J|A|C|S
ARTICLES

Published on Web 08/13/2008

Sugar-Assisted Glycopeptide Ligation with Complex Oligosaccharides: Scope and Limitations

Clay S. Bennett,[†] Stephen M. Dean,[†] Richard J. Payne,[†,‡] Simon Ficht,[†] Ashraf Brik,[†,§] and Chi-Huey Wong*[,†,⊥]

Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037 and Genomics Research Center, Academia Sinica, 128 Section 2, Academia Road, Nankang, Taipei 115, Taiwan

Received February 11, 2008; E-mail: wong@scripps.edu; chwong@gate.sinica.edu.tw

Abstract: We have previously shown sugar-assisted ligation (SAL) to be a useful method for the convergent construction of glycopeptides. However to date SAL has only been carried out on systems where the thiol auxiliary is attached to a monosaccharide. For SAL to be truly applicable to the construction of fully elaborated glycopeptides and glycoproteins, it must be possible to carry out the reaction when the thiol auxiliary is attached to more elaborate sugars, as these are frequently what are observed in nature. Here we examine the effects of glycosylation at C-3, C-4, and C-6 of the C-2 auxiliary-containing glycan. Model glycopeptides where synthesized chemoenzymatically and reacted with peptide thioesters used in our previous work. These studies reveal that SAL is sensitive to extended glycosylation on the auxiliary-containing sugar. While it is possible to carry out SAL with extended glycosylation at C-4 and C-6, the presence of glycosylation at C-3 prevents the ligation from occurring. Additionally, with glycosylation at C-4 the ligation efficiency is affected by the identity of the N-terminal AA, while the nature of the C-terminal residue of the peptide thioester does not appear to affect ligation efficiency. These studies provide useful guidelines in deciding when it is appropriate to use SAL in the synthesis of complex glycopeptides and glycoproteins and how to choose ligation junctions for optimal yield.

Introduction

Protein glycosylation is a complex co/post-translational modification which has been estimated to be present in greater than 50% of all human proteins.[1] The attachment of oligosaccharides to the protein backbone introduces a staggering array of structural and functional diversity, far beyond that which is available from the 20 canonical amino acids.[2] Accordingly, glycans have been shown to play important roles in a number of biological processes, including protein folding, cellular adhesion, cell targeting, and cell differentiation.[3−7] In addition, abnormal glycosylation of proteins is linked with several disorders including autoimmune diseases and cancer.[8,9] Despite its importance, very little is understood about the consequences of protein glycosylation at the molecular level, due to the fact that in biological systems glycoproteins are produced as heterogeneous mixtures of glycoforms and isoforms which are extremely difficult, if not impossible, to separate on a preparative scale. As a result, there has been a significant amount of research directed at the production of homogeneous glycoproteins through chemical and chemoenzymatic methods.[10−12]

One of the most common approaches to the chemical synthesis of proteins involves the union of smaller peptide fragments through native chemical ligation (NCL).[13,14] NCL has proved to be extremely powerful for the convergent synthesis of large glycopeptides and glycoproteins.[15−21] Al-

[†] The Scripps Research Institute.
[‡] Present Address: School of Chemistry, University of Sydney, NSW 2006, Australia
[§] Present Address: Department of Chemistry, Ben-Gurion University, Beer Sheva, Israel
[⊥] Academia Sinica.

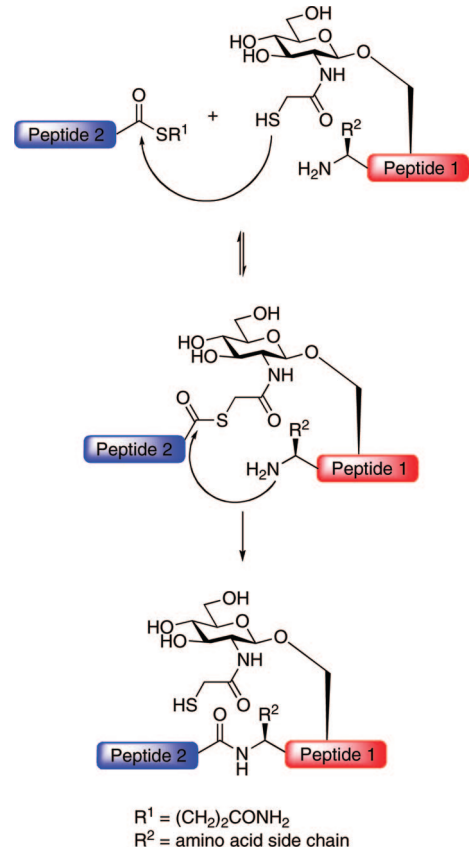
(1) Apweiler, R.; Hermjakob, H.; Sharon, N. *Biochem. Biophys. Acta* **1999**, *1473*, 4–8.
(2) Spiro, R. G. *Glycobiology* **2002**, *12*, 43R–56R.
(3) Sita, R.; Braakman, I. *Nature* **2003**, *426*, 891–894.
(4) Spiro, R. G. *Cell. Mol. Life Sci.* **2004**, *61*, 1025–1041.
(5) Helenius, A.; Aebi, M. *Annu. Rev. Biochem.* **2004**, *73*, 1019–1049.
(6) Dwek, R. A. *Chem. Rev.* **1996**, *96*, 683–720.
(7) Varki, A. *Glycobiology* **1993**, *3*, 97–130.
(8) Miller, L. H.; Good, M. F.; Milon, G. *Science* **1994**, *264*, 1878–1883.
(9) Dube, D. H.; Bertozzi, C. R. *Nat. Rev. Drug. Discovery* **2005**, *4*, 477–488.
(10) David, B. G. *Chem. Rev.* **2002**, *102*, 579–601.
(11) Pratt, M. R.; Bertozzi, C. R. *Chem. Soc. Rev.* **2005**, *34*, 58–68.
(12) Bennett, C. S.; Wong, C. H. *Chem. Soc. Rev.* **2007**, *36*, 1227–1238.
(13) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776–779.
(14) Dawson, P. E.; Kent, S. B. H. *Annu. Rev. Biochem.* **2000**, *69*, 923–960.
(15) Shin, Y.; Winans, K. A.; Backes, B. J.; Kent, S. B. H.; Ellman, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **1999**, *121*, 11684–11689.
(16) Marcaurelle, L. A.; Mizoue, L. S.; Wilken, J.; Oldham, L.; Kent, S. B. H.; Handel, T. M.; Bertozzi, C. R. *Chem.—Eur. J.* **2001**, *7*, 1129–1132.
(17) Warren, J. D.; Miller, J. S.; Keding, S. J.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2004**, *126*, 6576–6578.
(18) Mezzato, S.; Schaffrath, M.; Schaffrath, M.; Unverzagt, C. *Angew. Chem., Int. Ed.* **2005**, *44*, 1650–1654.
(19) Hackenberger, C. P. R.; Friel, C. T.; Radford, S. E.; Imperiali, B. *J. Am. Chem. Soc.* **2005**, *127*, 12882–12889.
(20) Chen, J.; Chen, G.; Wu, B.; Wan, Q.; Tan, Z.; Hua, Z.; Danishefsky, S. J. *Tetrahedron Lett.* **2006**, 8013–8016.
(21) Yamamoto, N.; Tanabe, Y.; Okamoto, R; Dawson, P. E.; Kajihara, Y. *J. Am. Chem. Soc.* **2008**, *130*, 501–510.

though useful, NCL is limited by the need for a N-terminal cysteine, alanine, or phenylalanine residue, the latter two of which are introduced following NCL through chemical desulfurization of cysteine and β-mercaptophenylalanine, respectively.[22−24] One approach to circumvent the fact that many glycoproteins do not possess these residues at strategically useful positions along the protein backbone is the use of cysteine-free ligation techniques. In this approach a thiol-containing auxiliary is placed at the N-terminus of a peptide to act as a cysteine surrogate.[25,26] While the use of auxiliaries has expanded the number of possible ligation junctions available for glycoprotein synthesis, this approach is limited to sterically unencumbered amino acids.[27−30]

Recently, our laboratory has introduced an alternative approach to glycopeptide ligation, termed sugar-assisted ligation (SAL).[31−35] This approach utilizes a glycopeptide in which the thiol auxiliary is attached through a sugar (*N*-acetylglucosamine or *N*-acetylgalactosamine) via either the C-2 acetamide or a 2-mercaptoacetate moiety on the C-3 hydroxyl group. Similar to other auxiliaries, these glycopeptides undergo thioester exchange with a peptide thioester, followed by an S → N acyl shift to afford a native peptide linkage (Scheme 1). The reaction shows high tolerance at the ligation junction and is orthogonal to functional groups present in all proteinogenic amino acids.[32] Following ligation, the thiol handle can be removed through hydrogenolysis, in the case of the 2-mercaptoacetamido auxiliary, or deacetylation, in the case of the 2-mercaptoacetyl auxiliary. While the use of chemical desulfurization is incompatible with unprotected cysteine residues, recent work from the Kent group, the Danishefsky group, and our own laboratory has shown that protection of cysteine residues with an acetamidomethyl (Acm) protecting group circumvents this problem.[23,36,37] The utility of this approach has been demonstrated through the synthesis of the glycoprotein antibiotic diptericin,[36] opening up new avenues for the synthesis of complex glycopeptides and possibly glycoproteins.[38]

Given our interest in the chemical synthesis of homogeneous glycoproteins, our next goal was to apply SAL to the construction of glycopeptides containing complex glycans. Although we had previously demonstrated that the auxiliary-containing glycan can be enzymatically elaborated following ligation,[32] this approach is always not practical for glycoprotein synthesis as the small differences in molecular weight between the starting material and product make purification exceedingly difficult if the reaction does not proceed to completion. Additionally, because it is not possible to selectively elaborate one glycan in the presence of others of the same type, the current approach is not amenable to the selective synthesis of glycoproteins containing multiple glycans with differences in their composition.[39] In order to circumvent these problems, it is necessary to take a more convergent approach, where the fully elaborated glycans are attached to the peptide backbone using chemical or enzymatic methods prior to ligation. We therefore sought to examine if it was possible to carry out SAL in systems where the thiol auxiliary was attached to more complex glycans.

Scheme 1. Mechanism of Sugar-Assisted Ligation (SAL)



In order to test the compatibility of SAL with complex auxiliary-containing glycans we chose to examine model glycopeptides **1a**−**d**, **2a**−**d**, **3a**−**d**, and **4** in the reaction (Figure 1). These peptides would allow us to probe the effects of glycosylation at C-3, C-4, and C-6 of the bridgehead sugar. By using peptide backbones that have been examined in previous studies, we could make direct comparison to the SAL reactions of glycopeptides bearing monosaccharides. Importantly, compounds **1a**−**d**, **2a**−**d**, and **3a**−**d** could be prepared enzymatically, providing us with an opportunity to examine the ability of glycosyltransferases to effectively elaborate the auxiliary-containing sugar. Here we report that extended glycosylation of the auxiliary-containing sugar can have a profound effect on

(22) Yan, L. Z.; Dawson, P. E. *J. Am. Chem. Soc.* **2001**, *123*, 526–533.
(23) Pentlute, B. L.; Kent, S. B. H. *Org. Lett.* **2007**, *9*, 687–690.
(24) Crich, D.; Banerjee, A. *J. Am. Chem. Soc.* **2007**, *129*, 10064–10065.
(25) Offer, J.; Boddy, C. N. C.; Dawson, P. E. *J. Am. Chem. Soc.* **2002**, *124*, 4642–4646.
(26) Low, D. W.; Hill, M. G.; Carrasco, M. R.; Kent, S. B. H.; Botti, P. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 6554–6559.
(27) Macmillan, D.; Anderson, D. W. *Org. Lett.* **2004**, *6*, 4659–4125.
(28) Wu, B.; Chen, J. H.; Warren, J. D.; Chen, G.; Hua, Z. H.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2006**, *45*, 4116–4125.
(29) Macmillan, D. *Angew. Chem., Int. Ed.* **2006**, *45*, 7668–7672.
(30) Chen, G.; Warren, J. D.; Chen, J.; Wu, B.; Wan, Q.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2006**, *128*, 7460–7462.
(31) Brik, A.; Yang, Y. Y.; Ficht, S.; Wong, C. H. *J. Am. Chem. Soc.* **2006**, *128*, 5626–5627.
(32) Brik, A.; Ficht, S.; Yang, Y. Y.; Bennett, C. S.; Wong, C. H. *J. Am. Chem. Soc.* **2006**, *128*, 15026–15033.
(33) Ficht, S.; Payne, R. J.; Brik, A.; Wong, C. H. *Angew. Chem., Int. Ed.* **2007**, *46*, 5975–5979.
(34) Payne, R. J.; Ficht, S.; Tang, S.; Brik, A.; Yang, Y. Y.; Wong, C. H. *J. Am. Chem. Soc.* **2007**, *129*, 13527–13536.
(35) Brik, A.; Wong, C. H. *Chem.—Eur. J.* **2007**, *13*, 5670–5675.
(36) Yang, Y. Y.; Ficht, S.; Brik, A.; Wong, C. H. *J. Am. Chem. Soc.* **2007**, *129*, 7690–7701.
(37) Wan, Q.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2007**, *46*, 9248–9252.
(38) Hsieh-Wilson, L. *Nature* **2007**, *445*, 31–33.
(39) For a recent example where enzymatic elaboration of a glycopeptide containing two *N*-acetylglucosamine residues led to mixtures of products, see: (a) Haneda, K.; Takeuchi, M.; Tagashira, M.; Inazu, T.; Toma, K.; Isogai, Y.; Hori, M.; Kobayashi, K.; Takeuchi, M.; Takegawa, K.; Yamamoto, K. *Carbohydr. Res.* **2004**, *341*, 181–190.

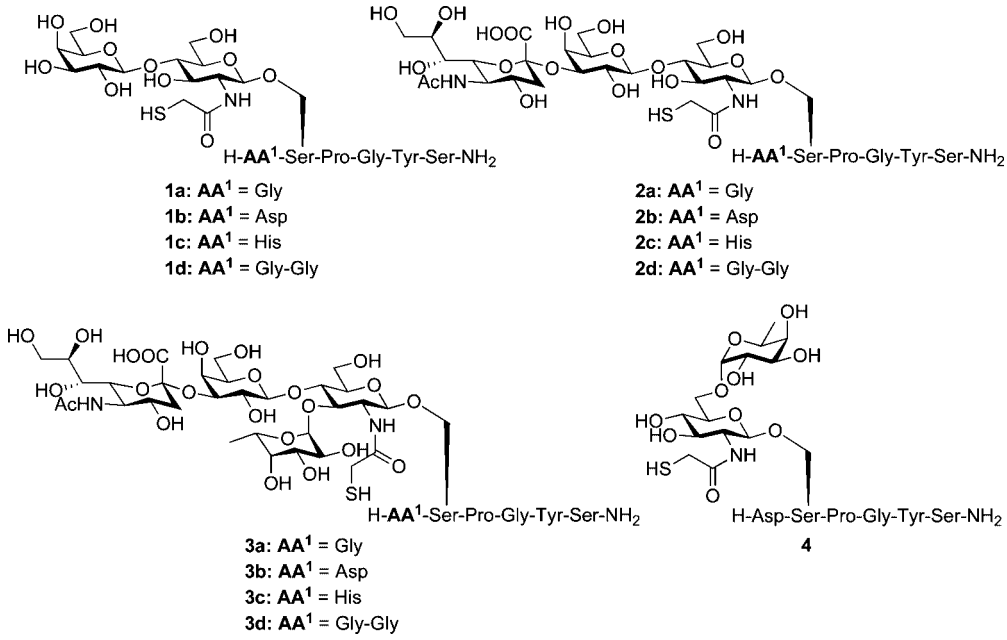


Figure 1. Model auxiliary-containing glycopeptides to be examined in this study.

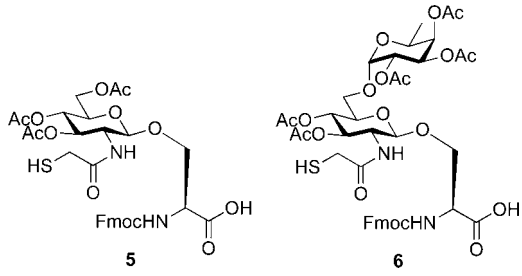


Figure 2. Auxiliary-containing glycosyl amino acids required for glycopeptide synthesis.

the SAL reaction. The efficiency of the reactions appears to be dictated by the position in which the bridgehead sugar is modified.

Results and Discussion

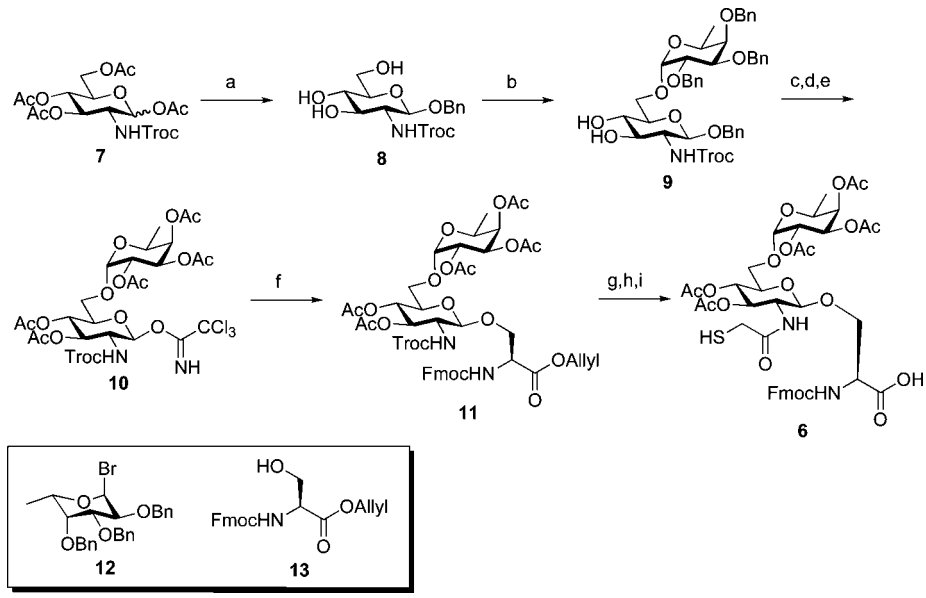
Synthesis of Glycosyl Amino Acid Building Blocks. The initial phase of this study involved the construction of the auxiliary-containing glycosyl amino building blocks for solid-phase peptide synthesis (SPPS) of the model glycopeptides (Figure 1). We envisioned that glycopeptides **1**−**3** could be constructed through enzymatic elaboration of a monosaccharide-containing glycopeptide following SPPS. As such most of the targets could be synthesized from the previously reported amino acid **5** (Figure 2).[34] For the construction of glycopeptide **4** it was necessary to synthesize fucose-containing amino acid **6** (Figure 2), as there is no readily available enzyme for modification at the C-6 position of the bridgehead GlcNAc.

Synthesis of **6** began with transformation of known glucosamine derivative **7**[40] into triol **8** through a three-step sequence (Scheme 2). Triol **8** was coupled to fucosyl bromide **12**[41] under halide ion conditions to afford disaccharide **9** in modest yield.[42,43] At this point it was necessary to replace the benzyl protecting groups with acetates in order to stabilize the glycosidic linkage during SPPS. Hydrogenolysis of the benzyl groups occurred with partial removal of the 2,2,2-trichloroethyl carbamate (Troc), necessitating its reinstallation prior to global acetylation. The resulting peracetylated disaccharide was reacted with benzylamine in THF to effect selective cleavage of the anomeric acetate, followed by treatment with 2,2,2-trichloroacetonitrile in the presence of potassium carbonate to afford glycosyl trichloroacetimidate **10**.[44] This was coupled to protected serine **13**[45] in the presence of TMSOTf and activated sieves to afford glycosyl amino acid **12** in excellent yield. Removal of the Troc protecting group was followed by HBTU/DIEA-mediated coupling of the free amine to *S*-trityl-2-mercaptoacetic acid[46] and removal of the allyl protecting group[47] to provide glycosyl amino acid **6** in high yield.

Solid-Phase Peptide and Glycopeptide Synthesis. Synthesis of glycopeptides **14a**−**d** (Scheme 3), which served as precursors for enzymatic elaboration, was achieved using SPPS on Rink-amide resin.[48] Due to the complexity of **5**, only 1 equiv of amino acid was used and the coupling time was extended to 6 h. As reported previously,[32,34] these conditions were sufficient for efficient coupling as determined by the UV absorption of the Fmoc/piperidine adduct at 302 nm. Following coupling of **5**, the resin was split into four equal parts for the synthesis of Gly, Asp, His, and Gly-Gly glycopeptides **14a**−**d**. These targets were chosen because the N-terminal Gly, Asp, and His glycopeptides perform particularly well in SAL, while the Gly-Gly peptide provides a simple extended glycopeptide model. Following the final coupling, the acetate protecting groups on the sugar were removed by hydrazinolysis prior to release from the resin using

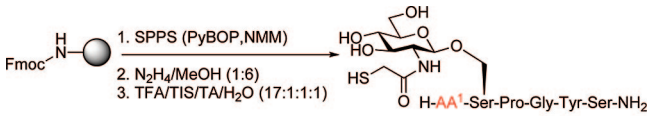
(40) Paulsen, H.; Krogmann, C. *Liebigs Ann. Chem.* **1989**, 1203–1213.
(41) Dejter-Juszynski, D.; Flowers, H. M. *Carboydr. Res.* **1971**, *18*, 215–226.
(42) Lemieux, R. U.; Hendriks, K. B.; James; Stick, R. V.; James, K. *J. Am. Chem. Soc.* **1975**, *97*, 4056–4062.
(43) For the use of halide-ion-mediated glycosylation on a similar system, see: (a) Lee, H. H.; Baptista, J. A. B.; Krepinsky, J. J. *Can. J. Chem.* **1990**, *68*, 953–957.
(44) Schmidt, R. R.; Michel, J. *Tetrahedron Lett.* **1984**, *25*, 821–824.
(45) Vorheer, T.; Bannwarth, W. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2661–2664.
(46) Kupihar, Z.; Schmel, Z.; Kele, Z.; Penke, B.; Kovacs, L. *Bioorg. Med. Chem. Lett.* **2001**, *9*, 1241–1247.
(47) Ciommer, M.; Kunz, H. *Synlett* **1991**, *8*, 593–595.
(48) Albericio, F.; Carpino, L. A. *Methods Enzymol.* **1997**, *289*, 104–126.

Scheme 2. Synthesis of Glycosyl Amino Acid **6**[a]



[a] Conditions: (a) (i) 30% HBr/AcOH, CH_2Cl_2; (ii) BnOH, Ag_2CO_3, AW300MS, CH_2Cl_2; (iii) 1:1 0.1 N NaOH (aq.)/MeOH, 50%, 3 steps; (b) TBAB, **12**, 2:1 CH_2Cl_2/DMF, 4 Å MS, 37%; (c) (i) H_2, 10% Pd/C, 3:1 5% formic acid in MeOH/ethyl acetate; (ii) TrocCl, $NaHCO_3$, H_2O; (iii) Ac_2O, pyridine, 71%, 3 steps; (d) $BnNH_2$, THF, 55%; (e) Cl_3CCN, K_2CO_3, CH_2Cl_2, 59%; (f) **13**, TMSOTf, AW300MS, CH_2Cl_2, −78 °C, 82%; (g) Zn, AcOH, 70%; (h) $TrtSCH_2COOH$, HBTU, DIEA, DMF, 95%; (i) $Pd(PPh_3)_4$, NMA, THF, 82%. AW300MS = acid washed 3 Å molecular sieves, TBAB = tetrabutylammonium bromide, TrocCl = 2,2,2-trichloroethyl chloroformate, HBTU = 2-(1*H*-benzotriazole-1y-l)-1,1,3,3-tetramethylaminium hexafluorophosphate, DIEA = *N*,*N*-diisopropylethylamine, NMA = *N*-methylaniline.

Scheme 3. Fmoc-Based SPPS of Glycopeptides **14a**−**d**



glycopeptide	AA[1]	isolated yield (overall after HPLC)
14a	Gly	82 %
14b	Asp	68%
14c	His	71%
14d	Gly-Gly	45%

trifluoroacetic acid/triisopropylsilane/thioanisole/water (17:1:1:1). The resulting products were purified by HPLC to afford the desired glycopeptides in 45−82% yield.

Synthesis of glycopeptide **4** was also achieved using Fmoc-based SPPS (Scheme 4). Due to the challenges associated with the construction of amino acid **6**, we chose to only synthesize a N-terminal Asp glycopeptide as glycopeptides possessing this residue at their N-terminus provided some of the best yields for SAL. Similar to the synthesis of **14a**−**d**, we found that acceptable yields could be achieved using 1 equiv of **6** and a coupling time of 6 h. Because we were concerned about the stability of the sensitive α-1,6-fucose linkage we chose to release the glycopeptide from the resin prior to removal of the carbohydrate protecting groups. Following removal from the resin under standard conditions and HPLC purification the glycopeptide, Zemplén deacetylation at pH 9 (to prevent β-elimination of the glycan)[49] afforded glycopeptide **4**.

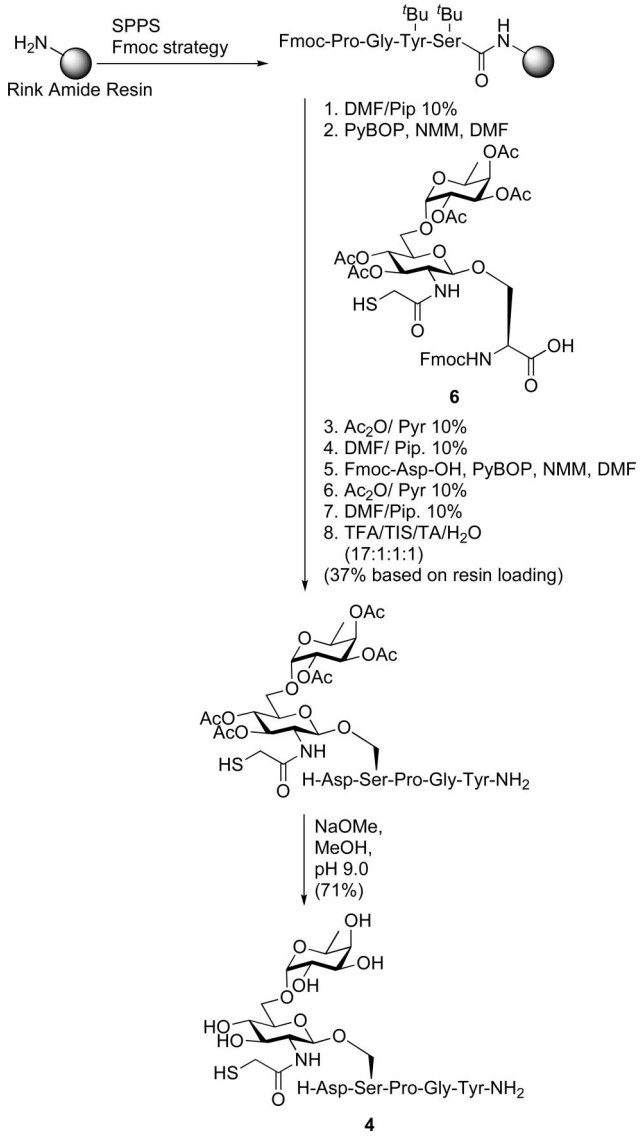
Peptide thioesters bearing amino acids Gly, Tyr, His, and Ala at the C-terminus were synthesized using the Boc-strategy, as reported previously.[32,34] These peptide thioesters were selected so that direct comparisons could be made with SAL reactions bearing monosaccharides which were reported previously. The N-termini of the peptide thioesters were capped with acetates prior to removal from the resin in order to prevent any unwanted aminolysis during the ligation reaction.[50−52]

Enzymatic Elaboration of Glycopeptides. Having synthesized glycopeptides **14a**−**d**, we next turned our attention to the enzymatic synthesis of glycopeptides **1**−**3a**−**d**. To achieve this, we chose to focus on glycosyltransferase-mediated elaboration of **14a**−**d**, as many of these enzymes are known to have a broad substrate tolerance with regard to the glycosyl acceptor.[53] Importantly, from previous studies we already knew that the presence of the sulfhydryl group at C-2 of the bridgehead sugar did not interfere with at least one glycosyltransferase, β-1,4-galactosyltransferase (GalT).[32] As such, treatment of glycopeptides **14a**−**d** with GalT and UDP-galactose (UDP-Gal) in the presence of alkaline phosphatase and $MnCl_2$ afforded the desired disaccharide products **1a**−**d** in 81−86% isolated yields (Scheme 5).[54−56] These products were readily converted to sialic acid containing trisaccharides **2a**−**d** upon treatment with CMP-sialic acid (CMP-sial) and α−2,3-(*N*)-sialyltransferase (SialT), under conditions similar to those described for the GalT reaction.[57] Conversion of **2a**−**d** to glycopeptides **3a**−**d** bearing the sialyl

(49) Brocke, C.; Kunz, H. *Synthesis* **2004**, 525–542.

(50) Kemp, D. S.; Bernstein, Z. W.; McNiel, G. N. *J. Org. Chem.* **1974**, *39*, 2831–2835.
(51) Kemp, D. S.; Choong, S. L. H.; Pekaar, J. *J. Org. Chem.* **1974**, *39*, 3841–3847.
(52) Payne, R. J.; Ficht, S.; Greenberg, W. A.; Wong, C. H. *Angew. Chem., Int. Ed.* **2008**, *47*, 4411–4415.
(53) Blixt, O.; Razi, N. *Methods Enzymol.* **2006**, *415*, 4411–4415.
(54) Schanbacher, F. L.; Ebner, K. E. *J. Biol. Chem.* **1970**, *245*, 5057–5061.
(55) Beyer, T. A.; Sadler, J. E.; Rearicj, J. I.; Paulson, J. C.; Hill, R. L. *Adv. Enzymol.* **1981**, *52*, 23–175.
(56) Wen, D. X.; Livingston, B. D.; Medzihradszky, K. F.; Kelm, S.; Burlingame, A. L.; Paulson, J. C. *J. Biol. Chem.* **1992**, *267*, 21011–21019.

Scheme 4. Synthesis of Glycopeptide **4**[a]



[a] PyBOP = benzotriazole-1-yl-oxy-*tris*-pyrrolidino-phosphonium hexafluorophosphate; Pip = piperidine; TFA = trifluoroacetic acid; TIS = triisopropylsilane; TA = thioanisole; NMM = *N*-methylmorpholine.

Lewis X motif using α-1,3-fucosyltransferase (FucT)[58] proved to be much more challenging, initially providing the desired target glycopeptides in poor yields. This was not entirely surprising given the proximity of the unnatural sulfhydryl group to the C-3 glycosylation site on the bridgehead sugar. Pleasingly, however, we found that it was possible to obtain target glycopeptides **3a**−**d** through the use of an excess of GDP-fucose, FucT, and prolonged reaction times (see Supporting Information).

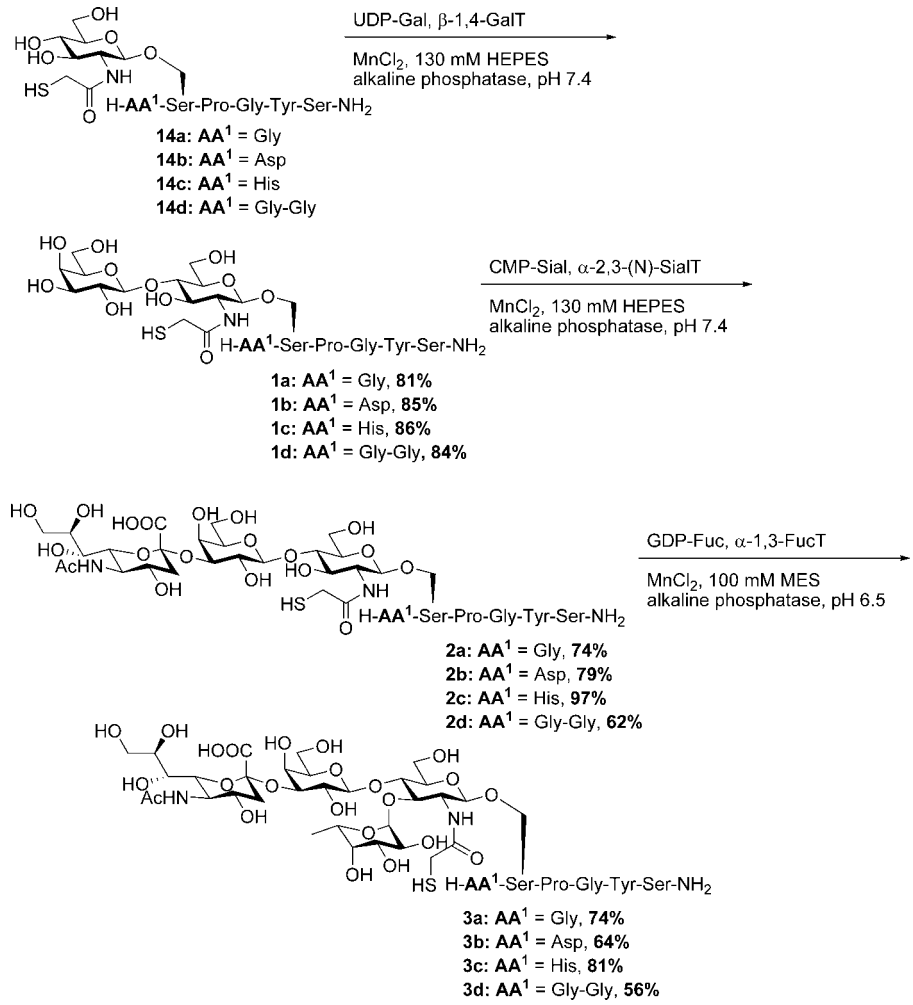
Scope of Sugar-Assisted Ligation with Complex Glycans. With the target glycopeptides in hand, we turned our attention to investigating the efficiency of SAL in the presence of more complex glycans. Our initial investigations focused on the ligation between glycopeptides **1a**-**d** with peptide thioester **15a**, bearing a C-terminal glycine residue, under mixed solvent system conditions (4:1 v/v *N*-methyl pyrrolidinone/6 M guanidine hydrochloride, 1 M HEPES, pH 8.5, 2% PhSH, 37 °C),[33] in order to minimize peptide thioester hydrolysis. Under these conditions glycopeptides **1a** and **1b**, possessing glycine and aspartic acid at their N-terminus, underwent ligation with **15a** in 63% and 70% yields, respectively (Table 1, entries 1 and 2). The fact that these yields were lower than those observed in previous studies with the C-2 auxiliary-containing monosaccharide (91% for glycine)[34] suggests that the increased steric bulk of the galactose moiety may effect the efficiency of the reaction. This effect is even more pronounced with glycopeptide **1c**, possessing a N-terminal histidine, which undergoes ligation with **15a** in 36% yield (entry 3). This result was surprising given that in previous studies glycopeptides bearing an N-terminal His gave good yields in SAL reactions. The lower yield observed with the more complex glycan is presumably due to unfavorable interactions between the larger His side chain and the auxiliary-containing disaccharide during the S → N acyl shift. Finally, extension of the N-terminus of the glycopeptide by an additional glycine residue (**1d**) results in a drop in yield, presumably due to the increased size of the proposed ring transition state (entry 4).

We next turned our attention to the effect of extended glycosylation on the reaction with more sterically demanding peptide thioesters. To this end, we examined the reaction of **1b** with peptide thioesters **15b**−**d**, possessing Tyr, His, and Ala at their C-terminus, respectively (table 1, entries 5−7). These peptide thioesters had been used in our previous studies and would therefore allow a better assessment of the effects of the larger glycan on the reaction. Pleasingly, we found that the presence of the sterically demanding Tyr residue in peptide thioester **15b** did not interfere with the reaction, providing the ligation product in yields comparable to those observed with peptide thioester **15a** (entry 5). Similarly, glycopeptide thioester **15c**, possessing a C-terminal His residue, also underwent ligation in good yield (entry 6). Consistent with our previous studies,[32−34] the ligation between **1b** and peptide thioester **15d**, possessing an C-terminal Ala residue, gave a slightly lower yield (55%); however this is still deemed to be synthetically useful (entry 7). These data suggest that the C-terminal residue of the peptide thioester is less sensitive to the presence of larger glycans than the N-terminus of the glycopeptide, in line with trends observed in previous SAL studies.[31−34]

In order to further probe the effects of glycosylation at C-4, we examined the efficiency of SAL with trisaccharide glycopeptides **2a**−**d**. Pleasingly, we found that the addition of a sialic acid residue to C-3 of the terminal galactose had little effect on the reaction between peptide thioester **15a** and glycopeptides **2a** and **2b** (Table 2, entries 1 and 2). By comparison, the reaction between glycopeptide **2c**, possessing a N-terminal His, and **15a** proceeded in poor (14%) yield (entry 3), with no observable increase in yield with prolonged reaction times. The large decrease in yield upon moving to the larger glycan, relative to that obtained with disaccharide-containing glycopeptide **1c** (Table 1, entry 3), further underscores our observation that larger amino acids experience unfavorable steric interactions with the extended glycans in the reaction. The increased bulk of the glycan did not affect the reaction between **15a** and extended glycopeptide **2d**, which gave the desired product in yields comparable to those obtained with the disaccharide-containing glycopeptide **1d**. From these results, we conclude that for sterically unencumbered amino acids extending glycosylation

(57) Wen, D. X.; Livingston, B. D.; Medzihradszky, K. F.; Kelm, S.; Burlingame, A. L.; Paulson, J. C. *J. Biol. Chem.* **1992**, *267*, 21011–21019.
(58) Lin, S. W.; Yuan, T. M.; Li, J. R.; Lin, C. H. *Biochemistry* **2006**, *45*, 8108–8116.

Scheme 5. Enzymatic Elaboration of Glycopeptides **14a**−**d**[a]



[a] Abbreviations: UDP-Gal = Uridine-5-diphosphate-α-D-galactose, disodium salt, β-1,4-GalT = β-1,4-galactosyltransferase, HEPES = 4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid, CMP-Sial = cytosine monophosphate sialic acid, monosodium salt, α-2,3-SialT = α-2,3-sialyltransferase, GDP-Fuc = Guanosine-5-diphosphate-β-L-fucose, disodium salt, α-1,3-FucT = α-1,3-fucosyltransferase, MES = 2-(*N*-morpholino)-1-ethanesulfonic acid.

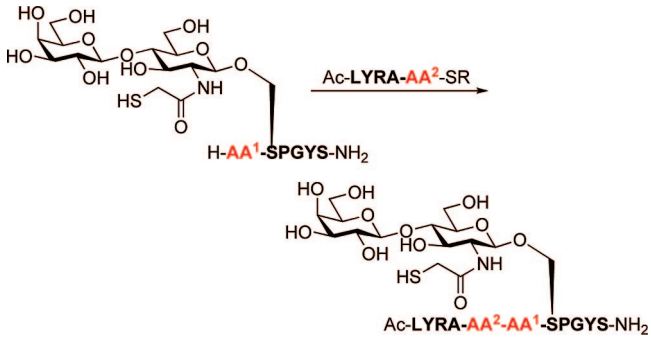
at C-4 beyond a disaccharide does not have a major impact on the reaction. Ligation products could be desulfurized in an efficient manner by treatment with 10% palladium on alumina in the presence of tris(2-carboxyethyl)phosphine hydrochloride (TCEP, see Supporting Information).[32]

We next turned our attention to the more sterically congested sialyl Lewis X glycopeptides **3a**−**d**. Given the effects of steric hindrance on the reaction observed with glycosylation at C-4, we were concerned that the introduction of the fucose residue onto the C-3 position of the bridgehead sugar would be deleterious to the reaction. This indeed proved to be the case, and we were unable to isolate any product from ligations between **3a**−**d** and peptide thioester **15a**, even after prolonged reaction times (Scheme 6). These results indicate that the SAL reaction is not compatible with extended glycosylation at positions adjacent to the thiol auxiliary. This observation provides valuable information about which glycan structures can be utilized for SAL reactions and should prove useful for planning syntheses of complex glycopeptides and glycoproteins in future studies.

We next examined the effect of glycosylation at C-6 of the bridgehead sugar using glycopeptide **4**. Given the distance from the auxiliary, we were hopeful that this modification would not have a significant negative effect on the reaction. To this end, we examined the reaction between glycopeptide **4** and peptide thioesters **15a** and **15d** (Scheme 7). In both cases the SAL reaction proceeded in moderate (44%) yield to afford glycopeptides **16a** and **16b**. While these yields are synthetically useful, they are lower than those observed with auxiliary-containing monosaccharides or C-4 substituted auxiliary-containing glycans. This suggests that the glycan at C-6 may be interacting with the peptide backbone. Importantly, given the use of the generally more reactive glycopeptide possessing a N-terminal Asp in the SAL reaction, these results indicate that care must be used in selecting N-terminal residues in SAL reactions when the auxiliary-containing glycan is substituted at C-6.

N-Linked Ligation Study. Having examined the effects of complex O-glycosylation on the efficiency of the SAL reaction, we sought to determine if complex N-linked auxiliary-containing glycopeptides were compatible with the reaction. To this end, we envisioned enzymatic elaboration of glycopeptide **17**[32] using endo-β-*N*-acetylglucosaminidase A or M (Endo A or M) mediated transglycosylation.[59,60] Unfortunately, all attempts to transfer a complex oligosaccharide to the auxiliary-containing glycan in **17** using Endo M failed. Rationalizing that the C-2 sulfhydryl group might be interfering with the reaction, we next examined Endo A mediated transfer of sugar oxazoline **18**,[61]

Table 1. Scope of SAL with Glycopeptides **1a**−**d**[a]



entry	glycopeptide (AA^1)	thioester (AA^2)	ligation junction -AA^2-AA^1-	isolated ligation yield[b]
1	**1a** (Gly)	**15a** (Gly)	Gly-Gly	63%
2	**1b** (Asp)	**15a**	Gly-Asp	70%
3	**1c** (His)	**15a**	Gly-His	36%
4	**1d** (Gly-Gly)	**15a**	Gly-Gly	49%
5	**1b**	**15b** (Tyr)	Tyr-Asp	70%
6	**1b**	**15c** (His)	His-Asp	70%
7	**1b**	**15d** (Ala)	Ala-Asp	55%

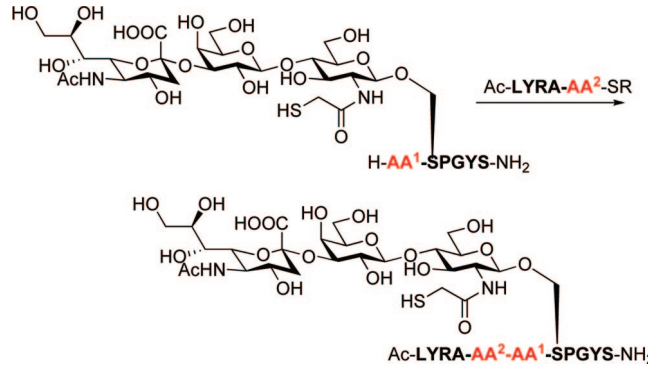
[a] Conditions: 4:1 v/v NMP/6 M Gn·HCl, 1 M HEPES, pH 8.5, 2% PhSH, 37 °C, 96 h. [b] Isolated yield after HPLC purification.

as this has been reported to be a much more efficient substrate for Endo mediated transfer.[62−64] Pleasingly, this strategy proved to be more effective, delivering the target glycopeptide **19** in 38% yield (Scheme 8). This glycopeptide underwent SAL with peptide thioester **15a** to afford glycopeptide **20** in 40% yield after HPLC purification.

Conclusion

The presence of extended glycosylation on the auxiliary-containing glycan in SAL can have a significant impact on the outcome of the reaction. Glycosylation at C-4 has the smallest effect on the reaction, where in many cases the reaction proceeds with high levels of efficiency. The effect of extended glycosylation at C-6 on the SAL reaction is more pronounced, resulting in lower, but still synthetically useful, ligation yields. In both of these cases, the selection of the N-terminal amino acid in the auxiliary-containing glycopeptide is critical, and sterically unencumbered amino acids are necessary for optimal yields. Importantly, it appears that the peptide thioester is much less sensitive to extended glycosylation at C-4 and C-6 on the auxiliary-containing glycan, as even peptide thioesters possessing bulky C-terminal residues smoothly undergo the reaction with glycopeptides bearing appropriate N-terminal residues. SAL is not tolerant of glycosylation at positions adjacent to the auxiliary, however, as glycosylation at C-3 prevents the reaction from taking place. Finally, both O-linked and N-linked auxiliary-containing glycopeptides can be extended, although it appears that the latter is more sensitive to larger glycans. Based on these results, we urge caution in using SAL when a novel glycosylation pattern is encountered in a glycoprotein, as structural changes in more complex glycans can have major and unexpected effects on the reaction, as evidenced by the results obtained with the C-6 substituted glycopeptide **4**. Nevertheless,

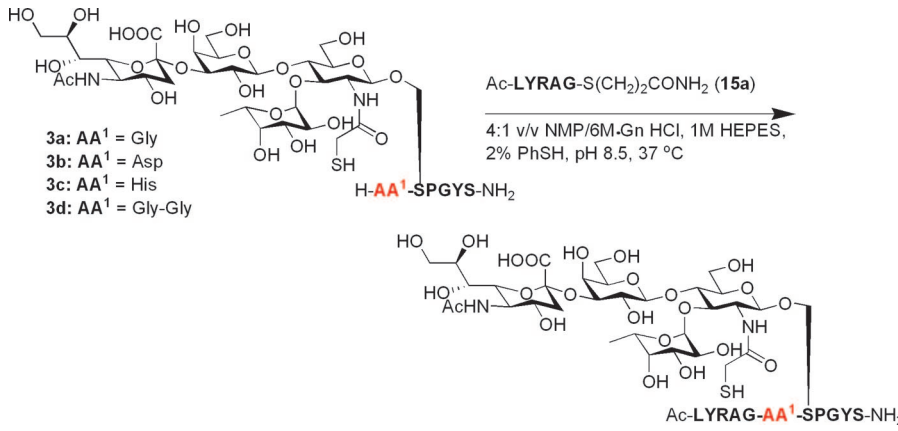
Table 2. Scope of SAL with Trisaccharide-Containing Glycopeptides **2a**−**d**[a]



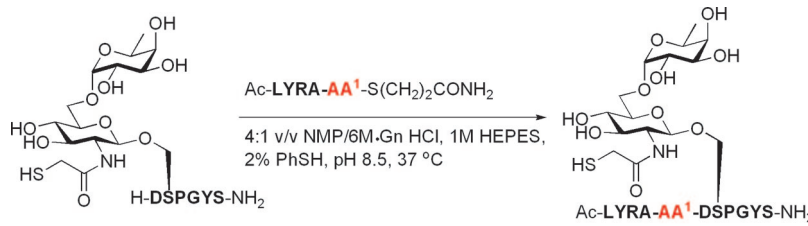
entry	glycopeptide (AA^1)	ligation junction -AA^2-AA^1-	isolated ligation yield[b]
1	**2a** (Gly)	Gly-Gly	57%
2	**2b** (Asp)	Gly-Asp	70%
3	**2c** (His)	Gly-His	14%
4	**2d** (Gly-Gly)	Gly-Gly	44%

[a] Conditions: 4:1 v/v NMP/6 M Gn·HCl, 1 M HEPES, pH 8.5, 2% PhSH, 37 °C, 96 h. [b] Isolated yield after HPLC purification.

Scheme 6. Attempted Ligation of Glycopeptides **3a**−**d** with Peptide Thioester **15a**

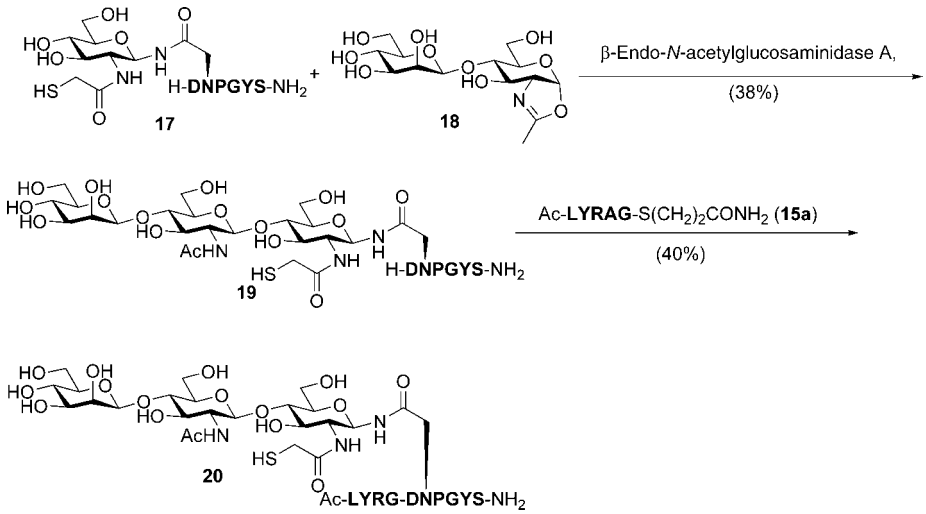


Scheme 7. SAL Reactions between Glycopeptide **4** and Peptide Thioesters **15a** and **15d**



Peptide thioester	AA^1	isolated yield (overall after HPLC)
15a	Gly	44 %
15d	Ala	44%

Scheme 8. Endo-β-*N*-acetylglucosaminidase A Mediated Elaboration of Auxiliary-Containing N-Linked Glycopeptide **17** and Use of the Resulting Product in SAL[a,b]



[a] Endo-mediated glycosylation conditions 20 mM phosphate buffer, pH 6.0. [b] SAL conditions 4:1 v/v NMP/6 M Gn·HCl, 1 M HEPES, pH 8.5, 2% PhSH, 37 °C, 96 h.

we feel that when appropriate SAL is a useful addition to the glycopeptide, and potentially glycoprotein, synthesis toolbox, as it permits access to ligation junctions that cannot be constructed using other ligation strategies.

Experimental Section

General procedure for sugar-assisted ligation: A 20 mM solution of the glycopeptide (1.5 equiv) in 4:1 v/v NMP/6 M Gn HCl, 1 M HEPES, pH 8.5 was added to the peptide thioester (1 equiv). The resulting solution was treated with thiophenol (2% v/v) and incubated at 37 °C for 4 days, before quenching with 300 μL of aqueous TCEP solution (10 mg/mL). The resulting solution was purified by preparative HPLC (0−35% A/B, A = 0.1% formic acid in acetonitrile, B = 0.1% formic acid in water) and analyzed by MALDI-TOF/MS (matrix, 2,5-dihydroxybenzoic acid).

Acknowledgment. We thank Professor Chun-Hung Lin (Academica Sinica) for providing us with fucosyltransferases and Professor Kaoru Takegawa (Kagawa University) for the gift of Endo A-GST fusion protein plasmid. This work was supported by the NIH and the Skaggs Institute of Chemical Biology. C.S.B. is grateful for a NIH postdoctoral fellowship. R.J.P. is grateful for funding provided by the Lindemann Trust Fellowship. S.F. thanks the Deutsche Akademische Austauschdienst (DAAD) for a postdoctoral fellowship. We thank Professor William A. Greenberg and Dr. Sarah R. Hanson for help in preparation of the manuscript.

Supporting Information Available: Experimental procedures, characterization of products, and other detailed results. This material is available free of charge via the Internet at http://pubs.acs.org.

JA8010513

(59) Fan, J. Q.; Takegawa, K.; Iwahara, S.; Kondo, A.; Kato, I.; Abeyunawardana, C.; Lee, Y. C. *J. Biol. Chem.* **1995**, *270*, 17723–17729.
(60) Akaike, E.; Tsutsumida, M.; Osumi, K.; Fujita, M.; Yamanoi, T.; Yamamoto, K.; Fujta, K. *Carbohydr. Res.* **2004**, *339*, 719–722.
(61) Li, B.; Zeng, Y.; Hauser, S.; Song, H.; Wang, L. X. *J. Am. Chem. Soc.* **2005**, *127*, 9692–9693.
(62) Li, H.; Li, B.; Song, H.; Breydo, L.; Baskakov, I. V.; Wag, L. X. *J. Org. Chem.* **2005**, *70*, 9990–9996.
(63) Zeng, Y.; Wang, J.; Li, B.; Hauser, S.; Li, H.; Wang, L. X. *Chem.—Eur. J.* **2006**, *12*, 3355–3364.
(64) Rising, T. W. D. F.; Claridge, T. D. W.; Moir, J. W. B.; Fairbanks, A. J. *ChemBioChem* **2006**, *7*, 1177–1180.