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Rapid Formation of N-Glycopeptides *via* Cu(II)-Promoted Glycosylative Ligation

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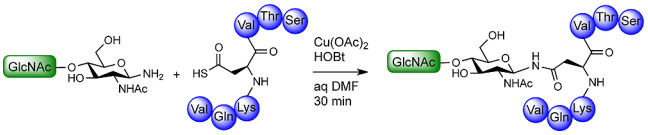
ABSTRACT



Herein is described the chemoselective Cu(II)-HOBt promoted chemical ligation of glycosylamines and peptide thioacids to give N-glycosylated peptides. The method is distinguished from other chemical approaches to peptide N-glycosylation in that (1) it can be employed in the presence of unprotected N-terminal and Lys side chain amines; (2) it is remarkably fast, going to completion in under 30 min; and (3) it produces glycopeptides without attendant aspartimide formation.

Protein N-glycosylation, wherein a glycan is attached to an asparagine residue, is an important co- and post-translational modification that influences a diverse array of biological processes ranging from protein quality control to cell recognition.[1,2] It has been hypothesized that the epigenetically controlled biosynthesis of glycoproteins has evolved in higher organisms as a relatively rapid means to modulate protein function in response to environmental changes.[3] Glycoproteins have also been implicated in disease states. For example, cancer cells undergo adaptive regulation of their cell surface glycosylation patterns in order to acquire a survival advantage.[4] Progress toward elucidating the glycoproteome has been hindered by the difficulty in obtaining homogeneous glycoproteins for characterization and biological studies. The challenges of glycopeptide synthesis using recombinant techniques make the case for developing chemical synthetic methods to access homogeneous glycoproteins.[5]

The covalent union of the carbohydrate and peptide domains of glycoproteins remains a formidable challenge. Recent synthetic approaches to N-linked glycoprotein constructs have generally utilized the native chemical ligation[6] of glycopeptide segments, which are prepared by incorporating the glycosylated amino acids during a solid phase peptide synthesis.[5,7] Syntheses of N-glycopeptides arising from the coupling of a glycosylamine such as **1** with an aspartic acid residue embedded in a peptide **2** to give the N-glycosylated peptide **3** (Scheme 1) have also been reported.[8−11]

Inherent limitations of these protocols necessitate the masking of free amino groups (N-terminus, Lys side chains) in the peptide, restricting their application to the middle and early stages of a projected glycoprotein synthesis.[12]

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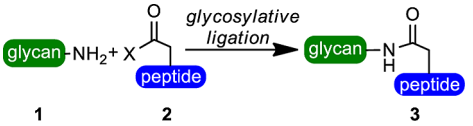
An aspartylation procedure that circumvents this chemoselectivity issue would permit the introduction of the glycan moiety at a later stage in the synthesis, thus making it more convergent. Finally, several protecting group schemes have been developed as potential solutions to the ubiquitous problem of aspartimide formation.[11] While these represent significant advances, the development of an aspartylation protocol which bypassed aspartimide formation altogether could reduce protecting group manipulations and simplify the chemical synthesis of glycopeptides. We now report a method that enables the rapid chemical ligation of a glycosylamine and a specific aspartic acid residue in an unprotected peptide to give a native N-linked glycopeptide fragment.

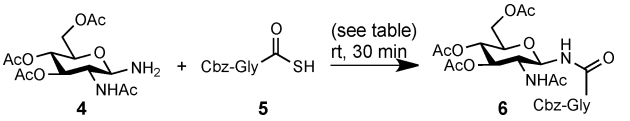
Scheme 1



Our approach is derived from our recently reported Cu(II)-promoted ligation of unprotected peptide thioacids and aziridine-2-carbonyl peptides.[13] This facile coupling reaction proceeds chemoselectively in the presence of unprotected primary amines, which we tentatively attribute to the unique properties of the aziridine lone pair (reduced basicity and reduced steric hindrance) working in concert with the cupric ion activated thioacid. We reasoned that a glycosylamine, with an estimated ammonium ion pK_a of approximately 6,[14] might exhibit similar behavior with unprotected peptide thioacids, specifically one derived from an aspartic acid side chain. If this logic held, these properties would enable the analogous Cu(II)-promoted N-glycosylative ligation to proceed chemoselectively in the presence of (protonated) free amines.

To test this idea, we initially examined the coupling of $Ac_3GlcNAc\beta$-NH_2 (**4**)[15] and Cbz-Gly-SH (**5**)[16] to provide the readily isolated product **6** (Table 1), confirming that the aziridine-mediated ligation conditions did, indeed, result in the desired amide bond formation (entry 1).[17] Reaction optimization studies (entries 2–6) indicated that the maximum yield of **6** was obtained when the thioacid was added to the Cu(II)–HOBt complex followed by a 4-fold excess of glycosylamine (entry 5). These conditions minimized the detrimental effect resulting from the known propensity of glycosylamines toward hydrolysis.[18] Only marginal improvement in yield accompanied a further increase in the amount of **6** used. This reaction was complete after 30 min at room temperature. Control experiments demonstrated the necessity for both Cu(II) and HOBt in the reaction (entries 7 and 8).[19] A known oxidative protocol[9] did not produce **6** after 30 min (entry 9). The desired reaction could be "rescued" by the inclusion of $Cu(OAc)_2$, but DMSO was deemed to be inferior to DMF (entry 10).

Table 1. Reaction Optimization and Control Experiments



entry	equiv of **4**	equiv of **5**	reaction conditions	yield of **6**
1	1.0	1.0	$Cu(OAc)_2$ (1.0), HOBt (2.0), aq. DMF[a]	64%[c]
2	1.0	1.0	$Cu(OAc)_2$ (1.0), HOBt (2.0), aq. DMF[b]	68%[d]
3	2.0	1.0	$Cu(OAc)_2$ (1.0), HOBt (2.0), aq. DMF[b]	75%[d]
4	3.0	1.0	$Cu(OAc)_2$ (1.0), HOBt (2.0), aq. DMF[b]	81%[d]
5	4.0	1.0	$Cu(OAc)_2$ (1.0), HOBt (2.0), aq. DMF[b]	90%[c]
6	5.0	1.0	$Cu(OAc)_2$ (1.0), HOBt (2.0), aq. DMF[b]	92%[d]
7	1.0	1.1	HOBt (2.2), DMF[b]	0%[e]
8	1.0	1.1	$Cu(OAc)_2$ (1.0), aq. DMF[b]	0%[e]
9	4.0	1.0	HOBt (2.0), DMSO	0%[d]
10	4.0	1.0	$Cu(OAc)_2$ (1.0), HOBt (2.0), aq. DMSO[b]	83%[d]

[a] Order of addition: aq $Cu(OAc)_2 \bullet H_2O$, HOBt, amine, thioacid. [b] Order of addition: aq $Cu(OAc)_2 \bullet H_2O$, thioacid, amine. [c] Isolated yield. [d] Quantitative HPLC analysis. [e] TLC analysis.

(12) (a) Davis and co-workers reported the coupling of GlcNAcβ-N_3 and Fmoc-Ser-Asp(OBt)-Leu-Thr-NH_2 using the Staudinger reaction: Doores, K. J.; Mimura, Y.; Dwek, R. A.; Rudd, P. M.; Elliott, T.; Davis, B. G. *Chem. Commun.* **2006**, 1401. (b) Damkaci, F.; DeShong, P. *J. Am. Chem. Soc.* **2003**, *125*, 4408.

(13) Dyer, F. B.; Park, C.-M.; Joseph, R.; Garner, P. *J. Am. Chem. Soc.* **2011**, *133*, 20033.

(14) Legler, G. *Biochim. Biophys. Acta* **1978**, *524*, 94.

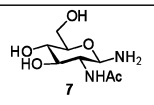
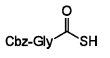
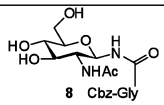
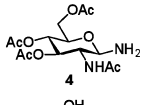
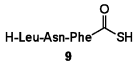
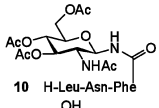
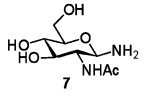
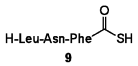
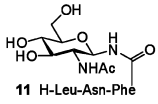
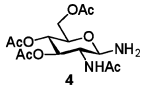
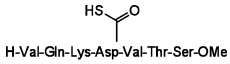
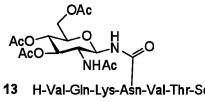
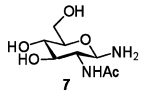
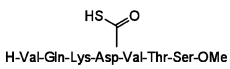
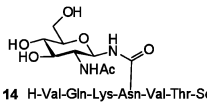
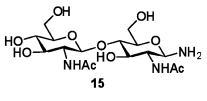
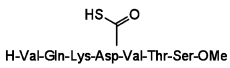
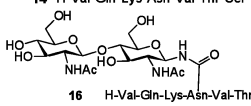
(15) Glycosylamine **4** was prepared from commercially available glucosamine hydrochloride (1a. NaOMe/MeOH; 1b. Ac_2O 0°C to rt, 92% yield; 2. AcCl, rt, 65% yield; 3. NaN_3, DMF, 70°C, 96% yield; 4. H_2 Pd/C, EtOAc, rt, 90% yield) using a known method: Cunha, A.; Pereira, L. c.; de Souza, R.; de Souza, M. C. l.; Ferreira, V. *Nucleosides, Nucleotides, Nucleic Acids* **2001**, *20*, 1555.

(16) Thioacid **5** was prepared from commercially available Cbz-Gly-OH (1. TrtSH, EDC, DMAP, CH_2Cl_2, rt, 90% yield; 2. TFA, Et_3SiH, CH_2Cl_2, rt, 85% yield) using a known method: Crich, D.; Sharma, I. *Angew. Chem., Int. Ed.* **2009**, *48*, 7591.

(17) While our work was already in progress, Gopi and coworkers reported the facile, Cu(II)-promoted coupling of thioacids and amines in MeOH. The method was applied to the synthesis of fully protected peptides. They also showed that this amidation was catalyzed by *in situ* generated copper sulfide. Mali, S. M.; Jadhav, S. V.; Gopi, H. N. *Chem. Commun.* **2012**, *48*, 7085–7087.

(18) Isbell, H. S.; Frush, H. J. *J. Org. Chem.* **1958**, *23*, 1309.

Table 2. Chemoselective N-Glycosylation Reactions[a]

entry	glycosylamine	thioacid	ligation product	isolated yield
1	 **7**	 **5**	 **8** Cbz-Gly	94%
2	 **4**	 **9**	 **10** H-Leu-Asn-Phe	72%[b]
3	 **7**	 **9**	 **11** H-Leu-Asn-Phe	81%[c]
4	 **4**	 **12**	 **13** H-Val-Gln-Lys-Asn-Val-Thr-Ser-OMe	55%[d]
5	 **7**	 **12**	 **14** H-Val-Gln-Lys-Asn-Val-Thr-Ser-OMe	65%[e]
6	 **15**	 **12**	 **16** H-Val-Gln-Lys-Asn-Val-Thr-Ser-OMe	53%[f]

[a] The optimized conditions from Table 1, entry 5 were used for these reactions. [b] 19% of hydrolyzed thioacid was isolated. [c] 5% of hydrolyzed thioacid was isolated. [d] 32% of hydrolyzed thioacid was isolated. [e] 21% of hydrolyzed thioacid was isolated. [f] 30% of hydrolyzed thioacid was isolated.

With an optimized glycosylation protocol in hand, we next examined the chemoselectivity issue employing our optimized reaction conditions and substrates that did not incorporate protecting groups (Table 2). The coupling of GlcNAcβ-NH_2 (**7**)[20] and thioacid **5** gave glycosylated amino acid **8** in 94% isolated yield (entry 1), demonstrating the feasibility of using unprotected glycosylamines in the reaction. The coupling of peracetylated glycosylamine **4** and the unprotected tripeptide thioacid H-Leu-Asn-Phe-SH (**9**)[21] provided our first test of peptide amine chemoselectivity, cleanly producing **10** in 72% isolated yield (entry 2). Careful HPLC-MS analysis of the reaction mixture showed no evidence of interference by the N-terminal amine, confirming the chemoselectivity.[22] This result was in line with our aziridine-mediated peptide ligation studies.[13] Building on these two experiments, the coupling of unprotected glycosylamine **7** and unprotected thioacid **9** afforded the product **11** in 81% isolated yield (entry 3).

Moving on to examples that are directly related to glycobiology, we next examined the glycosylative ligation in the context of aspartylation using the heptapeptide thioacid **12**,[23] a compound that contains unprotected amine and alcohol moieties. The coupling of **4** and **12** proceeded smoothly in the presence of both the N-terminal amine and unprotected Lys side chain to afford the glycopeptide **13** in 55% isolated yield (entry 4). As expected, the ligation of **7** and **12** gave the glycopeptide **14** in 65% isolated yield (entry 5). Finally, the reaction of chitobiosylamine (**15**)[24] and thioacid **12** produced the glycopeptide **16** in 53% isolated yield (entry 6). This example demonstrated the compatibility of glycosylative ligation with an intersaccharide acetal linkage. None of the aspartimide that often accompanies N-glycosylation procedures was observed in these ligation reactions.[25] The point of glycan attachment to the peptide in **16** (ω-aspartylation) was confirmed by means of a ROESY NMR experiment. In each case, the mass balance was accounted for by isolation of the thioacid hydrolysis byproduct. Products **14** and **16** correspond to truncated segments of the common α-subunit found in human

(19) The benefit of adding Cu(II) salts together with HOBt or $Cu(OBt)_2$ to standard peptide coupling reactions in order to suppress racemization has been known for some time: (a) Miyazawa, T.; Otomatsu, T.; Fukui, Y.; Yamada, T.; Kuwata, S. *Int. J. Peptide Protein Res.* **1992**, *39*, 308. (b) Gibson, F. S.; Rapoport, H. *J. Org. Chem.* **1995**, *60*, 2615. (c) Ryadnov, M. G.; Klimenko, L. V.; Mitin, Y. V. *J. Peptide Res.* **1999**, *53*, 322. (d) Van den Nest, W.; Yuval, S.; Albericio, F. *J. Peptide Sci.* **2001**, *7*, 115.

(20) Kiyozumi, M.; Kato, K.; Komori, T.; Yamamoto, A.; Kawasaki, T.; Tsukamoto, H. *Carbohyd. Res.* **1970**, *14*, 355.

(21) Known compound Boc-Phe-SFm was elongated to Fmoc-Leu-Asn-Phe-SFm with HATU/Boc chemistry and then globally deprotected with DBU and purified by HPLC. See: Wu, W.; Zhang, Z.; Liebeskind, L. S. *J. Am. Chem. Soc.* **2011**, *133*, 14256.

(22) Lack of selectivity would lead to peptide coupling products of the type H-Leu-Asn-Phe-Leu-Asn-Phe-X (X = SH, OH, Ac_3GlcNAcβ-NH), which were not detected in the crude reaction mixture.

(23) Synthesis of peptide thioacid **12**: Boc-Val-Gln(Tr)-Lys(Boc)-Asp(OAll)-Val-Thr(^{t}Bu)-Ser(^{t}Bu) on 2-chlorotrityl resin was synthesized using standard Fmoc-based solid phase synthesis protocols. Conversion to **12** was accomplished in solution (1. AcOH/TFE/DCM to obtain Boc-Val-Gln(Tr)-Lys(Boc)-Asp(OAll)-Val-Thr(^{t}Bu)-Ser(^{t}Bu)-OH; 2. CH_2N_2; 3. $Pd(PPh_3)_4$/pTolSO_2Na; 4. DIC, cat. DMAP, HS-Tmb; 5. TFA global deprotection).

(24) Hackenberger, C. P. R.; O'Reilly, M. K.; Imperiali, B. *J. Org. Chem.* **2005**, *70*, 3574.

(25) This conclusion was confirmed by comparing with a genuine sample of the expected aspartimide.

glycoprotein hormones (e.g., human chorionic gonadotropin, hCG).[26]

The N-glycosylative ligation provides a means for the chemoselective attachment of a glycosylamine to a specific aspartic acid residue in an unprotected peptide. Furthermore, this Cu(II)-mediated ligation reaction appears to proceed significantly faster than aspartylation protocols that have previously been reported. Finally, the Cu(II)-promoted glycosylative ligation formed the expected N-glycopeptides with no detectable amount of aspartimide side product. Although the mechanism of this reaction remains to be determined, the critical role played by the cuprate ion is established. While the N-glycosylative ligation is still at the earliest stages of development, this reaction shows promise for the convergent synthesis of complex N-glycopeptides. Extension of this reaction to larger systems will be the next challenge to face.

Acknowledgment. This research was supported by a grant from the National Science Foundation (CHE-1149327). We thank Dr. Greg Helms (WSU Center for NMR Spectroscopy) for his technical assistance with the 2D NMR experiments on glycopeptide **16**.

Supporting Information Available. Experimental procedures and characterization data for all new compounds are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

(26) Pierce, J. G.; Parsons, T. F. *Annu. Rev. Biochem.* **1981**, *50*, 465.

The authors declare no competing financial interest.