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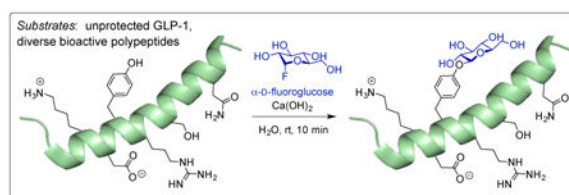
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Rapid Phenolic *O*-Glycosylation of Small Molecules and Complex Unprotected Peptides in Aqueous Solvent

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Abstract



Glycosylated natural products and synthetic glycopeptides represent a significant and growing source of biochemical probes and therapeutic agents. However, methods that enable the aqueous glycosylation of endogenous amino acid functionality in peptides without the use of protecting groups are scarce. Here, we report a transformation that facilitates the efficient aqueous *O*-glycosylation of phenolic functionality in a wide range of small molecules, unprotected tyrosine, and tyrosine residues embedded within a range of complex, fully unprotected peptides. The transformation, which uses glycosyl fluoride donors and is promoted by $\text{Ca}(\text{OH})_2$, proceeds rapidly at room temperature in water with good yields and selective formation of unique anomeric products depending on the stereochemistry of the glycosyl donor. High functional group tolerance is observed, and the phenol glycosylation occurs selectively in the presence of virtually all side chains of the proteinogenic amino acids with the singular exception of Cys. This method offers a highly selective, efficient, and operationally simple approach for the protecting group-free synthesis of *O*-aryl glycosides and Tyr-*O*-glycosylated peptides in water.

Glycosylation can modulate the physiological properties of small molecules and peptides, with specific impacts such as improved metabolic stability,¹ membrane permeability,² biodistribution,³ and ligand-target interactions.^{4,5} Thus, numerous glycosylated natural products and synthetic glycopeptides are extant as important biochemical probes and therapeutic agents (some examples are shown in Figure 1a).^{6,7,8,9,10} While methods for the

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glycosylation of organic compounds are numerous, glycosylation methods typically require protection of glycosyl donors and acceptors.^{11,12,13} The most common exceptions generally involve the application of glycosyl transferases in the enzymatic context.^{14,15,16,17} The optimization of glycosylation methodology for reactions conducted in organic solvents has made applications on highly polar compounds such as native oligopeptides rare.¹⁸ Perhaps for related reasons, despite the introduction of many new methods for selective peptide/protein synthesis, strategies for the selective glycosylation of endogenous amino acid functionality in unprotected peptides, and in aqueous solvents, remain scarce.^{19,20,21,22,23,24} To date, such transformations have been limited to *S*-glycosylation of cysteine (Cys)^{25,26,27,28} and amide coupling of aspartic acid (Asp) to form *N*-glycosides.²⁹ Reported herein is an efficient *O*-glycosylation of phenolic functionality in a wide range of small molecules, the amino acid tyrosine (Tyr), and of the Tyr residue embedded within a range of complex, water-soluble, fully unprotected peptides. Features of this method include rapid reaction rates and good yields with high anomeric selectivity, even as the glycosylation occurs in bulk aqueous solvent. At the same time, high functional group tolerance is observed such that the desired reaction occurs in the presence of virtually all side chains of the proteinogenic amino acids (with the singular, but potentially exploitable exception of Cys), with attendant high chemoselectivity for phenolic *O*-glycosylation, including Tyr-*O*-glycosylation, even in the presence of other nucleophilic, potential acceptor functionality.

Naturally occurring Tyr-*O*-glycosides have been observed in the active site of glycogenin,³⁰ certain prokaryotic surface-layer glycoproteins,³¹ and amyloid β -peptides detected in human cerebrospinal fluid.^{32,33} Currently, strategies to access Tyr-*O*-glycopeptides entail solid phase peptide synthesis incorporating protected-glycosyl Tyr residues that are prepared via aromatic *O*-glycosylation prior to peptide chain assembly (Figure 1b).^{34,35,36} Biosynthetic approaches with synthetically preglycosylated amino acids have also been reported.³⁷ A wide range of methods for aromatic *O*-glycosylation have been developed since the coupling of peracetyl glucopyranosyl chloride and potassium phenolate was reported by Michael in 1879,³⁸ and many of these methods reflect mild reaction conditions, tolerance of various functional groups, and high anomeric selectivity (Figure 1c) so long as the chemistry occurs in organic solvents with protected substrates.^{39,40,41,42,43,44} Major advances in catalytic control of glycosylations, the complexity of oligosaccharides that are available, and our mechanistic understanding of key reactions are all emerging.^{45,46,47} At the same time, current aromatic methods share two features that might be viewed as limitations for the goal of convergent Tyr-*O*-glycosylation of complex peptides: each requires multiple protecting groups to ensure reaction specificity, and most are based on chemistry that functions well only in a non-aqueous solvent or co-solvent. The development of new, phenolic *O*-glycosylation strategies for substrates soluble only in water demand reactions that proceed with high efficiency in water, which is a potentially competitive nucleophile for most activated glycosyl donors. An additional, highly desirable feature would be efficient glycosylation that occurs in the presence of the diverse functionality characterizing unprotected polypeptide chains of natural or synthetic origin.

To enable aqueous glycosylation, glycosyl donors that do not undergo hydrolysis or hydrolyze slowly are required. Glycosyl fluorides, developed and studied by Mukaiyama and Nicolaou, exhibit relative stability toward hydrolysis and have emerged as useful

glycosyl donors in conventional chemical glycosylation.^{48,49,50} Glycosyl fluorides have also seen wide usage as transition state-analogue substrates (TSAS) for hydrolases^{51,52} and as agents for proteomic profiling.⁵³ From the physical organic perspective, Jencks and co-workers reported the aqueous substitution of α -fluoroglucose by sodium azide, notably avoiding hydrolysis and observing complete inversion of stereochemistry to produce the β -anomer.^{54,55} Capitalizing on this observation, our laboratories developed a method to effect the regioselective glycosylation of unprotected sucrose and sucrose analogues in an aqueous medium. This reaction employed α -fluoroglycosides as the glycosyl donors and proceeded efficiently for a range of sucrose analogues as glycosyl acceptors in the presence of Ca^{2+} additives (Figure 1d).⁵⁶ Mechanistic studies of this intriguing process, including nuclear magnetic resonance (NMR) studies and examination of the exhaustive set of mono-deoxysucrose analogues, revealed that the glycosylation of sucrose requires a discrete array of substrate hydroxyl groups that presumably engage both Ca^{2+} and the glycosylation substrates with multiple contacts. The reaction is remarkably specific. However, inspired once again by the stability of glycosyl fluorides, their classical reactivity toward anionic nucleophiles, and our prior results, we investigated whether these glycosyl donors could be utilized for the fully aqueous *O*-glycosylation of phenols, including peptide-embedded Tyr.

Results and Discussion

Our experiments began by examining the glycosylation of *N*-Boc-tyrosine (**1**) under conditions previously developed for the selective glycosylation of sucrose (Table 1, entry 1). The reaction proceeded with complete conversion to generate *N*-Boc-*O*- β -D-glucopyranosyl-L-tyrosine (**2**). NMR analysis of the reaction mixture indicated glycosylation exclusively at the hydroxyl group of **1**, with no products of aromatic *C*-glycosylation or carboxylic acid *O*-glycosylation. We then investigated whether conditions without trimethylamine could be developed in order to improve compatibility with more complex substrates. However, exclusion of trimethylamine, in aqueous solvent and $\text{Ca}(\text{OTf})_2$ as a promoter, led to no conversion, highlighting the need for alkaline conditions (Table 1, entry 2). Exchanging $\text{Ca}(\text{OTf})_2$ with $\text{Ca}(\text{OH})_2$ proved effective, generating the *O*-glucosyl (*O*-Glc) product with near complete conversion (95%; Table 1, entry 3). Interestingly, investigation of other alkali and alkaline earth metal hydroxides underscored the special reactivity enabled by Ca^{2+} (Table 1, entries 4–9). Omitting a metal salt altogether preempted product formation (Table 1, entry 10), and only slow hydrolysis of the glycosyl fluoride was observed. Optimization of the reagent loading and concentration (entries 11–17) culminated in efficient conditions to produce products in nearly quantitative yield (entry 16). Finally, time-course studies revealed the fast rate of the reaction, showing that the reaction is nearly complete after only 10 minutes (Table 1, entries 16 and 17).

Notably, the optimized glycosylation conditions generate exclusively the *O*- β -glucosyl product, providing evidence for a $\text{S}_{\text{N}}2$ -type mechanism. This observation is analogous to those recorded in the $\text{Ca}^{2+}/\text{NMe}_3$ -promoted sucrose glycosylation using α -D-fluoroglycosides under aqueous conditions;⁵⁶ so too, this observation recalls that of Jencks in the study of displacement of α -D-fluoroglucose by azide ion, later studied computationally.^{55,57,58,59} The observed increase in conversion as a function of increased salt concentration is also consistent with the ionic-strength dependence of a generic $\text{S}_{\text{N}}2$

reaction mechanism, also observed by Jencks.⁵⁵ The stereochemical purity of the product is independent of reaction duration; under 1.0 M reaction conditions, only the β -anomer product was observed after 10 min and after 24 h reaction times. No evidence of epimerization of the Tyr α -stereocenter after 24 h was detected under these conditions (see Supplementary Pages 18–22).

With suitable reaction conditions for Tyr *O*-glycosylation established, we evaluated a range of phenols as glycosyl acceptors (Figure 2). Protected *O*-glucosyl tyrosine products **2** and **3**, generated from Boc-Tyr-OH and Ac-Tyr-NH₂, respectively, were each isolated in 91% yield. Fully unprotected *O*-glucosyl tyrosine **4** was isolated in 92% yield, with exclusive glycosylation of the phenolic hydroxyl group of tyrosine despite the presence of a primary amine. Conversion of phenol to **5** proceeded in 92% yield, and substituted aryl glucosides including 4-methoxy (**6**; 92% yield), 4-chloro (**7**; 93% yield), 4-cyano (**8**; 80% yield), and 4-trifluoromethyl (**9**, 60% yield) were all isolated in good to excellent yields. Higher yields with more electron-rich (less acidic) phenols may result from greater nucleophilicity of conjugate bases as well as more rapid *in situ* hydrolysis of electron-deficient aryl glucoside products. Substituted aryl glucosides bearing aniline (**10**; 72% yield), primary amine (**11**; 83% yield), primary alcohol (**12**; 82% yield), and ketone (**13**; 94% yield) functionality were isolated in good yields, as well, and in each case glycosylation was only observed at the phenolic hydroxyl group. The impact of sterics on the glycosylation of substituted phenols was evaluated through formation of 2,6-dimethyl (**14**) and 3,5-dimethyl (**15**) phenyl glucosides. Significantly diminished yield for **14** (42%) compared to **15** (77%), despite similar substrate solubility, revealed the lower reactivity of substrates with *ortho* substituents; this effect parallels diminished isolated yields obtained for *O*- β -aryl glucosides resulting from thymol (**16**; 23% yield) and 2,3-dimethoxyphenol (**17**; 80% yield). In addition to *ortho* substitution, substrate insolubility under the reaction conditions significantly decreased yield; benzyloxy (**18**, 10% yield) and alkenyl (**19**, 10% yield) substituted *O*- β -aryl glucosides were each isolated in low yield due to low substrate solubility. In addition to α -D-fluoroglucose, the glycosyl donors α -D-fluoromaltose and α -D-fluorogalactose underwent glycosylation with Boc-Tyr-OH under the reaction conditions, providing *O*-glycosyl tyrosine products **20** and **21**, respectively, in good yields (74% yield and 46% yield, respectively). The case of α -D-fluoromannose is also notable in that, while **22** can be isolated (~25% yield), the reaction is a bit less efficient and the major product is the α -anomer (see Supplementary Pages 32–33 and 114–115), consistent with a double inversion process involving the axial C2-OH group. We also examined the reaction of β -D-fluoroglucose under analogous reaction conditions, employing 4-methoxyphenol as the glycosyl acceptor. Once again, this reaction takes place with net retention of anomeric configuration, and **6** is formed in 26% yield, also consistent with a double inversion. In this case, the greater instability of β -fluoroglucose may account for the lower yield of **6**, and its reactivity at the anomeric position. Double inversion with β -fluoroglucose may involve intramolecular displacement as a prelude to glycosylation,⁶⁰ proceeding through glycosyl epoxide intermediates when the vicinal 2-hydroxyl group and 1-fluoride are disposed *trans* in the starting material⁶¹ (see Supplementary Page 90 for details.) In any event, taken together, these results above reflect a significant scope of phenolic compounds that may be subjected to the rapid, stereoselective *O*-glycosylation reaction in bulk aqueous solvent.

We then turned our attention to the question of biocompatible functional group tolerance. The interrogation of chemoselective Tyr-*O*-glycosylation within fully unprotected peptides seemed a compelling context to explore. To examine reactions on a scale of relevance to the study of complex bioactive peptides, practical reaction conditions for smaller quantities of substrate were developed. Optimization of dilute reaction conditions for the glycosylation of Boc-Tyr-OH revealed that a 1.0 M loading of both α -D-fluoroglucose and Ca(OH)₂ allowed complete conversion to **2** at 100 mM, 10 mM, and 1.0 mM substrate loading (see Supplementary Pages 11–17).

We then applied these conditions to a range of bioactive peptides. Glucagon-like peptide-1 (GLP-1₇₋₃₇; **23**; Figure 3)⁶² is a 30-residue peptide hormone bearing a single Tyr residue, and functions to stimulate and inhibit the secretion of insulin and glucagon, respectively.⁶³ GLP-1 also possesses a host of other side chain functionalities, setting up *in situ* evaluation of functional group tolerance during the projected Tyr-*O*-glycosylation. Upon reaction of **23** for 10 min, high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LCMS) analysis indicated 46% conversion to a mono-glucosyl product (**24**) with 31% remaining **23** (Figure 3). Of the remaining 23%, HPLC analysis of the unpurified reaction mixture shows a mixture of various mono- and bis-glucosyl products, indicating that a small amount of non-phenolic glycosylation as well as peptide decomposition may be occurring under the reaction conditions. Purification of the major component is straightforward, and MS/MS analysis of the isolated mono-glucosyl product supports assignment of Tyr as the site of glycosylation (see Supplementary Pages 37–48). A GLP-1 mutant lacking the phenolic hydroxyl, Y19F GLP-1₇₋₃₇ (**25**), was also prepared by solid-phase peptide synthesis. Upon reaction of **25** for 10 min, HPLC and LCMS analysis revealed 86% remaining starting material along with minor mono-glucosyl product peaks (Figure 3), further supporting Tyr as the site of glycosylation.

Next, we assessed the generality of the aqueous Tyr-*O*-glycosylation reaction with additional biologically active peptides that each carried a single Tyr residue (Figure 4). Endomorphin-2, a highly selective agonist for the μ -opioid receptor,⁶⁴ was glycosylated to generate **26** in 100% HPLC yield (40% isolated yield). While the crude reaction mixture once again revealed the presence of some bis-glycosylated material (as indicated by LCMS), this side product could be removed such that a sample of mono-glycosylated endomorphin-2 could be characterized by ¹H and ¹³C NMR spectroscopy. Interestingly, endomorphin-1, when subjected to the same reaction conditions delivered **27** with near quantitative conversion. In this case, however, the isolated material revealed the presence of a second species, which could not be removed. While racemization was not observed in the control experiments described above, it cannot not be excluded in this case. Since the only difference between endomorphin-2 and endomorphin-1 is the swap of a tryptophan (Trp) for a phenylalanine (Phe) residue, we performed a control/competition experiment in which *N*-Boc-Tyr-OH and *N*-Boc-Trp-OH were exposed to glycosylation conditions, and only phenolic glycosylation was observed (see Supplementary Page 85 for details; conformational isomerism may be responsible for the observation of a second species in the case of glycosylated endomorphin-1.)⁶⁵ Nevertheless, the opioid receptor ligand leu-enkephalin was an excellent substrate for the glycosylation, with **28** generated in 81% HPLC

yield (36% isolated yield). Compound **28** could be characterized as a mono-glycosylated species by ^1H and ^{13}C NMR spectroscopy, as in the case of endomorphin-2.⁶⁶ We then returned to examination of some more complicated peptides. Luteinizing-hormone releasing-hormone (LH-RH) was glycosylated to generate analogue **29** in 83% HPLC yield.⁶⁷ The neurohypophysial hormones vasopressin and oxytocin were glycosylated in 67% HPLC yield and 68% HPLC yield, respectively, to generate analogues **30** and **31**.⁶⁸ Finally, the 32-amino acid polypeptide hormone, calcitonin, was glycosylated in 49% HPLC yield to generate **32**.⁶⁹ In all cases, varying amounts of off-target glycosylation and/or decomposition were observed by HPLC analysis. We note that the identification of off-target glycosylation products by tandem mass spectrometry (MS/MS) was nontrivial based on the small quantities observed and due to inhomogeneity of the peaks observed in the LC traces for these minor products. For example, we analyzed the byproducts obtained during glycosylation of GLP-1 and its Phe mutant. Manually searching for glycosylated fragments lacking a tyrosine residue revealed multiple such species (see Supplementary Page 48). While we were not able to determine the definitive sites of glycosylation of these side products, this additional level of MS/MS analysis revealed that the minor levels of glycosylation were likely to the N-terminal side of the peptide. Notably, there are three serine (Ser) and two threonine (Thr) residues in GLP-1 to the N-terminal side of Tyr, in addition to the N-terminus itself. We speculate that these could be the off-target glycosylation sites. Like GLP-1, calcitonin incorporates multiple Ser, Thr, and lysine (Lys) residues, and, like vasopressin and oxytocin, calcitonin features a disulfide bond, demonstrating the high chemoselectivity of aqueous Tyr-*O*-glycosylation mediated by Ca^{2+} salts and glucosyl fluorides.

These experiments imply that the side chains of Ser, Thr, Lys, Trp, Asp, glutamic acid (Glu), asparagine (Asn), glutamine (Gln), histidine (His), arginine (Arg), as well as disulfide bonds, are relatively inert under these glycosylation conditions. None of the bioactive peptides evaluated contained a free Cys side chain, and one might expect reactivity from Cys due to its high nucleophilicity and $\text{p}K_{\text{a}}$ not wildly different than that of Tyr.⁷⁰ While Cys *S*-glycosides are rare in nature, chemists have prepared and utilized synthetic *S*-glycosides for various purposes.^{71,72,73,74} Indeed, under the glycosylation conditions optimized for Boc-Tyr-OH, we observed full conversion of Boc-Cys-OH (**33**) to *N*-Boc-*S*- β -D-glucopyranosyl-L-cysteine (**34**), and the product was isolated in 98% yield (Figure 5a). In light of future applications toward complex molecule glycosylation, a model dipeptide **35** bearing both Tyr and Cys was prepared. Under the standard reaction conditions, HPLC analysis revealed 36% conversion to the bis-glycosylated product **36**, 33% conversion to Boc-Tyr-Cys(*S*-Glc)-OH **37**, 10% conversion to Boc-Tyr(*O*-Glc)-Cys-OH **38**, and 21% starting material (**35**), indicating low chemoselectivity between Tyr and Cys (Figure 5b). Ongoing efforts seek to better differentiate the reactivity of Tyr and Cys and explore strategies for site-selective glycosylation.

Conclusion

The experiments described above culminate in a highly efficient phenolic *O*-glycosylation method of significant potential. Fundamentally, these results extend the versatility of Ca^{2+} -promoted glycosylation reactions that employ glucosyl fluorides beyond the idiosyncratic

case of sucrose and its carefully adjusted derivatives. Indeed, through extension of the classical literature, we found that the lower pK_a phenols provide a strong analogy to the results of Jencks concerning nucleophilic azide, and that wide generality of phenolic structural types may undergo rapid glycosylation in water under these conditions. Moreover, the reactions often occur with a predictable stereochemical outcome. The protocol is straightforward, and the reactions proceed generally with high efficiency, within ten minutes, and at ambient temperature. The reactions also proceed in entirely aqueous solvent. Substrates, including highly complex oligopeptides, may be employed without the use of protecting groups. The functional group tolerance of the reaction appears to be quite high, as exemplified by a number of complex, bioactive oligopeptide substrates. Numerous amino acid side chains are spectators when a Tyr residue is present in the sequence, and macrocyclic rings including side-chain disulfide bridges are tolerated, with no significant degradation through either hydrolysis or β -elimination pathways. Cys residues, on the other hand, appear to react competitively at the $-SH$ in addition to the $-OH$ position of Tyr, revealing a mode of reactivity to be considered when applying this method. On the one hand, this side reaction represents a current shortcoming of the approach. On the other hand, numerous applications of the present chemistry seem possible, and the site-selective Tyr versus Cys glycosylation points to one challenge we now hope to address, among others such as the site-selective glycosyl transfer to unique Tyr (or other residues) in polyfunctional cases where more than one Tyr or Cys is present. Of course, we remain interested in whether the chemistry described herein may be adapted to the selective glycosylation of polypeptides at other, for the moment-unreactive positions, such as Ser, Thr, and the other functionalized amino acid side chains.

Data availability

The characterization data for new chemical compounds are given in Supplementary Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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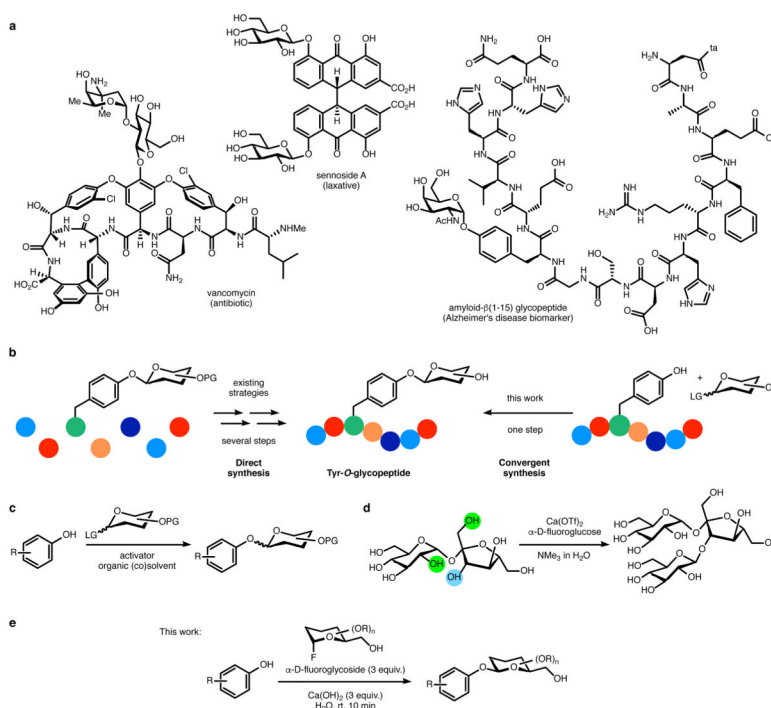
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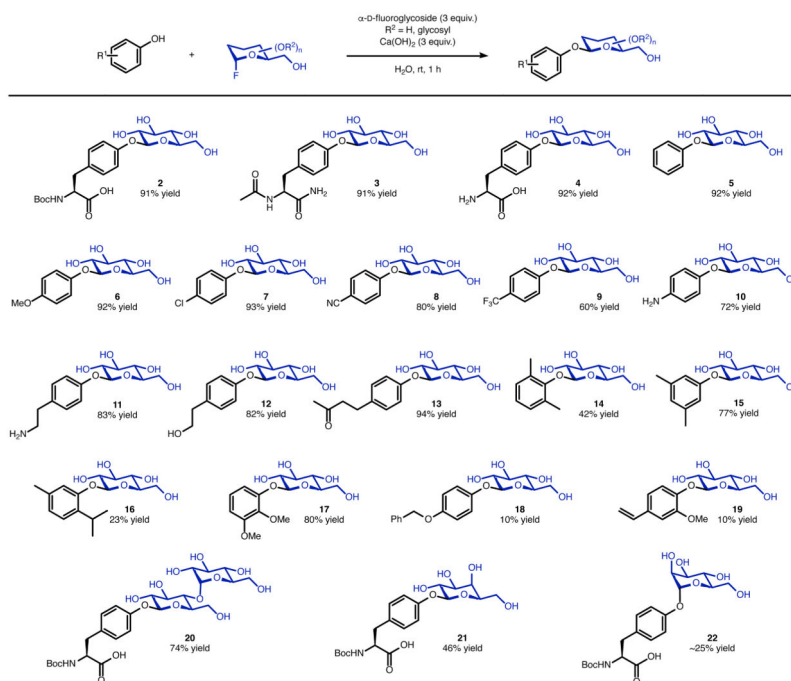
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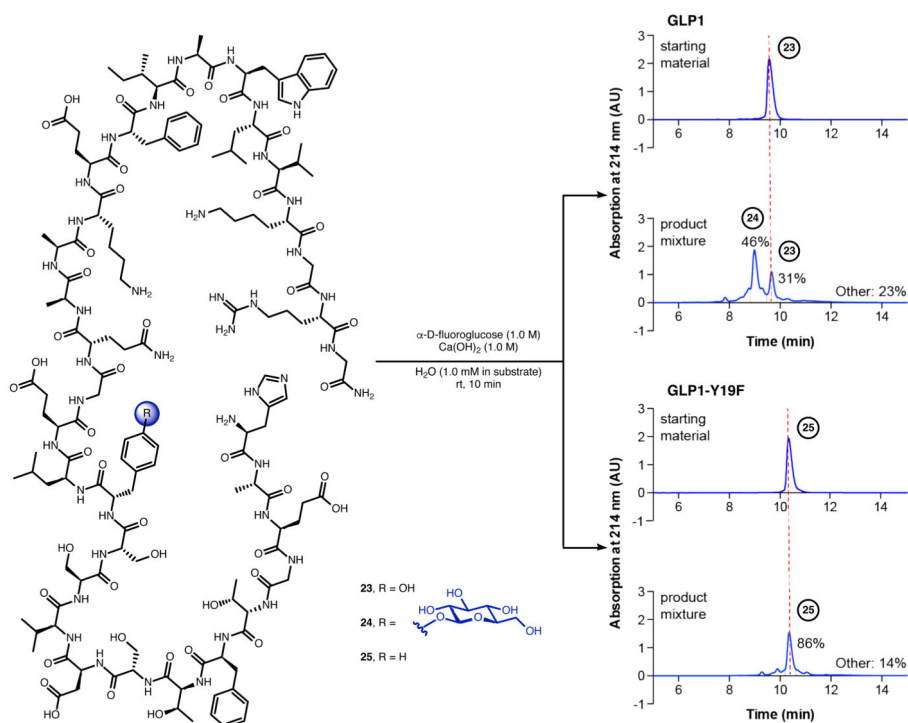
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**Figure 1.**

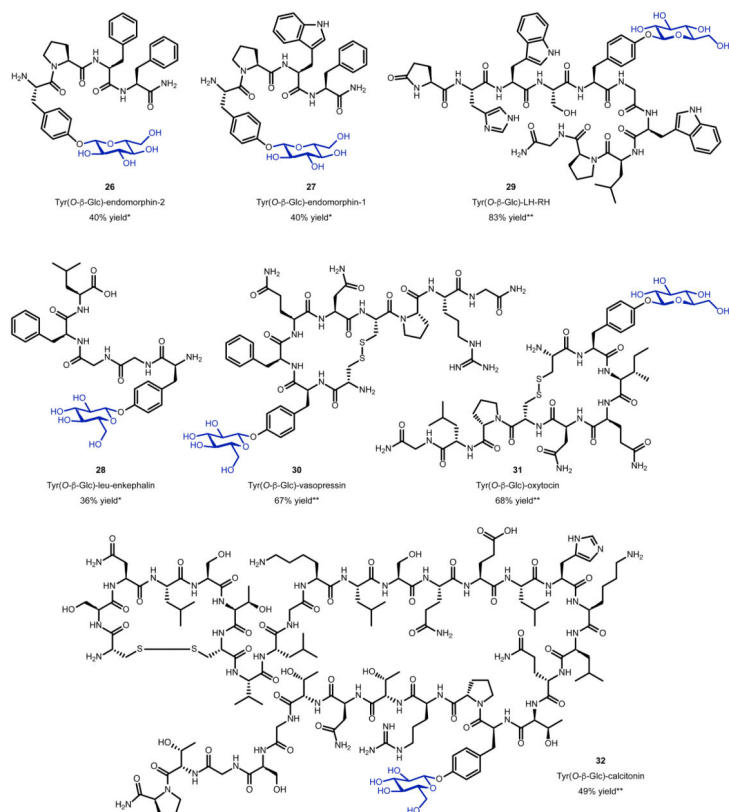
Natural products containing phenolic *O*-glycosyl moieties and strategies to access phenolic *O*-glycosyl linkages in small and complex molecules **(a)** Selected examples of glycosylated natural products exhibiting varied biological activity. **(b)** Schematic representation of direct vs. convergent synthetic methodologies to access glycopeptides. Coloured circles represent amino acids. **(c)** Conditions for previously reported aromatic *O*-glycosylation methods, discussed and referenced in the text; those require a protecting group for the oxygen sites (OPG). **(d)** Previously reported protecting-group-free aqueous glycosylation of sucrose promoted by Ca^{2+} and NMe_3 (ref. 56). The OH groups required for reaction are highlighted in green, the OH reaction site is shown in blue. **(e)** This work: *O*-glycosylation in aqueous solvent.

**Figure 2.**

Evaluating substrate scope. The reactions were carried out at a substrate concentration of 1.0 M, the yields shown refer to isolated products. Reaction conditions: 0.5 mmol substrate, α -D-fluoroglycoside (1.5 mmol, 3 equiv.), $\text{Ca}(\text{OH})_2$ (1.5 mmol, 3 equiv.), H_2O (1.0 M in substrate), rt, 1 h. The products were purified via reversed-phase flash column chromatography.

**Figure 3.**

Tyrosine-selective glycosylation of glucagon-like peptide 1. HPLC yield reported (calculated by integrating peak corresponding to monosubstituted peptide at 214 nm). Reaction conditions: 0.5 or 1.0 mg substrate, α -D-fluoroglucose (1.0 M, 1000 equiv.), $\text{Ca}(\text{OH})_2$ (1.0 M, 1000 equiv.), H_2O (1.0 mM in substrate), rt, 10 min. Reactions quenched with 0.5 M EDTA solution (pH = 8.0) and purified using desalting and buffer exchange column (SpinOUT™ GT-100).

**Figure 4.**

Tyrosine-selective glycosylation of biologically active peptides. *Isolated yield reported (Reaction conditions specified in Supplementary Page 49). **HPLC yield reported (calculated by integrating peak corresponding to monosubstituted peptide at 214 nm), reaction conditions: 0.5 or 1.0 mg substrate, α -D-fluoroglucose (1.0 M, 1000 equiv.), $\text{Ca}(\text{OH})_2$ (1.0 M, 1000 equiv.), H_2O (1.0 mM in substrate), rt, 10 min. Reactions quenched with 0.5 M EDTA solution (pH = 8.0) and purified using desalting and buffer exchange column (SpinOUT™ GT-100).

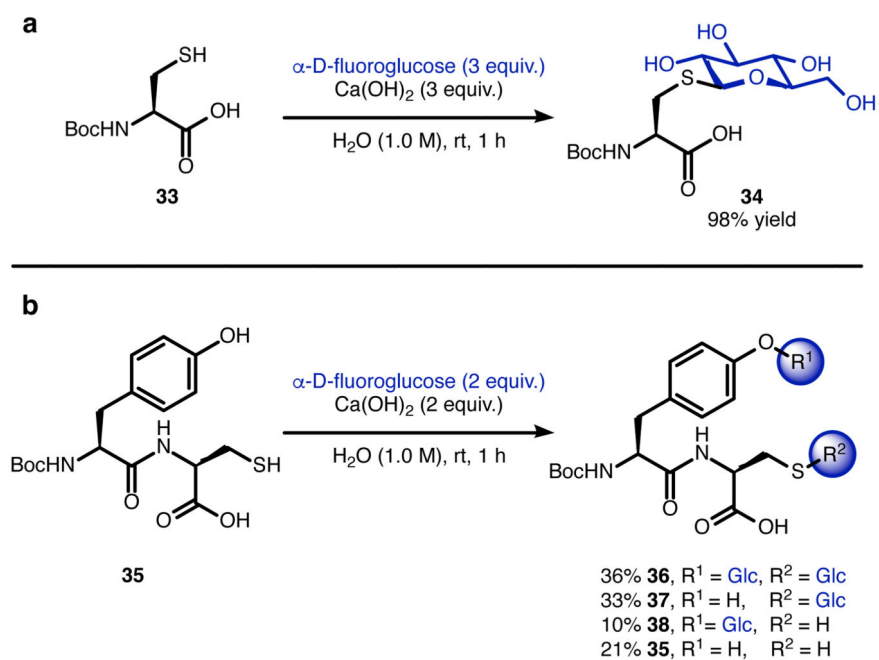
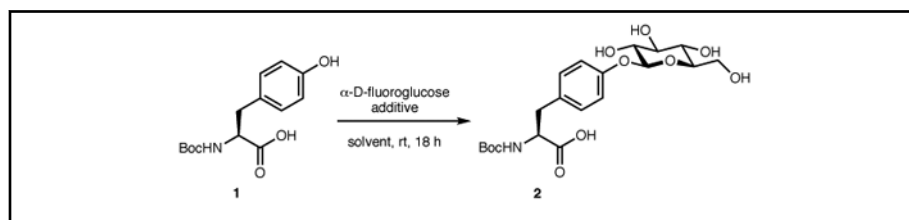


Figure 5. Reactivity of cysteine under the aqueous glycosylation conditions. **(a)** Glycosylation of Boc-Cys-OH (**33**). **(b)** Glycosylation of Boc-Tyr-Cys-OH (**35**).

Table 1Optimization of reaction conditions for the *O*-glycosylation of Boc-L-Tyr-OH (**1**).


Entry	Solvent (conc.)	α -D-fluoroglucose (equiv.)	Additive	NMR yield (%)
1	45% NMe ₃ /H ₂ O (0.2 M)	6	Ca(OTf) ₂ (6 equiv.)	100
2	H ₂ O (0.2 M)	6	Ca(OTf) ₂ (6 equiv.)	0
3	H ₂ O (0.2 M)	6	Ca(OH) ₂ (6 equiv.)	95
4	H ₂ O (0.2 M)	6	LiOH (6 equiv.)	8
5	H ₂ O (0.2 M)	6	NaOH (6 equiv.)	6
6	H ₂ O (0.2 M)	6	KOH (6 equiv.)	6
7	H ₂ O (0.2 M)	6	CsOH (6 equiv.)	5
8	H ₂ O (0.2 M)	6	Mg(OH) ₂ (6 equiv.)	0
9	H ₂ O (0.2 M)	6	Ba(OH) ₂ (6 equiv.)	4
10	H ₂ O (0.2 M)	6	none	0
11	H ₂ O (0.2 M)	3	Ca(OH) ₂ (3 equiv.)	73
12	H ₂ O (0.5 M)	3	Ca(OH) ₂ (3 equiv.)	92
13	H ₂ O (1.0 M)	3	Ca(OH) ₂ (3 equiv.)	97
14	H ₂ O (1.0 M)	2	Ca(OH) ₂ (2 equiv.)	93
15	H ₂ O (1.0 M)	1	Ca(OH) ₂ (1 equiv.)	16
16 ^a	H ₂ O (1.0 M)	3	Ca(OH) ₂ (3 equiv.)	95
17 ^b	H ₂ O (1.0 M)	3	Ca(OH) ₂ (3 equiv.)	94

^aReaction time = 1 h.^bReaction time = 10 min.