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Ready protease-catalyzed synthesis of carbohydrate–amino acid conjugates†

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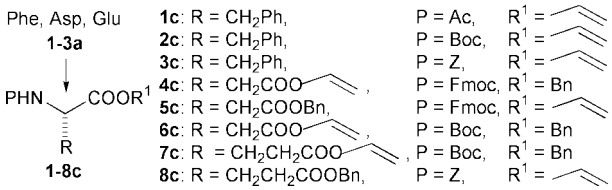
The protease-catalyzed synthesis of amino acid ester–carbohydrate conjugates as glycopeptide analogues has been achieved in a highly regioselective and carbohydrate-specific manner using amino acid vinyl ester acyl donors and minimally or completely unprotected carbohydrate acyl acceptors, which together probed active sites of proteases to reveal yield efficiencies that are modulated by the carbohydrate C-2 substituent, and that may be exploited to allow selective one-pot syntheses.

Many carbohydrate–peptide conjugates display a wide variety of potent biological activities of potential therapeutic and commercial value.[1,2] For example, glycoproteins act as critical cell surface communication markers,[3] glycopeptide motifs such as the Thomsen–Friedenreich (Tf) antigen are associated with cancer cell lines[4] and an oligomeric sequence of the glycopeptide motif (AAT[Galβ(1,3)GalNAcα])$_n$ displays unusual non-colligative antifreeze properties.[5] Access to well-defined carbohydrate–peptide conjugates and their analogues to probe the nature of these properties is essential. A large number of elegant methods have been developed for the synthesis and assembly of *N*- and *O*-linked glycopeptides[2,6] but these methods may be complicated by low glycosylation efficiencies and extensive protection regimes to ensure regioselectivity. To avoid these potential problems we have investigated the utility of enzyme-catalyzed regioselective acylation of carbohydrates as a one-step method. Despite the ready construction of ester–carbohydrate linkages, there have been remarkably few syntheses of amino acid esters of carbohydrates.[7] Furthermore, although the utility of hydrolases as powerful catalysts for regioselective acylation of carbohydrates is well demonstrated,[8] their use in the transfer of amino acids to carbohydrates is, with very few exceptions,[9] neglected. This is all the more surprising given that several biofunctional molecules, such as enkephalin–carbohydrate conjugates that modulate fibroblast and melanoma growth,[10] are themselves α-amino esters of carbohydrates. Moreover, carbohydrate–peptide conjugates connected by potentially metabolisable, sacrificial linkages, such as esters, have high potential utility as prodrugs in which the glycan moiety affords both protection and specific transport properties.[11] We therefore set ourselves the goal of establishing a ready, short route for the creation of such ester-linked glycopeptides.

Initially, we chose the serine protease subtilisin *Bacillus lentus* (SBL, EC 3.4.21.14) as a powerful catalyst for ester synthesis[12] and the representative amino acids phenylalanine **1a**, aspartic acid **2a** and glutamic acid **3a**. Amino acid derivatives (Scheme 1)† were chosen to probe not only the amino acid specificity of SBL but also its tolerance for a variety of amine (Ac, Boc, Fmoc, Z) and ester (Bn) protecting groups. Pd(OAc)$_2$-mediated transesterification[13] of **1–8b** with vinyl acetate (Scheme 1) allowed the preparation of the corresponding Phe, Asp and Glu; α and side-chain vinyl esters **1–8c** as acyl donors that render transesterifications essentially irreversible.[14]

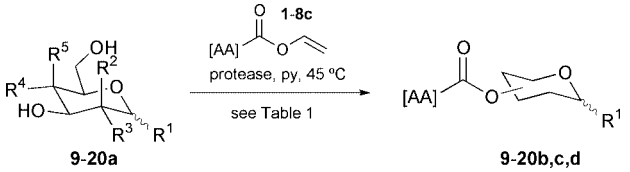
† Electronic supplementary information (ESI) available: experimental details. See http://www.rsc.org/suppdata/cc/b1/b104137c/



Scheme 1 For reagents and conditions, see ESI.

With these acyl donors in hand, we investigated their utility in transesterification reactions with a representative range of carbohydrate acyl acceptors **9a–20a** (Scheme 2, Table 1). After exploring a range of conditions, the use of SBL lyophilised from phosphate buffer (pH 8.0) in anhydrous pyridine at 45 °C proved optimal. Initial variation of parent carbohydrate in the completely deprotected series **9a–12a** revealed exclusive *O*-6 regioselectivity but only low isolated yields of either D-glucose **9b** or D-galactose **10b** 6-*O*-phenylalaninate esters.[15] However, a higher yield of the 6-*O*-phenylalaninate ester of D-mannose **11b** indicated an exciting preference based only on the stereochemistry of the parent carbohydrate. This crucial dependency on carbohydrate acceptor was yet more dramatically confirmed by the complete absence of product from the attempted esterification of *N*-acetylglucosamine **12a** from which only **12a** and the product of acyl donor hydrolysis **1b** were recovered. Next the effect of anomeric substituent was probed. Introduction of a methyl substituent at *O*-1 increased yield only slightly in the case of D-galactose and D-glucose acyl acceptors **13–15a**. Moreover, the near identical yields of α- and β-glucosides **13**, **14b** indicated that, at least in the D-gluco series, anomeric stereochemistry had little or no effect on overall yield. Most notably, the apparent specificity preference of SBL for D-manno acyl acceptors was further confirmed by the higher yield (76%) of ester **16b** obtained here from α-D-mannoside **16a**.

Thioglycosides and selenoglycosides are important glycosyl donors[16] and we next investigated their esterification to provide potential glycopeptide donors, in which the glycosyl unit might be further extended, and as further probes of the effect of anomeric substituent. Consistent with both their larger size and the potential for aromatic aglycones in carbohydrate substrates to interact with protein surfaces,[17] more dramatic results were obtained for the thioglycosides **17–20a**. A trend in the efficiencies of the formation of 6-*O*-phenylalaninate products in the order D-manno > D-gluco > D-galacto > *N*-acetyl-D-gluco emerged. In addition, for the first time, reduced regioselectivity was observed for D-thioglucoside **17a** (3:2, 6-*O* **17b**: 3-*O* **17c**).[18]



Scheme 2

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Table 1 Carbohydrate–amino acid coupling reactions

Coupling pair	R^1	R^2	R^3	R^4	R^5	Yield of 6-*O*-acyl (%)[c]
9a–1c[a]	OH	H	OH	OH	H	24 **9b**
10a–1c[a]	OH	H	OH	H	OH	24 **10b**
11a–1c[a]	OH	OH	H	OH	H	49 **11b**
12a–1c[a]	OH	H	NHAc	OH	H	—
13a–1c[a]	α-OMe	H	OH	OH	H	25 **13b**
14a–1c[a]	β-OMe	H	OH	OH	H	28 **14b**
15a–1c[a]	β-OMe	H	OH	H	OH	30 **15b**
16a–1c[a]	α-OMe	OH	H	OH	H	76 **16b**
17a–1c[a]	β-SPh	H	OH	OH	H	44 **17b** + 29 **17c**[e]
18a–1c[a]	β-SPh	H	OH	H	OH	36 **18b**
19a–1c[a]	α-SPh	OH	H	OH	H	62 **19b**
20a–1c[a]	β-SePh	H	NHAc	OH	H	23 **20b**
16a–1c[b]	α-OMe	OH	H	OH	H	48 **16b**
16a–2c[a]	α-OMe	OH	H	OH	H	32 **16c**
16a–2c[d]	α-OMe	OH	H	OH	H	63 **16c** + 17 **16d**[e]
16a–3c[d]	α-OMe	OH	H	OH	H	60 **16e**

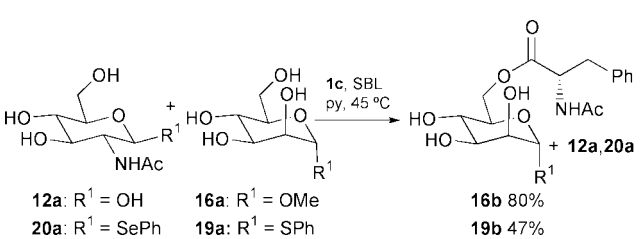
[a] 2 mg ml^{-1} of lyophilized (from pH 8.0, 0.1 M phosphate) SBL preparation, 45 °C, anhydrous pyridine, 120 h. [b] 1 mg ml^{-1} of TL-CLEC, 45 °C, 1:25 water–pyridine. [c] All yields are for isolated, purified, single compounds. [d] As for footnote *a* but for 500 h. [e] Yield of 3-*O*-acyl.

Next we investigated the effect of varying the amino acid acyl donor. Disappointingly, but consistent with the observed low affinity of SBL for other amino acid esters,[19] none of the aspartate or glutamate acyl donors were accepted as substrates. In all cases only vinyl esters **4**–**8c** were recovered indicating an absence of acyl–enzyme intermediate formation. This contrasted with the reactions of **1c** from which only transesterification or hydrolysis products were recovered. In order to further assess the utility of **1**, **4**–**8c** as acyl donor probes, we also screened their reactivity with CLEC-thermolysin (TL-CLEC)[20] as a protease with a different substrate specificity profile, that includes β-aspartate esters.[21] However, TL-CLEC also failed to accept **4**–**8c** and again only **1c** was accepted, allowing the preparation of **16b** from **16a** in 48% yield.

Next, the effect of *N*-protection in the acyl donor was investigated using Boc- and Z-protected phenylalanine donors **2**, **3c**, respectively. For **2c** much lower rates of reaction were observed than for **1c** and after a comparable period of time lower yields (32%) for the esterification of **16a** were obtained. However, extended reaction times gratifyingly allowed the preparation of 6-*O*-phenylalaninates **16c**, **e** from **2**, **3c** in 63 and 60% yields, respectively. The utility of **16c**, **e** as glycopeptide building blocks was confirmed through their quantitative *N*-deprotection to methyl 6-*O*-phenylalaninyl-α-D-mannopyranoside **21**, which may be extended at its *N*-terminus.

Finally, the valuable specificity information obtained in these screens was exploited to allow selective one-pot couplings. We were delighted to find that different carbohydrate acyl acceptors successfully competed in one-pot reactions to allow carbohydrate-selective esterification. Thus, in 1:1 mixtures of **12a** + **16a** and **19a** + **20a** (Scheme 3) mannosides reacted over *N*-acetylglucosaminides with **1c** in SBL-catalyzed acylations to yield mannoside esters **16**, **19b** exclusively. In both reactions no trace of **12b** or **20b**, respectively, was detected during this highly selective process.

In summary, we have described a ready method for the construction of glycan–peptide conjugates by exploiting a highly regioselective protease-catalyzed transesterification process. The yields for this selective carbohydrate–peptide conjugation of 23–76%, compare well with overall yields of <34% for alternative routes employing protection–deprotection strategies.[7] The glycopeptides formed are powerful building blocks that will allow sugar reducing end (*e.g.* **17**–**20b**) or peptide *N*-terminal (*e.g.* **21**) extension. In addition, we have probed the substrate specificity of the proteases SBL and TL-CLEC in this reaction using the novel vinyl esters **1**–**8c** and this has indicated a strong preference for phenylalanine but flexibility in the *N*-protection that may be used. Furthermore, we have successfully exploited striking differences in the rate of reaction of carbohydrate acyl acceptors in this system to perform exclusively mannose over *N*-acetylglucosamine selective one-pot acylations. We have recently reported greatly broadened substrate amino acid ester specificities for glycosylated variants of SBL[22] and we are currently exploring transesterifications catalyzed by these glyco-SBLs with **4**–**8c** and other donors the results of which will be reported in due course.



Scheme 3

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