Organic LETTERS

Letter

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The Rapid and Facile Synthesis of Oxyamine Linkers for the Preparation of Hydrolytically Stable Glycoconjugates

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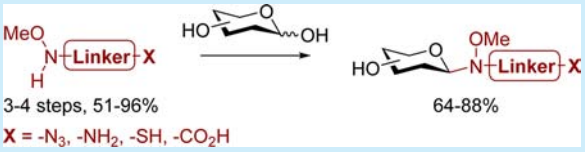
Supporting Information

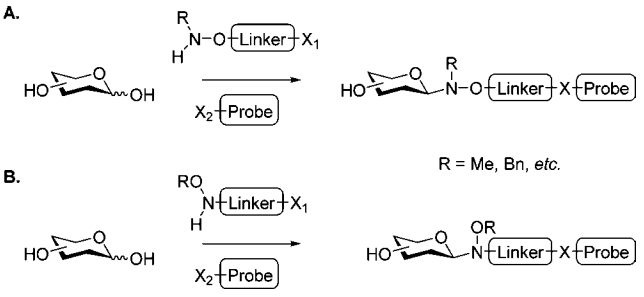
ABSTRACT: The synthesis of a number of *N*-glycosyl-*N*-alkyl-methoxyamine bifunctional linkers is described. The linkers contain an *N*-methoxyamine functional group for conjugation to carbohydrates and a terminal group, such as an amine, azide, thiol, or carboxylic acid, for conjugation to the probe of choice. The strategy for the linker synthesis is rapid (3−4 steps) and efficient (51−96% overall yield), and many of the linkers can be synthesized using a three-step one-pot strategy. Moreover, the linkers can be conjugated to glycans in excellent yield and they show excellent stability toward hydrolytic cleavage.



Glycoconjugates, in the form glycolipids and glycoproteins, are ubiquitous in Nature and serve a number of biological functions.[1] Accordingly, there has been much incentive for the development of efficient methodology for the construction of glycoconjugate mimetics,[2] glycopeptides,[3] and carbohydrate arrays[4] and the synthesis of fluorescent or biotinylated glycoconjugate probes.[5] To this end, strategies have been developed which allow for the conjugation of the carbohydrate of interest to a bifunctional linker that can then be further functionalized as is required. These linkers can be introduced during the synthesis of oligosaccharides. However, this requires additional synthetic steps and, moreover, does not allow for the conjugation of carbohydrates from natural sources. Accordingly, much effort has been devoted toward the development of linker strategies that can be performed on the reducing end of unprotected sugars. These approaches include the use of reductive amination,[6] Kochetkov amination,[7] and the use of oxyamines.[8] The latter strategy has particular merit, as it allows for the facile introduction of a variety of functionalized linkers without affecting the structural integrity of the reducing end sugar. Oxyamines are also readily introduced (Scheme 1) and are quite stable to hydrolysis.

N-Glycosyl oxyamines were first synthesized in the late 1970s to study *N*-aryl-*N*-hydroxy-glucuronylamine metabolites of anticancer drugs.[9] However, it was not until 20 years later that the use of oxyamines as bifunctional linkers was realized when Peri et al. prepared neoglycopeptides and neoglycolipids.[10] Other elegant applications of oxyamines include their use in the synthesis of glycan and glycolipid analogues,[11] in the neoglycorandomization of methoxyamine-appended drug targets,[8] and in the conjugation of carbohydrates to amino acids.[12] Seminal examples include work by the groups of Blixt,[13] Carraso,[14] Nitz,[15] and Jensen,[16] and more recently, Boons and co-workers demonstrated the power of oxime linkers by attaching complex *N*-glycans to microarrays[17,18] through the use of a 2-[(methylamino)oxy]ethylamine linker.[13] In all previously described studies, the oxyamine linkers were of "Type A" (Scheme 1). Moreover, while the linkers clearly showed much potential in the synthesis of glycoconjugates, their preparation could be improved, particularly in terms of yield (10−34%) and number of steps (4−6 steps). The strategies employed also require different approaches for different terminal functional groups. To this end, we explored methodology for the efficient, scalable, and preferably one-pot synthesis of a series of differently functionalized oxyamine linkers with good hydrolytic stability.

Scheme 1. Two General Strategies for the Conjugation of Carbohydrates to Oxyamine Linkers and Further Functionalization with the Probe of Interest

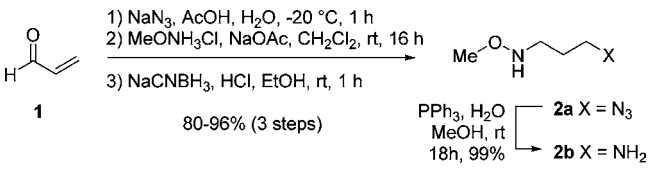


Key to our synthetic approach was the use of acrolein as a bifunctional reagent, and to explore our synthetic strategy, we first set out to prepare an azide-functionalized oxyamine linker (Scheme 2). Here, acrolein (**1**) was subjected to a Michael addition using NaN_3 to generate 3-azidopropanal, which was subsequently condensed with methoxyamine. The resultant

Received: December 17, 2014
Published: January 16, 2015

DOI: 10.1021/ol503634j
Org. Lett. 2015, 17, 624−627

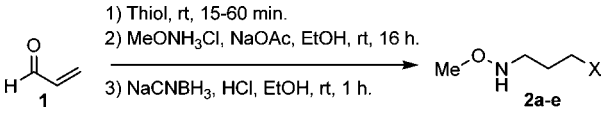
Scheme 2. Synthesis of Azide- and Amine-Functionalized Oxyamine Linkers

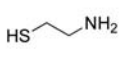
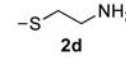
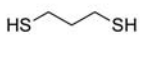
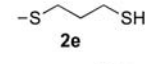
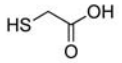
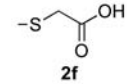
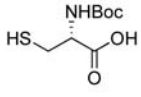
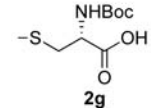


imine was then reduced using $NaCNBH_3$ to yield the target oxyamine linker **2a** in three steps and in 80% overall yield. Purification of the intermediate products, however, is not required, and methoxyamine **2a** can indeed be prepared in a higher (96%) yield if the intermediates are not isolated. Moreover, amine **2a** can either be distilled or purified by silica gel column chromatography. The azide in **2a** can then be reduced using a Staudinger reaction[19] to prepare amine functionalized linker **2b**. Formation of linker **2b** is high yielding (99% from **2a**), and the product can be readily purified by a reversed phase plug to remove the phosphine byproducts.

Having established the potential of our methodology, we then sought to extend the repertoire of oxyamine functionalized linkers that could be prepared. First, we explored the construction of a thiol-functionalized linker (entry 1, Table 1). To this end, thioacetic acid was reacted with acrolein (**1**) to

Table 1. Three-Step One-Pot Synthesis of Oxyamine Linkers



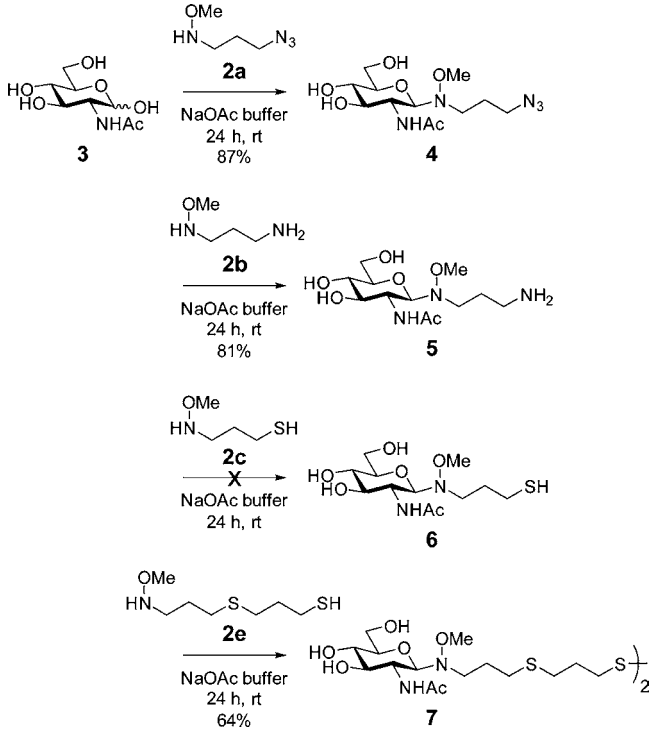
entry	thiol	product	yield
1		 **2c**	79% (4 steps)
2		 **2d**	66%
3		 **2e**	76%
4		 **2f**	79%
5		 **2g**	51%

[a]Intermediate imine was deacetylated using NaOMe/MeOH prior to reduction.

provide the 1,4-adduct, which was again condensed with methoxyamine to give the corresponding oxime. Initial attempts to reduce the oxime in a one-pot approach led to the formation of the amine; however, *S*-to-*N* acyl migration from the thiol could not be prevented and the acetamide product was isolated in high yield. Deprotection of the intermediate thioacetate followed by reduction, however, allowed for the successful synthesis of thiol-functionalized linker **2c** in 79% yield (four steps). With the goal of developing a one-pot procedure, we then turned to the use of functionalized thiols for the Michael addition (entries 2−5). Reaction of cysteamine with acrolein, followed by the addition of methoxyamine, led to *in situ* oxime formation. The oxime was subsequently reduced by the addition of $NaCNBH_3$, leading to the one-pot three-step synthesis of the amine-functionalized linker **2d** in 66% overall yield (entry 2). Extension of the methodology to the use of propane-1,3-dithiol (entry 3) then allowed for the rapid synthesis of thiol-functionalized linker **2e** in 76% yield. In order to produce a carboxy-modified linker, 2-mercaptoacetic acid was used (entry 4), and again, the required linker **2f** could be readily prepared via the three-step one-pot strategy and in 79% overall yield. Due to the high water solubility of linker **2f**, an aqueous workup procedure was not viable and instead the reaction was quenched by the addition of 1 M NaOH (aq.) before concentration and purification by silica gel flash column chromatography. To determine if the methodology was amenable to the use of more complex substrates, acrolein (**1**) was subjected to a Michael addition with *N*-Boc-cysteine, followed by oxime formation and reduction (entry 5). While the overall yield for the synthesis of cysteine linker **2g** was modest (51% yield over three steps), this route nonetheless provides a rapid entry to an alkoxyamine functionalized amino acid.

With a series of oxyamine linkers in hand, we then set out to determine the efficiency of glycan conjugation. Given the importance of GlcNAc as an N-linked reducing end terminal sugar,[20] this substrate was used for linker conjugation. To this end, GlcNAc (**3**) and the appropriate linker (10 equiv) were stirred in an aqueous AcOH/NH_4OAc buffer at pH 4.5 for 24 h at room temperature (Scheme 3). The solvent conditions were chosen as they allow for the solubilization of most glycans. The conjugation of azide linker **2a** and amine linker **2b** proceeded smoothly and in excellent yields to give *N*-glycans **4** and **5** in 87% and 81% yield, respectively. The conjugation of the sugar to the linker was confirmed by HMBCs between the CH_2-1 of the linker and the CH-1′ of the glycan. Moreover, exclusive

Scheme 3. Conjugation of Oxyamine Linkers to GlcNAc



DOI: 10.1021/ol503634j
Org. Lett. 2015, 17, 624−627

formation of the β-pyranose configuration was confirmed by NMR analysis, which revealed a $J_{1',2'}$ of 9.8 Hz and an HMBC between H-1′ and C-5′. The conjugation of **2c**, however, did not lead to the desired glycosylamine **6** and instead gave a diastereomeric mixture of thioaminals, which were formed via the nucleophilic attack of the thiol onto the oxime intermediate. Notwithstanding, the extension of the thiol-linker chain with the use of **2e** saw the successful formation of the disulfide glycosylamine **7** in 64% yield. Here, it should be noted that tris(2-carboxyethyl)phosphine (TCEP) or 1,3-propanedithiol can also be added to the reaction mixture to prevent the formation of disulfides. While it is difficult to directly compare the coupling yields of our linker (Type B) with those of type A, on the whole we observe excellent yields, which are comparable to, if not better than, those previously reported in literature. Herein, it is important to note that the best yields are obtained when the concentration of the reaction mixture is kept high (1 M), with at least 10 equiv of linker.

As oxime-linkers are hydrolyzed under acidic aqueous conditions, we investigated the rates of hydrolysis of glycoconjugate **4** at varying pH. Previous work in this field has shown that the substitution pattern of the nitrogen and oxygen in oxyamines affects their hydrolytic stability, with the electron-donating Bn group on either the oxygen or nitrogen of the oxime decreasing the rate of hydrolysis.[16] The disadvantage of the bulky benzyl group, however, is that conjugation to the glycan is not as efficient and lectin binding can be compromised.[16] Thus, we subjected glycoconjugate **4**, with the OMe group on the nitrogen, to aqueous conditions at pH 4.75, 5, 6, 7, and 9 to evaluate linker stability (Figure 1). As

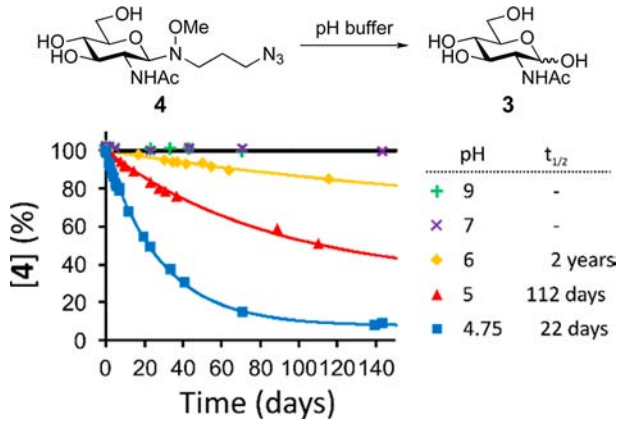
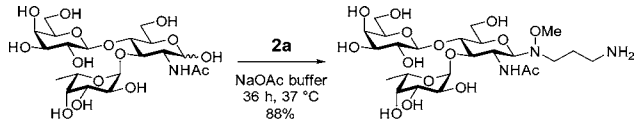


Figure 1. Rates of hydrolysis of oxyamine linked GlcNAc **4** at pH 4.75, 5, 6, 7, and 9. As determined by ^{1}H NMR analysis of 50 mM solutions of glycoside **4** in sodium phosphate buffered D_2O.

anticipated, the rates of hydrolysis were pH dependent, with the glycoconjugate **4** showing no appreciable cleavage at pH 7 and 9 over a 100 day period. At pH 6, only limited cleavage was observed with $t_{1/2}$ = 2 years, which indicates that the linker is well suited for use under physiological conditions. At pH 5 and 4.5, $t_{1/2}$ = 112 and 22 days, respectively, which was 2- to 5-fold better than the analogous benzylated oxyamine of Type A (Figure 1).[16] Accordingly, the *N*-linked methoxy amine linker (Type B) can be readily prepared and, in addition, shows excellent hydrolytic stability.

Finally, to illustrate the versatility of the linker strategy, conjugation to a more sterically hindered and complex carbohydrate of biological relevance was undertaken. Thus, linker **2a** and Lewis^x (**8**) were stirred in a 2 M aqueous $NaOAc/NH_4OAc$ buffer at 37 °C (Scheme 4). The reaction

Scheme 4. Conjugation of Lewis^x (8) to the Bifunctional Methoxyamine Linker 2a



proved sluggish, and only ca. 25% conversion was observed after 24 h. Increasing the temperature to 40 °C, however, led to a marked increase in reaction rate, with complete conversion to glycan **9** being observed after 36 h. Purification by direct loading of the reaction mixture onto a Bio-Gel P-2 column for size exclusion chromatography then allowed for the isolation of conjugate **9** in 88% yield and as only the β-pyranosyl glycoside, as evidenced by $^1J_{CH\text{-}1'}$ = 154 Hz, and HMBC between H-1′and C-5′.

In conclusion, we have successfully synthesized a variety of oxyamine linkers in high yield and in a few steps. Many of the linkers can be synthesized using a one-pot protocol and can be prepared on a gram scale. The linkers can be readily coupled to glycans in excellent yields, and hydrolytic stability studies revealed that the methoxyamine linker is stable over a large pH range, with exceptional stability at physiological pH. Accordingly, the coupling efficiencies and hydrolytic stabilities of our "Type B" linker appear to be comparable to, if not better than, those of "Type A", and moreover, our linkers can be prepared in high yields with the functional group of choice. The use of the linker for conjugation to more complex glycans has been demonstrated by the highly efficient coupling to Lewis^x. We will further explore the application of this methodology in due course.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the Wellington Medical Research Foundation for financial support (2014/253).

■ REFERENCES

(1) *Glycoconjugates: Composition: Structure, and Function*; Allen, H. J., Kisailus, E. C., Eds.; Marcel Dekker, Inc: New York, 1992.
(2) (a) Davis, B. G. *J. Chem. Soc., Perkin Trans. 1* **1999**, 3215−3237. (b) Peri, F.; Cipolla, L.; La Ferla, B.; Nicotra, F. *C. R. Chim.* **2003**, *6*, 635−644. (c) Boltje, T. J.; Buskas, T.; Boons, G.-J. *Nat. Chem.* **2009**, *1*, 611−622.
(3) Specker, D.; Wittmann, V. *Top. Curr. Chem.* **2007**, 65−107.

DOI: 10.1021/ol503634j
Org. Lett. 2015, 17, 624−627

(4) (a) Horlacher, T.; Seeberger, P. H. *Chem. Soc. Rev.* **2008**, *37*, 1414−1422. (b) Laurent, N.; Voglmeir, J.; Flitsch, S. L. *Chem. Commun.* **2008**, 4400−4412. (c) Oyelaran, O.; Gildersleeve, J. C. *Curr. Opin. Chem. Biol.* **2009**, *13*, 406−413.
(5) (a) Stocker, B. L.; Timmer, M. S. M. *ChemBioChem* **2013**, *14*, 1164−1184. (b) Timmer, M. S. M.; Stocker, B. L.; Seeberger, P. H. *Curr. Opin. Chem. Biol.* **2007**, *11*, 59−65.
(6) Song, X.; Xia, B.; Stowell, S. R.; Lasanajak, Y.; Smith, D. F.; Cummings, R. D. *Chem. Biol.* **2009**, *16*, 36−47.
(7) Likhosherstov, L. M.; Novikova, O. S.; Derevitskaja, V. A. *Carbohydr. Res.* **1986**, *146*, C1−C5.
(8) For a recent review, see: Goff, R. D.; Thorson, J. S. *MedChemComm* **2014**, *5*, 1036−1047.
(9) (a) Moreno, H. R.; Radomski, J. L. *Cancer Lett.* **1978**, *4*, 85−88. (b) Poupko, J. M.; Hearn, W. L.; Radomski, J. L. *Toxicol. Appl. Pharmacol.* **1979**, *50*, 479−484.
(10) Peri, F.; Dumy, P.; Mutter, M. *Tetrahedron* **1998**, *54*, 12269−12278.
(11) (a) Peri, F.; Deutman, A.; La Ferla, B.; La Nicotra, F. *Chem. Commun.* **2002**, 1504−1505. (b) Peri, F.; Jiménez-Barbero, J.; García-Aparicio, V.; Tvaroska, I.; Nicotra, F. *Chem.—Eur. J.* **2004**, *10*, 1433−1444. (c) Ishida, J.; Hinou, H.; Naruchi, K.; Nishimura, S.-I. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 1197−1200.
(12) (a) Carrasco, M. R.; Nguyen, M. J.; Burnell, D. R.; Maclaren, M. D.; Hengel, S. M. *Tetrahedron Lett.* **2002**, *43*, 5727−5729. (b) Filira, F.; Biondi, B.; Biondi, L.; Giannini, E.; Gobbo, M.; Negri, L.; Rocchi, R. *Org. Biomol. Chem.* **2003**, *1*, 3059−3063. (c) Carrasco, M. R.; Brown, R. T.; Serafimova, I. M.; Silva, O. *J. Org. Chem.* **2003**, *68*, 195−197. (d) Carrasco, M. R.; Brown, R. T. *J. Org. Chem.* **2003**, *68*, 8853−8858. (e) Jiménez-Castells, C.; de la Torre, B. G.; Gutiérrez Gallego, R.; Andreu, D. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 5155−5158. (f) Seo, J.; Michaelian, N.; Owens, S. C.; Dashner, S. T.; Wong, A. J.; Barron, A. E.; Carrasco, M. R. *Org. Lett.* **2009**, *11*, 5210−5213. (g) Zevgiti, S.; Zabala, J. G.; Darji, A.; Dietrich, U.; Panou-Pomonis, E.; Sakarellos-Daitsiotis, M. *J. Pept. Sci.* **2012**, *18*, 52−58.
(13) Bohorov, O.; Andersson-Sand, H.; Hoffmann, J.; Blixt, O. *Glycobiol.* **2006**, *16*, 21−27.
(14) Seo, J.; Michaelian, N.; Owens, S. C.; Dashner, S. T.; Wong, A. J.; Barron, A. E.; Carrasco, M. R. *Org. Lett.* **2009**, *11*, 5210−5213.
(15) Leung, C.; Chibba, A.; Gómez-Biagi, R. F.; Nitz, M. *Carbohydr. Res.* **2009**, *344*, 570−575.
(16) Clo, E.; Blixt, O.; Jensen, K. J. *Eur. J. Org. Chem.* **2010**, 540−554.
(17) Wang, Z.; Chinoy, Z. S.; Ambre, S. G.; Peng, W.; McBride, R.; de Vries, R. P.; Glushka, J.; Paulson, J. C.; Boons, G.-J. *Science* **2013**, *341*, 379−383.
(18) Prudden, A. R.; Chinoy, Z. S.; Wolferta, M. A.; Boons, G.-J. *Chem. Commun.* **2014**, *50*, 7132−7135.
(19) Gololobov, Yu. G.; Zhmurova, I. N.; Kasukhin, L. F. *Tetrahedron* **1981**, *37*, 437−472.
(20) Werz, D. B.; Ranzinger, R.; Herget, S.; Adibekian, A.; von der Lieth, C.-W.; Seeberger, P. H. *ACS Chem. Biol.* **2007**, *2*, 685−691.

DOI: 10.1021/ol503634j
Org. Lett. 2015, 17, 624−627