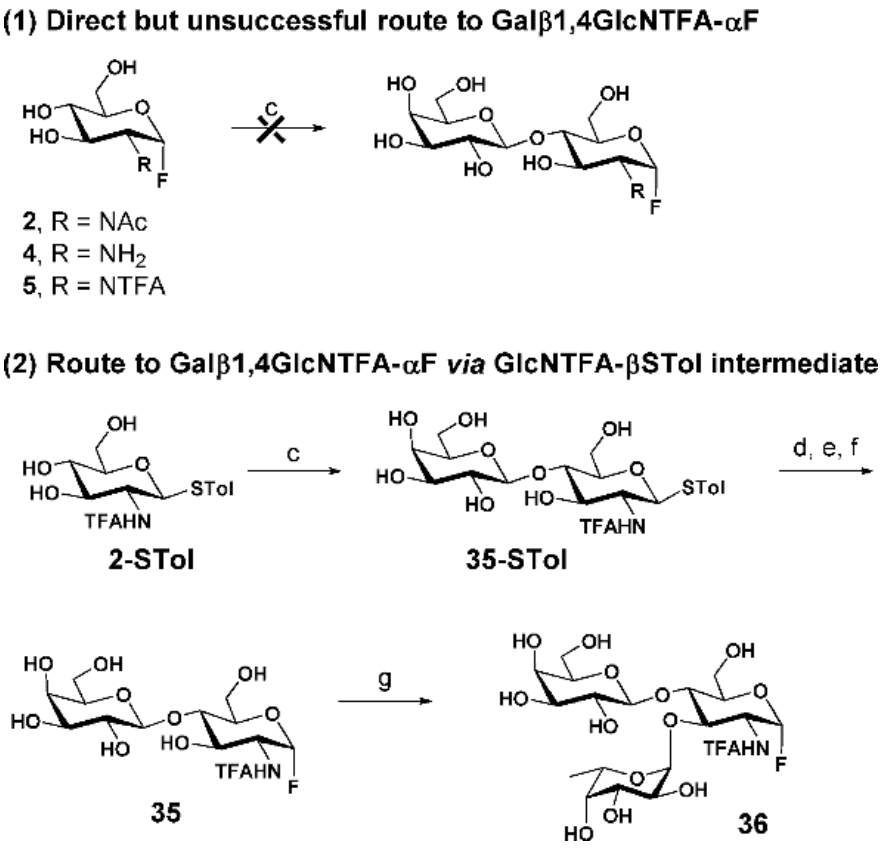
Table 2 Tyr O-glycosylation of α-fluorides (**1-18**) with YGGFL peptide: CaI_2 /NMe_3 vs. $Ca(OH)_2$

α-fluoride donor	α-fluoride donor	Glyco-peptide product Product structure	Glyco-peptide product HPLC yield (%) CaI_2[a]	Glyco-peptide product HPLC yield (%) $Ca(OH)_2$[a]
Glc- type	**1**	β-Glc-YGGFL (**1P**)[b]	100	85
	2	β-GlcNAc-YGGFL (**2P**)	22	N.D.[d]
	3	N/A[e]	N.D.[d]	N.D.[d]
	4	β-$GlcNH_2$-YGGFL (**4P**)	37	10
	5	β-$GlcNH_2$-YGGFL(**4P**)[c]	31	30
Gal- type	**6**	β-Gal-YGGFL (**6P**)	72	30
	7	N/A[e]	N.D.[d]	N.D.[d]
	8	N/A[e]	N.D.[d]	N.D.[d]
	9	β-$GalNH_2$-YGGFL (**9P**)	31	N.D.
	10	β-$GalNH_2$-YGGFL(**9P**)[c]	26	11
Man- type	**11**	α-Man-YGGFL (**11P**)	49	53
	12	N/A[e]	N.D.[d]	N.D.[d]
	13	N/A[e]	N.D.[d]	N.D.[d]
	14	N/A[e]	N.D.[d]	N.D.[d]
deoxy type	**15**	N/A[e]	N.D.[d]	N.D.[d]
	16	α-Rha-YGGFL(**16P**)	N.D.[d]	42
	17	β-Fuc-YGGFL (**17P**)	69	57
GlcA	**18**	β-GlcA-YGGFL (**18P**)	31	N.D.



[a] Yields were calculated based on $\lambda_{214\ nm}$-peak areas of peptide **19** and corresponding product in HPLC spectra.[b]Reaction time=10 min. [c] For donors **5**and **10** , NTFA group was converted to amino group under the reaction conditons, and therefore their glycosylation products were the same to donors **4** and **9** respectively.[d] N.D. refers to not detected.[e] N/A refers to not applicable.

F igure 2 Synthesis of oligosaccharide coupled leu-enkephalin



Scheme 2 Synthesis of α-fluoroLacNAc (**35**) and α-fluoroLewis X (**36**)

Reaction conditions: (c) One-pot multienzyme: Gal, SpGalK, SpGalU and NmlgtB; (d) Ac_2O and Pyridine; (e) NIS, DAST and DCM; (f) NaOMe and MeOH; (g) One-pot multienzyme: Fuc, BfFKP and Hp3/4FT.

With the optimized condition established, a number of biologically important glycans were ready to prepared by using α-fluorolactosyl or suitable α-fluoromonosaccharides as starting materials. Glycosyltransferases that catalyze glycosidic bond formation in high regio- and stereo-selectivity often remain relative strict substrate specificity as well. In this study, most enzymes except for BiGalHexNAcP and LgtB can well recognize α-fluoroglycosides. BiGalHexNAcP, being a galactosyl β1,3-N-acetyl-hexosamine phosphorylase from*Bifidobacterium infantis* , is capable of reversely taking both GlcNAc and GalNAc as acceptors to form β1,3-linked galactosides.[23] Herein, we unexpectedly found that neither α-fluoroGlcNH_2 nor α-fluoroGalNH_2 was acceptable for BiGalHexNAcP. However, α-fluoroGlcNTFA and α-fluoroGalNTFA that bearing a larger trifluoroacetamido group at C2 were able to be efficiently glycosylated by the enzyme.

At the same time, we found that CaI_2-mediated glycosylation reactions that employ either α-fluorohexosamines or their α-fluoro N-trifluoroacetyl derivatives performed in similar conversions and generated identical products, namely, hexosamine modified leu-enkephalins (Table 2). Therefore, carbohydrate determinants of TF (**29**), sialyl TF (**32**), Sialyl-Tn (**31**), Lacto-N-biose I (**33**), Lewis a (**34**) and H antigen (type 3, **30**) were prepared with α-fluoroGlcNTFA or α-fluoroGalNTFA as starting materials (Supplementary pages 27-32). Because *Neisseria meningitidis* derived β1,4-galactosyltransferase LgtB can only recognize β-STolGlcNTFA but β-STolGlcNH_2, α-fluoroGlcNTFA or α-fluoroGlcNH_2, the Galβ1,4GlcNTFAβSTol disaccharide was firstly synthesized and then converted to Galβ1,4GlcNTFAαfluoride (**35**) which can be further fucosylated to form Lewis X fluoride (Scheme 2). Other Kdn, Neu5Ac or Neu5Gc containing sialosides were synthesized starting from α-fluorolactose (Supplementary pages 23-27). All reactions were carried out in a one-pot multienzyme fashion where sugar nucleotides were generated *in situ* by using nucleotidyltransferases and/or glycokinases and transferred to individual acceptors by corresponding glycosyltransferases.[24]

As shown in Figure 2, both α-fluorolactose (**20**) and α-fluoroGb3 (αF-Galα1,4Lac, **24**) involved glycosylation proceeded in excellent yields in the presence of $Ca(OH)_2$. However, for α-fluorosialosides, $Ca(OH)_2$ and CaI_2-promoted glycosylation showed distinct compatibility or efficiency. With $Ca(OH)_2$, α2,6KDN- and α2,6Neu5Gc-modified Lactosyl leu-enkephalins were obtained in relative low yields (19% and 39%, respectively). α-fluorosialosides containing Neu5Ac even failed to generate glycosyl products regardless of the sialyl linkages. However, exchanging $Ca(OH)_2$ with CaI_2 led to near complete conversion of leu-enkephalin to GM3 (**25**) modified glycopeptide. α2,6sialylated lactosyl leu-enkephalins decorated by all three natural forms including KDN (**23**), Neu5Ac (**21**) and Neu5Gc (**22**) also formed in moderate conversations (43%-54%) under the promotion of CaI_2. Apparently, the hydrogen-bonding network of substrate is more dependable for $Ca(OH)_2$-mediated glycosylation. Notably, α2,6Neu5Ac(α2,3Neu5Ac)lacosyl fluoride (**28**) was an efficient glycosyl donor in comparison of totally inactive α2,8Neu5Acα2,3Neu5Aclacosyl fluoride (**27**) under the optimized condition, suggesting that stereo-configuration of glycosyl donor might also affect the glycosyl transfer.

All α-fluorooligosaccharides initiating with α-fluoroGlcNTFA or α-fluoroGalNTFA underwent glycosylation with CaI_2 in spite of relative low yields. The moieties directly linked to leu-enkephalins were identified as GlcNH_2 or GalNH_2. The hexosamine containing glycosyl leu-enkephalins were further acylated to give the final products almost quantitatively. Thus, TF (**29**), sialyl TF (**32**), sialyl Tn (**31**), Lacto-N-biose I (**33)** , Lewis a (**34**) and H antigen (type 3, **30**) coupled leu-enkephalins were synthesized in two sequential steps. All oligosaccharide coupled glycopeptides were characterized by high resolution mass spectroscopy (Supplementary pages 32-39). α-fluoroGM2 (**26**) that is a branched ganglioside with residues of Neu5Ac and GalNAc at the non-reducing end was not an active glycosyl donor in the Ca^{2+}-promoted reaction.

Besides most abundant Asn N-glycosylation and Ser/Thr O-glycosylation, natural glycosyl modification also occurs at Trp, Tyr and hydroxylated lysine. Enzymes that catalyze peptide glycosylation have mostly evolved stringent selectivity on both glycosyl donor and acceptor. For example, a N-glycosyltransferase de-

rived from *Actinobacillus pleuropneumoniae* (ApNGT) is capable of transferring Glc but substituted $GlcNH_2$, $GlcN_3$ and GlcNAc to an asparagine residue in the conserved Asn-X-Ser/Thr (where X [?] Pro) sequon.[25] The Glc-tagged peptide/protein can be further remodelled by various endo-β-N-acetylglucosaminidases (ENGases) to exclusively add a preassembled high-mannose type or complex type N-glycan.[12] Another example is *Legionella* effector SetA that site-specifically introduces O-glucose onto a Ser/Thr-X-Leu-Pro/Gly sequence motif.[13] In contrast, CaI_2-mediated glycosylation exhibits excellent tolerance toward an array of complex glycosyl donors, especially for Neu5Ac attached glycosyl fluorides. Glycopeptides conjugated with N-acetyl-hexosamine initializing carbohydrates can alternately achieved by employing its hexosamine or N-trifluoroacetyl hexosamine derivatives as donors followed by an acetylation reaction. Thus, Ca^{2+}-promoted glycosylation was able to be further generalized and turned out to be an alternate and facile glycosylation mean other than chemical or enzymatic formation of Cys-O-, Asn-N- or Ser/Thr-O-glycosides. CaI_2-mediated coupling approach offers the exceptional flexibility between glycosyl donors and peptides, while the regio- and stereo-selectivity within oligosaccharides was well defined by glycosyltransferases. In addition, optimized reaction condition with CaI_2 instead of $Ca(OH)_2$ allowed to lower the equivalent of glycosyl donors used in reaction. For example, reducing lactosylfluoside by half did not obviously impair the yield (Supplementary page 33).

Conclusions

Though the synthesis of structure-defined complex oligosaccharides has been greatly facilitated by chemoenzymatic based approaches, the selectively coupling of these oligosaccharides to a given peptide remains challenging. Herein, a series of α-fluorooligosaccharides were readily produced in a one-pot multienzyme fashion, and 14 of them excepting for GD3 (**27**), GM2 (**26**) and Lewis X (**36**) fluoroglycosides were successfully used to generate their corresponding glycopeptides in Ca^{2+}-promoted glycosylation reaction.

Experimental

One pot multienzyme synthesis of α-fluorooligosaccharides: A mixture of the glycosyl donor, acceptor of α-fluorosaccharide, nucleoside 5'-triphosphate, a suitable glycokinase, nucleotidyltransferase and glycosyltransferase was buffered with Tris-HCL and incubated at 37 °C. The product formation was monitored by TLC. The reaction was stopped by adding the same volume of ice-cold ethanol and incubated at 4 °C for 30 min. The mixture was centrifuged and the precipitates were removed. The supernatant was concentrated and passed through a silica gel column followed by a Bio-Gel P-2 gel filtration column purifcation to give the product.

General synthesis of glycopeptides procedure: To a flask were sequentially added YGGFL peptide (0.3 μmol, 1.0 equiv), a fluoro donor (0.3 mmol, 1000 equiv.), CaI_2 (0.3 mmol, 1000 equiv.) and 45 wt% NMe_3/H_2O (300 μL). The flask was sealed with a rubber septum, and the reaction mixture was stirred at room temperature for 12 h. The resulting mixture was then centrifuged at 12000 rpm for 10 min. Supernatant was analyzed by HPLC (Hypersil Gold C18 5 μm 4.8 mm × 250 mm, 1 mL/min flow rate; gradient = 5 % B for 5 min, linear gradient of 5 % B to 50 % B in 11.5 min, held at 50 % for a further 5 min; buffer A = 0.5 0.5 $Ca(OH)_2$ (0.3 mmol, 1000 equiv.) in 300 μL water was used to replace CaI_2 in 45 wt% NMe_3/H_2O.

Supporting Information

The supporting information for this article is available on the WWW under https://doi.org/10.1002/cjoc.2023xxxxx.

Acknowledgement

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Calcium iodide mediated coupling of complex fluorooligosaccharides to phenolic peptide Pei Liu, Xing Quan, H
α-fluorooligosaccharides were chemo-enzymatically synthesized and further coupled with peptide-embedded Tyr under the p