ORGANIC LETTERS

2012
Vol. 14, No. 15
3958–3961

Divergent Behavior of Glycosylated Threonine and Serine Derivatives in Solid Phase Peptide Synthesis

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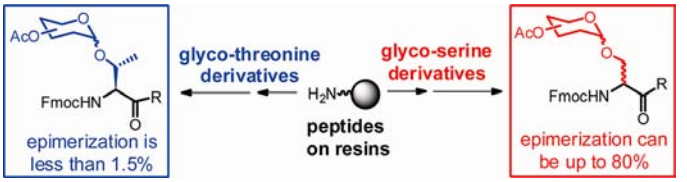
Received June 22, 2012

ABSTRACT



Solid phase peptide coupling of glycosylated threonine derivatives was systematically evaluated. In contrast to glycosylated serine derivatives which are highly prone to epimerization, glycosylated threonine derivatives produce only negligible amounts of epimerization. Under forcing conditions, glycosylated threonine analogs undergo β-elimination, rather than epimerization. Mechanistic studies and molecular modeling were used to understand the origin of the differences in reactivity.

Glycopeptides have been used extensively in basic and clinical research.[1−5] Glycopeptides can serve as structurally defined models of complex glycoproteins, and several are in development as therapeutic agents. For example, a glycopeptide isolated from urine has antiproliferative activity,[6] and glycosylation of an opioid peptide enhances its *in vivo* performance.[1] Glycopeptides are also being developed as vaccines for cancer and HIV, and several have progressed into clinical trials.[2,7−9]

Chemical synthesis is an important tool for obtaining structurally defined glycopeptides, since homogeneous glycopeptides are difficult to obtain from natural sources.[1,10] Chemical synthesis allows precise control of the chemical structure and access to large quantities of material. Moreover, one can obtain unnatural derivatives for studying structure−activity relationships.

The synthesis of glycopeptides can be significantly more challenging than standard peptides. Glycopeptides are commonly synthesized by coupling protected glyco-amino acids to a growing peptide chain via solid phase glycopeptide synthesis.[1] These couplings are often slow and inefficient. In addition, we recently demonstrated that many commonly used peptide coupling conditions produce high levels of epimerization for glyco-amino acids.[11] In fact, epimerization could produce as high as 80% of the unnatural epimer. At present, however, the factors that influence efficiency and epimerization are not well understood, and additional studies are needed to develop efficient and general peptide coupling conditions for glycopeptides.

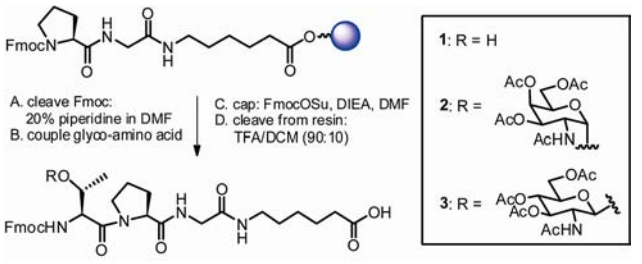
Our previous study focused on coupling glycosylated serine derivatives to a growing peptide chain.[11] In this study, we evaluated efficiency and epimerization when coupling glycosylated threonine derivatives. Nonglycosylated threonine derivatives are known to react more slowly

(1) Pratt, M. R.; Bertozzi, C. R. *Chem. Soc. Rev.* **2005**, *34*, 58.
(2) Westerlind, U.; Kunz, H. *Carbohydr. Chem.* **2010**, *36*, 1.
(3) Wilkinson, B. L.; Stone, R. S.; Capicciotti, C. J.; Thaysen-Andersen, M.; Matthews, J. M.; Packer, N. H.; Ben, R. N.; Payne, R. J. *Angew. Chem., Int. Ed.* **2012**, *51*, 3606.
(4) Gamblin, D. P.; Scanlan, E. M.; Davis, B. G. *Chem. Rev.* **2009**, *109*, 131.
(5) Payne, R. J.; Wong, C.-H. *Chem. Commun.* **2010**, *46*, 21.
(6) Kaczmarek, P.; Keay, S. K.; Tocci, G. M.; Koch, K. R.; Zhang, C.-O.; Barchi, J. J., Jr.; Grkovic, D.; Guo, L.; Michejda, C. J. *J. Med. Chem.* **2008**, *51*, 5974.
(7) Dalgleish, A. G. *Expert Rev. Vaccines* **2004**, *3*, 665.
(8) Zhu, J.-L.; Warren, J. D.; Danishefsky, S. J. *Expert Rev. Vaccines* **2009**, *8*, 1399.
(9) Springer, G. F. *Science* **1984**, *224*, 1198.
(10) Buskas, T.; Ingale, S.; Boons, G.-J. *Glycobiology* **2006**, *16*, 113R.
(11) Zhang, Y.; Muthana, S. M.; Farnsworth, D.; Ludek, O.; Adams, K.; Barchi, J. J.; Gildersleeve, J. C. *J. Am. Chem. Soc.* **2012**, *134*, 6316.

10.1021/ol301723e
Published on Web 07/20/2012

than the corresponding serine derivatives in solid phase peptide coupling reactions.[12] Since a slower coupling rate would allow more time for epimerization, it was possible that glycosylated threonine residues could be even more prone to side reactions.

Scheme 1. Coupling Threonine and Glycosylated Threonine Derivatives to ProGlyHex Resin



We carried out a systematic comparison by coupling glyco-amino acids to a growing peptide chain, Pro-Gly-Hex (Hex: 6-aminohexanoic acid), on solid phase. Two commonly used glyco-amino acids, Fmoc-Thr-(Ac_3GalNAcα)-OH and Fmoc-Thr(Ac_3GlcNAcβ)-OH, were selected for this study, along with the nonglycosylated counterpart as shown in Scheme 1. The desired glycopeptides, the unnatural epimers, and the truncated (uncoupled) peptides were chemically synthesized and used as standards for the development of HPLC assays to evaluate efficiency and epimerization (see Supporting Information (SI)). We selected seven coupling conditions that had previously been used to prepare glycopeptides (see Table 1 for references). They contain the most widely used activation agents, bases, and solvents, as well as variations in reaction times and equivalents of bases and reagents.

The results are summarized in Table 1. Little or no truncated peptide was observed in any of the reactions. Moreover, only negligible epimerization (≤1.5%) was detected for all of the coupling reactions. This result was in stark contrast to our previous results with glycosylated serine analogs. For example, coupling of Fmoc-Ser-(Ac_3GalNAcα)-OH using condition 2 produced ~70% epimerization, whereas < 1% epimerization was observed for glycosylated threonine derivatives. Under several

Table 1. Summary of Yields, Epimerization, and β-Eliminaion for Various Peptide Coupling Conditions

			Fmoc-Thr(R)-OH, where R =		
no.	coupling conditions[a]		Trt	Ac_3GalNAcα	Ac_3GlcNAcβ
1	AAs: 1.5 equiv	yields (%)[b]	98.3	98.2	98.8
	HATU/HOAt: 1.2/1.2 equiv	epimerization (%)[b]	<0.2	0.4	0.3
	NMM: 2.4 equiv	β-elimination (%)[b]	<0.2	<0.2	0.5
	0/8 h in DMF[13]				
2	AAs: 4.4 equiv	yields (%)[b]	99.9	84.1	40.4
	HATU: 4.4 equiv	epimerization (%)[b]	1.5	0.9	0.8
	NMM: 8.8 equiv	β-elimination (%)[b]	<0.2	<0.2	59.4
	3/12 h in NMP[14]				
3	AAs: 2 equiv	yields (%)[b]	98.6	99.1	94.1
	HBTU/HOBt: 4.5/4.5 equiv	epimerization (%)[b]	0.3	0.6	<0.2
	DIEA: 9 equiv	β-elimination (%)[b]	<0.2	0.4	5.0
	0/1 h in NMP[15]				
4	AAs: 3 equiv:	yields (%)[b]	99.8	99.2	97.9
	DCC/HOBt: 18/18 equiv	epimerization (%)[b]	0.2	0. 7	0.3
	1/64 h in NMP[16]	β-elimination (%)[b]	<0.2	0.7	1.9
5	AAs: 1.5 equiv	yields (%)[b]	92.5	99.3	95.3
	HBTU/HOBt: 1.5/1.5 equiv	epimerization (%)[b]	<0.2	0.5	0.6
	DIEA: 1.5 equiv	β-elimination (%)[b]	<0.2	0.3	2.2
	0/0.33 h in DMF[17]				
6	AAs: 2.5 equiv	yields (%)[b]	98.3	99.0	98.6
	BOP/HOBt: 2.5/2.5 equiv	epimerization (%)[b]	0.2	0.4	0.3
	DIEA: 2.5 equiv	β-elimination (%)[b]	<0.2	0.3	1.1
	0/4 h in DMF[18]				
7	AAs: 2.0 equiv	yields (%)[b]	99.1	99.0	98.2
	HATU/HOAt: 2.0/2.0 equiv	epimerization (%)[b]	<0.2	0.4	0.5
	TMP: 2.0 equiv	β-elimination (%)[b]	<0.2	0.3	0.3
	0/2 h in DMF				

[a] Glyco-amino acids were coupled to Pro-Gly-Hex resin. Preincubation time and coupling times are listed as x/y h (e.g., 3/12 h = 3 h preincubation followed by 12 h reaction time). [b] All data have an error of less than 0.3%. The yield refers to the percentage of D+L products relative to the total peptide (D+L products, β-elimination product, and truncated peptide). Abbreviations are as follows: 2-(7-Aza-1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyl aminium hexafluorophosphate (HATU), 2-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethylaminium hexafluorophosphate (HBTU), *N*,*N*′-dicyclohexylcarbodiimide (DCC), benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (BOP), *N*,*N*-diisopropylethylamine (DIEA), *N*-methylmorpholine (NMM), 2,4,6-trimethylpyridine (TMP), dimethylformamide (DMF), and *N*-methyl-2-pyrrolidone (NMP).

conditions, however, an unknown peptide was produced accounting for up to 60% of the mass. Further analysis revealed the side product to be Fmoc-dehydroaminobutyrate-Pro-Gly-Hex-OH, which results from β-elimination of the glycan moiety (Scheme 2, route b). Based on this result, we assessed β-elimination for all three amino acids and all seven conditions. β-Elimination primarily occurred for Fmoc-Thr(Ac_3GlcNAcβ)-OH.

The difference in behavior of glycosylated serine and threonine derivatives in peptide coupling reactions could be due to many factors. First, we anticipated that glycosylated threonine derivatives would react more slowly than serine derivatives; however, the relative rates had not been previously measured. If glycosylated threonine derivatives actually react faster, then there would be less time for epimerization. To test this possibility, we measured the relative coupling rates of other amino acids against threonine in a competition assay. Fmoc-Thr(Trt)-OH was mixed with the competing amino acid at an equivalent amount. The mixture was activated and then captured with the resin. The relative amounts of each peptide produced after 5 min were measured via an HPLC assay. As summarized in Table 2, the overall coupling rates of threonine-based amino acids were slower than serine derivatives. Therefore, a faster rate of peptide coupling does not account for the large difference in epimerization levels.

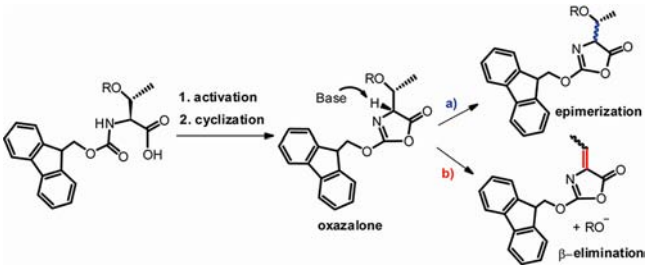
Table 2. Relative Overall Coupling Rate vs Threonine

amino acid[a]	relative reaction rate AA/Thr
Fmoc-Thr(Trt)-OH	1
Fmoc-Ser(Trt)-OH	3.33 ± 0.53
Fmoc-Thr(Ac_3GalNAcα)-OH	0.70 ± 0.03
Fmoc-D-Thr(Ac_3GalNAcα)-OH	0.90 ± 0.02
Fmoc-Thr(Ac_3GlcNAcβ)-OH	2.62 ± 0.06
Fmoc-D-Thr(Ac_3GlcNAcβ)-OH	1.01 ± 0.04

[a] All reactions were carried out by mixing Fmoc-Thr(Trt)-OH (2 equiv) and the listed amino acid (2 equiv) with HATU (4 equiv), HOAt (4 equiv), and TMP (4 equiv) in DMF. The assays were conducted in triplicate.

A second possible explanation is that for glycosylated threonine derivatives, the equilibrium between the natural and unnatural epimer lies heavily in favor of the natural epimer. If this was the case, epimerization could be occurring in the reaction, but it would not produce significant amounts of the unnatural epimer. To test this hypothesis, we preincubated the unnatural epimers of Fmoc-D-Thr(Trt)-OH and D-glyco-amino acids with HATU/NMM for 3 h and then coupled them to ProGlyHex resin (condition 2). The long preincubation step permits epimerization. If the natural epimer is energetically favored and the rate of epimerization is sufficient, extensive amounts of the natural epimer would be formed in these reactions. In actuality, very little epimerization was observed in these reactions. Fmoc-D-Thr(Trt)-OH gave ~4% epimerization, and Fmoc-D-Thr(Ac_3GalNAcα)-OH gave $< 0.2\%$ epimerization. Since epimerization was not observed for either the natural or unnatural epimer, we concluded that the rate of epimerization was too slow to reach equilibrium. Interestingly, Fmoc-D-Thr(Ac_3GlcNAcβ)-OH produced 92.4% of the β-elimination product under these conditions. Epimerization may be occurring for this substrate, but rapid β-elimination prevents measurement of the equilibrium.

Scheme 2. Potential Routes for Epimerization (a) and β-Elimination (b)



To better evaluate the equilibria, we next attempted to increase the rate of epimerization by varying the type of base and increasing the number of equivalents of base. As shown in Figure 1, we preincubated Fmoc-Thr(Ac_3GlcNAcβ)-OH (a) or Fmoc-Thr(Ac_3GalNAcα)-OH (b), and HATU for 3 h with different equivalents of NMM (a) or DIEA (b), respectively. Even under forcing conditions, little D-epimer was detected. Instead the β-eliminated peptide was observed as the major product for both glyco-amino acids. For example, incubation of Fmoc-Thr(Ac_3GlcNAcβ)-OH with 12 equiv of NMM produced ~90% β-eliminated product. Although we were unable to measure the equilibrium ratio of epimers, it is clear that glycosylated serine and threonine analogs produce different side products in these coupling reactions.

In our previous study on peptide couplings of glycosylated serine analogs, we found that the mild base, TMP, provides high yields with little or no epimerization. To further test the utility of this base, we evaluated the use of higher equivalents of TMP, analogous to the studies above with NMM and DIEA. Remarkably, epimerization and β-elimination were $< 5\%$ with up to 8 equiv of TMP for all three amino acids. Therefore, TMP produces at least a 10-fold lower level of side products as compared to NMM and DIEA. Based on these results and our previous results, we consider condition 7 (2 equiv of glyco-amino acid, 2 equiv of HATU and HOAt, and 2 equiv of TMP) to be the best conditions we have examined for solid phase peptide couplings involving glycosylated amino acids. Other mild

(12) Ragnarsson, U.; Karlsson, S.; Sandberg, B. *Acta Chem. Scand.* **1971**, *25*, 1487.
(13) Karch, F.; Hoffmann-Roder, A. *Beilstein J. Org. Chem.* **2010**, *6*, 47.
(14) Becker, T.; Kaiser, A.; Kunz, H. *Synthesis* **2009**, 1113.
(15) Hojo, H.; Matsumoto, Y.; Nakahara, Y.; Ito, E.; Suzuki, Y.; Suzuki, M.; Suzuki, A.; Nakahara, Y. *J. Am. Chem. Soc.* **2005**, *127*, 13720.
(16) Nakahara, Y.; Nakahara, Y.; Ogawa, T. *Carbohydr. Res.* **1996**, *292*, 71.
(17) Matsushita, T.; Hinou, H.; Kurogochi, M.; Shimizu, H.; Nishimura, S. *Org. Lett.* **2005**, *7*, 877.
(18) Chen, L.; Jensen, K. J.; Tejbrant, J.; Taylor, J. E.; Morgan, B. A.; Barany, G. *J. Pept. Res.* **2000**, *55*, 81.

peptide coupling conditions may also be useful for making glycopeptides.[19]

While the above experiments provided interesting insight into the reaction pathways, it was still not clear why glyco-threonine analogs have much lower levels of epimerization than glyco-serine analogs. Previous NMR studies have shown that glyco-serine and glyco-threonine can have significantly different preferred conformations in water,[20] but little information was available for protected derivatives in organic solvents. Therefore, we carried out molecular modeling to determine if differences in conformational preferences might contribute to differences in reactivity. To mimic the reaction solvent, a distance dependent dielectric constant of 38 was used. Since epimerization and β-elimination are thought to proceed through an oxazolone intermediate[21] (see Scheme 2), the corresponding oxazolone intermediates of Fmoc-Thr(Ac_3GalNAcα)-OH and Fmoc-Ser(Ac_3GalNAcα)-OH were modeled using methods reported previously.[11]

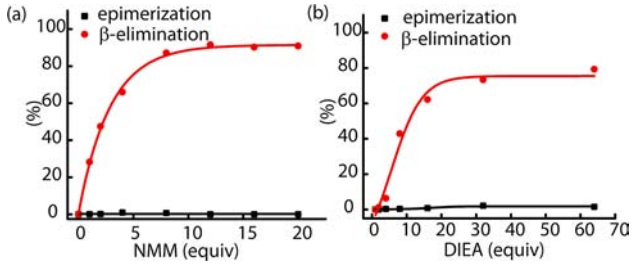


Figure 1. Epimerization and β-elimination as a function of base. (a) Fmoc-Thr(Ac_3GlcNAcβ)-OH; (b) Fmoc-Thr(Ac_3GalNAcα)-OH. Glyco-amino acids were coupled using the following coupling agents and ratios: AAs/HATU/base = 1/1/x. Each amino acid was preincubated for 3 h, and then the extent of epimerization and β-elimination were measured as described in the SI.

The preferred conformations of the glyco-serine and glyco-threonine oxazolones showed distinct differences (see Figure 2). In particular, the Hα−Cα−Cβ−Oβ dihedral angle for the threonine derivative was ~60° (Figure 2D), placing the β-methyl and the *O*-GalNAc groups *gauche* to the α-hydrogen. In contrast, the Hα−Cα−Cβ−Oβ dihedral angle for the serine derivative was ~180° (Figure 2, C), placing the *O*-GalNAc group *anti* to the α-hydrogen. The difference has two key effects. First, the α-hydrogen of glyco-threonine analogs is more sterically shielded than that of glyco-serine derivatives, which could significantly hinder abstraction of the α-hydrogen. Second, overlap of the C_β−O σ*-antibonding orbital with the C_α−H σ-bond in the glyco-serine derivative, but not the threonine derivative, could facilitate abstraction of this

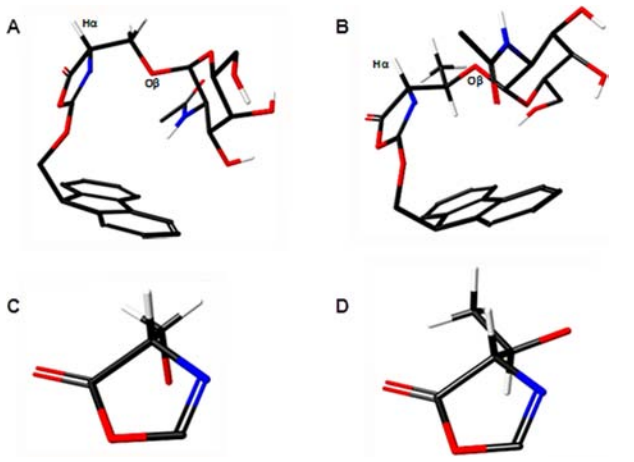


Figure 2. Low-energy models of the oxazolone intermediates derived from (A, C) Fmoc-Ser(GalNAcα)-OH and (B, D) Fmoc-Thr(GalNAcα)-OH generated from the Conformational Search utility in Macromodel 2011. Panels (C) and (D) focus on the oxazolone ring and the Hα−Cα−Cβ−Oβ dihedral angle.

hydrogen. Therefore, the combination of steric and stereoelectronic effects may contribute to the low epimerization of glyco-threonine analogs compared with the glyco-serine ones. When abstraction of the α-hydrogen is possible in a threonine derivative, formation of a more highly substituted alkene relative to serine derivatives likely contributes to the preference for β-elimination over epimerization.

In summary, we demonstrate that serine and threonine based glyco-amino acids display significantly different behaviors in solid phase peptide coupling reactions. Glyco-threonine derivatives produce little or no epimerization but, occasionally, give rise to β-elimination. In contrast, glyco-serine derivatives are highly prone to epimerization. The difference in reactivity is likely due to distinct differences in conformational preferences of the reactive intermediates. Fortunately, use of TMP as a base in the peptide coupling reactions significantly reduces both epimerization and β-elimination side reactions. Finally, our studies show that even small structural changes to glyco-amino acids, such as the presence or absence of a single methyl group, can have a dramatic effect on side reactions. Therefore, a more detailed understanding of the factors that contribute to epimerization and β-elimination will significantly improve our ability to synthesize glycopeptides with high efficiency and predictability.

Acknowledgment. This research was supported by the Intramural Research Program of the NIH, NCI. We gratefully acknowledge James A. Kelley (NIH/NCI) for obtaining mass spectrometry data for all the compounds.

Supporting Information Available. Experimental details for the syntheses of glyco-amino acids and supplemental HPLC data. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

(19) Mitchell, S. A.; Pratt, M. R.; Hruby, V. J.; Polt, R. *J. Org. Chem.* **2001**, *66*, 2327.
(20) Corzana, F.; Busto, J. H.; Jimenez-Oses, G.; Garcia de Luis, M.; Asensio, J. L.; Jimenez-Barbero, J.; Peregrina, J. M.; Avenoza, A. *J. Am. Chem. Soc.* **2007**, *129*, 9458.
(21) Albericio, F.; Carpino, L. A. *Methods Enzymol.* **1997**, *289*, 104.