Erschienen in: Chemistry - A European Journal ; 22 (2016), 48. - S. 17359-17365
https://dx.doi.org/10.1002/chem.201603369

Real-Time NMR Studies of Oxyamine Ligations of Reducing Carbohydrates under Equilibrium Conditions

Oliver R. Baudendistel[+], Daniel E. Wieland[+], Magnus S. Schmidt, and Valentin Wittmann*[a]

Abstract: Ligation reactions at the anomeric center of carbohydrates have gained increasing importance in the field of glycobiology. Oxyamines are frequently used in labeling, immobilization, and bioconjugation of reducing carbohydrates. Herein, we present a systematic investigation of these ligation reactions under aqueous conditions. A series of four unprotected monosaccharides (glucose, *N*-acetylglucosamine, mannose, and 2-deoxyglucose) and one disaccharide (*N*,*N*′-diacetylchitobiose) was reacted with three primary and one secondary oxyamine. We monitored the concentrations of the starting materials and products by ^{1}H NMR spectroscopy and determined reaction times and equilibrium yields. Our experiments show that the outcome of the ligation reaction is not only dependent on the sugar and oxyamine used but also strongly on the reaction conditions. In the case of glucose, lowering the pH from 6 to 3 led to steadily increasing reaction rates, whereas the yields were decreasing at the same time. Variation of the temperature did not only influence the product ratio in equilibrium but can also have a strong impact on the equilibrium yield. In the case of reactions of a primary oxyamine, increased temperatures led to a higher proportion of acyclic products. Reaction of the secondary oxyamine with glucose unexpectedly led to lower yields at higher temperatures.

Introduction

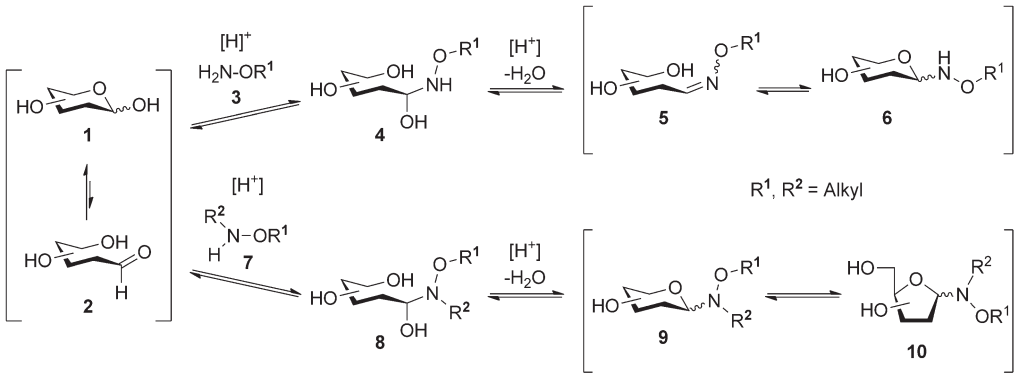
Carbohydrates belong to the structurally most diverse biomolecules and are heavily involved in post-translational protein modifications.[1] The molecular recognition of carbohydrates by proteins is the basis of fundamental biological processes such as fertilization, cell–cell communication, host–pathogen interactions, immune response, and cancer metastasis.[2] To study these recognition processes, methods to efficiently conjugate unprotected saccharides have gained increasing interest.[3] The aldehyde group present in the open-chain form of reducing carbohydrates can serve as a unique functionality enabling chemoselective ligation reactions. This is exploited in ligation reactions of carbohydrates with oxyamines and has been applied to introduce fluorescent tags,[4] to immobilize carbohydrates on surfaces for purification,[5] for the generation of microarrays,[6] and for surface plasmon resonance studies,[7] to functionalize carbohydrates in solution for subsequent immobilization,[8] to modify gold nanoparticles[9] and biomaterial scaffolds,[10] and to generate glycodendrimers,[11] neoglycolipids,[8a] neoglycopeptides and -proteins,[12] and therapeutic agents.[13]

[a] *O. R. Baudendistel,[+] D. E. Wieland,[+] Dr. M. S. Schmidt, Prof. Dr. V. Wittmann*
Department of Chemistry
Konstanz Research School Chemical Biology (KoRS CB)
University of Konstanz, 78457, Konstanz (Germany)
E mail: mail@valentin wittmann.de

[+] *These authors contributed equally to this work and are listed in alphabeti cal order.*

During ligations with primary oxyamines **3** the aldehyde of the open-chain form **2** of the carbohydrate is attacked to give hemiaminal **4** (Scheme 1). Subsequent water elimination leads to acyclic oximes **5** (with both an (*E*)- and (*Z*)-configuration) that are in equilibrium with cyclic *N*-glycosides **6** (with α- and/or β-configuration). All steps during oxyamine ligations are reversible. Carbohydrate-binding proteins often only recognize the cyclic conjugates **6**. Consequently, the state of equilibrium is highly important and has been investigated by various groups. Oxime formation was, for example, investigated by HPLC[4a,8b,14] or by NMR spectroscopy after a certain reaction time.[7b,8,14,15] These studies, which were carried out under different conditions, showed that the formation of acyclic oximes **5** is favored over cyclic **6**, which can be disadvantageous if carbohydrate–protein interactions are to be investigated and equilibration is not possible under the conditions of the binding assay.[8a]

Application of secondary oxyamines **7** circumvents this problem because they provide exclusively cyclic *N*-glycosides (Scheme 1). Therefore, secondary oxyamines have been applied in the preparation of neoglycosphingolipids,[16] therapeutic neoglycoconjugates,[17] in the formation of glycosyl acceptors in enzymatic synthesis,[18] for the immobilization on glass slides,[8b,19] or in the preparation of mucin analogues.[20] Investigation of reaction products of secondary alkylhydroxylamines **7** showed that cyclic pyranosides **9** and furanosides **10** can be formed depending on the carbohydrate used.[8b,16,17,19,21] In some cases, such as glucose and galactose, the *N*-glycosides are predominantly β-configured, whereas mannose gives both α- and β-pyranosides as well as α-furanosides. The above-mentioned studies were carried out either in aqueous buffers or in



Scheme 1. Reaction of reducing carbohydrates **1** with primary and secondary oxyamines (**3** and **7**, respectively) via an intermediate hemiaminal (**4** and **8**) to give, after dehydration, the respective oximes **5** and *N* glycosides (**6**, **9**, and **10**). The starting material and all reaction products are in equilibrium with each other.

organic solvents at different pH values and temperatures. Secondary oxyamines react slower[22] and result in lower yields compared to primary oxyamines.[21a,23] Consequently, nucleophilic catalysis of secondary (but also primary) oxyamines with aniline derivatives has been investigated.[22,24] Aniline catalysis improves the reaction rate but the equilibrium state is not affected.[24a] Besides oxyamines, hydrazides have also been applied in ligation reactions of reducing carbohydrates because they provide almost exclusively cyclic sugar conjugates.[6a,25] However, due to the lower stability of hydrazones compared to oximes,[26] the use of hydrazides will not be considered here.

The reactions of primary and secondary oxyamines have been investigated by various groups under very diverse reaction conditions, for example, in different solvents and at varying temperature and pH. Here, we report the first systematic investigation of kinetics and equilibrium states of oxyamine-based ligation reactions under aqueous conditions. Monitoring the progress of the reaction of primary and secondary oxyamines with a series of unprotected (reducing) sugars by NMR spectroscopy, we were able to follow the consumption of starting materials and the formation of individual reaction products and to determine reaction times and product compositions in equilibrium. We show that the outcome of the ligation reaction is not only dependent on the sugar and oxyamine used, but also strongly on the reaction conditions, specifically pH and temperature.

Results and Discussion

Experimental setup to monitor glycoconjugate formation

To determine kinetics and yields of the reaction between reducing carbohydrates and different oxyamines, we mixed all components in NMR tubes and followed the course of the reactions by ^{1}H NMR spectroscopy. For calibration of integrals we added [D_4]sodium 3-(trimethylsilyl)propanoate-2,2,3,3 (TSP) as an internal standard in a known concentration. A sugar concentration of 36 mM gave sufficient signal/noise ratio within a short measurement time. With this approach we were able to monitor all reactions in real time. Once the equilibrium was reached, all components in the reaction mixture were characterized by homo- and heteronuclear 2D NMR spectra (HSQC, COSY, HMBC, NOESY). To prevent a decrease of pH resulting from oxyamine consumption during the ligation, we carried out the reactions in a deuterated acetate buffer. Initial experiments showed that a buffer concentration of 100 mM was not sufficient to keep the pH constant, especially when working outside the optimal buffer range. For example, starting the reaction of glucose and *O*-ethylhydroxylamine (ethoxyamine) at pH 4 resulted in a reduced pH of 3 at the end of the reaction. A buffer concentration of 500 mM, on the other hand, provided a stable pH and thus was used throughout all reactions.

We first investigated the reaction of glucose (Glc) with 1.2 equivalents of ethoxyamine **11** at pH 4.5 (Figure 1 A). ^{1}H NMR spectra were recorded at defined time points that are exemplarily shown in Figure 1 B. Integration of the recorded spectra allowed us to monitor the decrease of glucose and formation of products over time. Based on the integrals, the corresponding yields were calculated and plotted against the reaction time *t* as shown in Figure 1 C. Additionally, exponential curve fitting of the data is shown. The appearance of (*E*)-oxime **12**, (*Z*)-oxime **13**, and β-*N*-pyranoside **14** is shown over time. Formation of the α-*N*-pyranoside was not observed. Furthermore, the calculated combined yield of **12**–**14** is depicted. One can clearly see that after 60 h no noticeable change in yields is observed. The obtained curves allowed quantification of the reaction times in the following manner. Since $t_{0.5}$ half-lives are not constants for bimolecular reactions but concentration-dependent, we decided to calculate $t_{0.9}$ values as a descriptive value of the reaction time under the used conditions. We define the $t_{0.9}$ value as the time after which the combined yield reaches 90% of its maximum obtained in equilibrium. In Figure 1 C we show both the $t_{0.9}$ and $t_{0.5}$ times. In the depicted example, $t_{0.5}$ is 5.47 h, $t_{0.9}$ is 19.3 h, and the combined equilibrium yield is 78%.

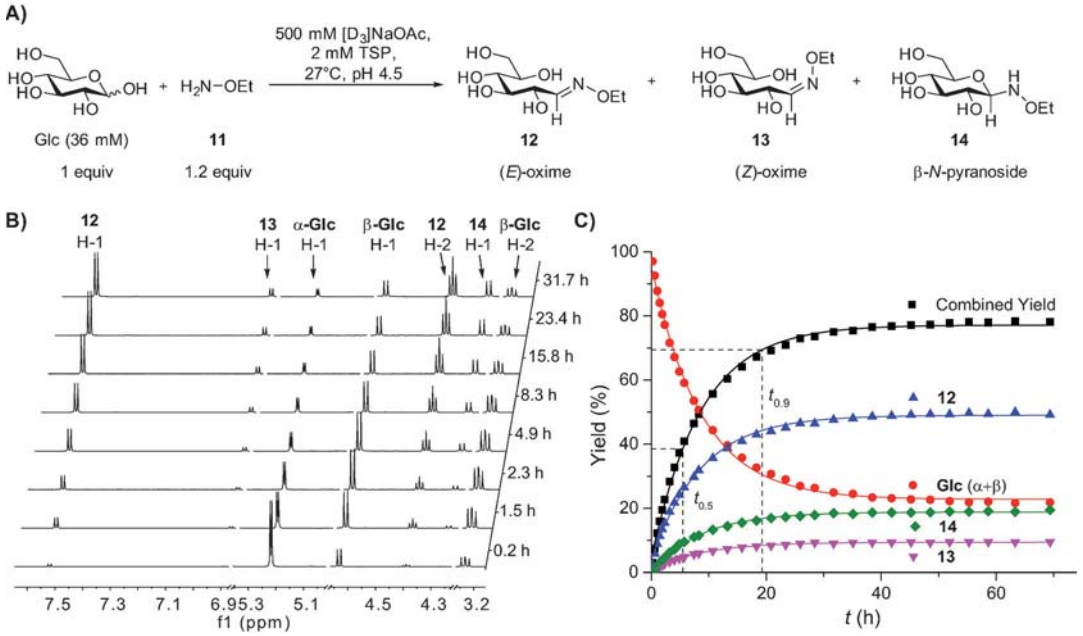


Figure 1. A) Reaction of glucose with ethoxyamine **11**. B) Stack of ^{1}H spectra recorded for this reaction at given time points. C) Course of the reactions ob tained from the integrals of spectra shown in (B). The combined yield in equilibrium is 78% and at $t_{0.9}$ (19.3 h) the yield is almost 70%.

Case study glucose: Influence of pH on reaction time and yield

First, we studied the influence of the pH on the ligation reaction. The reaction of glucose with 1.2 equivalents ethoxyamine **11** at 27 °C was investigated as described above at pH 3–6 (Table 1 and Figure 2 A). Whereas the ratio of acyclic to cyclic products did not change substantially over the whole pH range from 3 to 6, we observed a clear dependency of $t_{0.9}$ on pH with faster reactions at lower pH (Table 1, entries 1–5). For example, the reaction time decreased by a factor of 20 from pH 6 ($t_{0.9}$ = 88 h) to 3 ($t_{0.9}$ = 4.48 h). In contrast, the combined yield increased drastically with pH from 14% at pH 3 to 90% at pH 6. The two opposite effects of pH variation on reaction rate and yield are depicted in Figure 2 B. With respect to yield and acceptable reaction time, pH 5 seems to be the best compromise. Although at a pH of 6 slightly higher yields are obtained, the reaction times become unacceptably long. Therefore, all future experiments were carried out at pH 5.

Jencks reported reaction rates for the reaction of acetone with hydroxylamine at different pH values and found a rate maximum at pH 4.5.[27] This rate maximum was rationalized by the assumption that at higher pH water elimination becomes rate-determining, whereas at lower pH the addition of the oxyamine to the carbonyl group is rate-determining due to protonation of the oxyamine. Interestingly, in the case of glucose, in which a more reactive aldehyde group is attacked, the reaction rate increased steadily until pH 3. Since the combined yield dropped drastically under these acidic conditions, it was not possible to further lower the pH in order to observe a drop of reaction rate after having reached the maximum as expected from Jencks' results. In our case it is not the low reaction rate but the low yield that disfavors such acidic conditions.

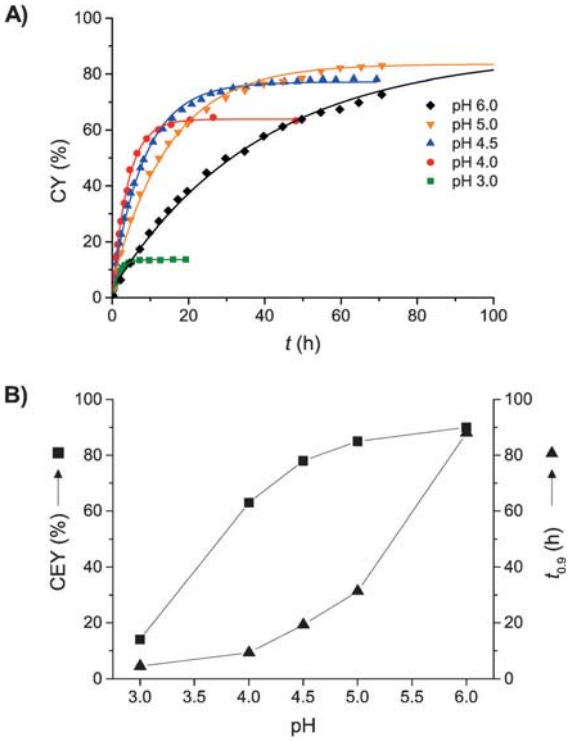
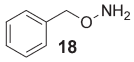
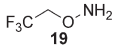


Figure 2. A) Combined yield (CY) of glycoconjugates **12** **14** over reaction time *t* for the reactions of glucose with 1.2 equivalents of ethoxyamine **11** at different pH values. B) Influence of pH on combined equilibrium yield (CEY) (■) and $t_{0.9}$ (▲) (values obtained from Table 1, entries 1 5).

Variation of the sugar and oxyamine

Next, we used different sugars in the ligation reaction. Replacement of Glc with *N*-acetylglucosamine (GlcNAc) resulted in

Table 1. Results of ligation reactions with primary oxyamines.

Entry	Sugar	Oxyamine	Oxyamine [equiv]	pH	*T* [°C]	Ratio[a] *E*/*Z*/α Pyr/β Pyr[b]	Ratio acycl./cycl.[c]	$t_{0.5}$ [h][d]	$t_{0.9}$ [h][d]	Combined equilibrium yield [%]
1	Glc	 **11**	1.2	3	27	2.8:0.6:0:1	3.4:1	1.36 ± 0.03	4.48 ± 0.08	14
2	Glc	**11**	1.2	4	27	2.7:0.5:0:1	3.2:1	2.78 ± 0.10	9.30 ± 0.27	63
3	Glc	**11**	1.2	4.5	27	2.5:0.5:0:1	3.0:1	5.47 ± 0.16	19.3 ± 0.47	78
4	Glc	**11**	1.2	5	27	2.5:0.5:0:1	3.0:1	8.98 ± 0.41	31.3 ± 1.17	85
5	Glc	**11**	1.2	6	27	2.5:0.5:0:1	3.0:1	25.5 ± 0.95	88.0 ± 2.65	90
6	Glc	**11**	2	5	27	2.5:0.5:0:1	3.0:1	6.14 ± 0.18	21.8 ± 0.54	95
7a	Glc	**11**	2	5	39	3.7:0.8:0:1	4.5:1	1.56 ± 0.03	5.34 ± 0.07	94
7b	Glc	**11**	2	5	60	5.5:1.3:0:1	6.8:1			92
8	GlcNAc	**11**	1.2	5	27	2.3:0.8:0:1	3.1:1	38.5 ± 1.21	132 ± 3.27	81
9	GlcNAc	**11**	2	5	27	2.3:0.8:0:1	3.1:1	25.5 ± 0.03	88.2 ± 1.43	92
10a	GlcNAc	**11**	2	5	39	3.6:1.2:0:1	4.8:1	6.01 ± 0.04	20.6 ± 0.12	90
10b	GlcNAc	**11**	2	5	21	2.2:0.7:0:1	2.9:1			90
11	2dGlc	**11**	1.2	5	27	1.5:1:0:0		0.21 ± 0.01	0.81 ± 0.02	98
12	Man	**11**	1.2	5	27	15.5:2.3:1:0	17.8:1	1.03 ± 0.12	5.76 ± 0.40	89
13	Man	**11**	2	5	27	15.9:2.3:1:0	18.2:1	0.62 ± 0.02	2.41 ± 0.04	94
14	Man	**11**	2	5	39	16.6:2.7:1:0	19.3:1	0.22 ± 0.01	0.84 ± 0.01	94
15	$(GlcNAc)_2$	**11**	2	5	27	2.7:1:0:1	3.7:1	12.9 ± 0.30	44.4 ± 0.87	90
16	Glc	 **18**	1.2	5	27	2.4:0.6:0:1	3.0:1	8.44 ± 0.36	32.9 ± 1.07	89
17	GlcNAc	**18**	1.2	5	27	1.8:0.8:0:1	2.6:1	34.7 ± 1.21	124 ± 3.35	86
18	2dGlc	**18**	1.2	5	27	39:30:0:1	69:1	0.18 ± 0.01	0.72 ± 0.02	99
19	Man	**18**	1	5	27	15.1:2.7:1:0	17.8:1	0.73 ± 0.03	3.03 ± 0.07	88
20	Glc	 **19**	1.2	5	27	1.8:0.4:0:1	2.2:1	87.1 ± 1.74	294 ± 4.64	76

[a] Ratio observed in equilibrium. Ratio slightly changes during the reaction since *N* glycosides are obtained from the oximes. [b] (*E*) oxime/(*Z*) oxime/α *N* glycopyranoside/β *N* glycopyranoside. [c] Ratio of acyclic (acycl.) to cyclic (cycl.) glycoconjugates. [d] Errors were determined according to the guide to the expression of uncertainty in measurement.[28]

a significantly slower reaction, but no substantial changes in product ratio and yield were observed (Table 1, entries 4 and 8). A possible explanation could be the altered electronegativity of the C2 substituent (NHAc vs. OH). Therefore, we also investigated 2-deoxyglucose (2dGlc), which lacks an electron-withdrawing group (EWG) at C2. This 2-deoxy sugar resulted in an extremely fast reaction ($t_{0.9}$ = 0.81 h, entry 11). With respect to product ratio, the situation is completely different and no cyclic product was observed at all. However, it is not only the presence or absence of an EWG at C2 that influences the outcome of a reaction but also the stereochemistry at C2. Mannose (Man), for example, reacted significantly faster than Glc but not as fast as 2dGlc, and a high acyclic to cyclic product ratio of 18:1 was observed (entry 12). As an example for an oligosaccharide we also investigated *N*,*N*′-diacetylchitobiose ($(GlcNAc)_2$) (entry 15). This disaccharide is the terminal structure present in all N-linked glycans and behaves very similar in the ligation with **11** as the monosaccharide GlcNAc with respect to product ratio and equilibrium yield; however, interestingly, the ligation is much faster.

We also checked the influence of the nature of the oxyamine. *O*-Benzylhydroxylamine **18** generally resulted in similar yields and reaction rates compared to ethoxyamine **11** (Table 1, entries 16–19). Also the acyclic to cyclic ratio is similar with slightly higher amounts of the cyclic product. Even more cyclic product (β-*N*-glycoside) is obtained using 2,2,2-trifluoroethoxyamine **19** (entry 20). However, the reaction is much slower with a 10-fold increased $t_{0.9}$ value compared to Glc (entry 4) and also gives a lower yield. In summary, we observed major differences regarding $t_{0.9}$, yield, and also product ratio (acycl/cycl) especially with variation of the sugar.

Variation of stoichiometry and reaction temperature

When carbohydrate ligations are carried out, high yields, short reaction times, and easy product isolation are desired. So far we used only a slight excess of oxyamine (1.2 equiv) facilitating product purification. As expected, increasing the amount of added oxyamine from 1.2 to 2 equivalents led to nearly quantitative yields in less time while the product ratios were not affected (Table 1, entries 6, 9, and 13). We also carried out the reaction at an increased temperature, which led to the expected acceleration of the reaction. Interestingly, we also observed a significant change in the ratio of acyclic to cyclic products. Carrying out the reaction of glucose and ethoxyamine at 39 instead of 27 °C, we noticed not only a four times faster reaction but also an increase of the acyclic/cyclic ratio from 3:1 to 4.5:1 (entries 6 and 7a). To check if this trend continued, we heated the same sample from 39 to 60 °C and observed that the ratio rose to 6.8:1 (entry 7b). After cooling the sample down to

27 °C, the original product ratio was obtained again. Similar trends were also observed for GlcNAc and Man. In the case of GlcNAc, the ratio increased from 3:1 at 27 °C to 4.8:1 at 39 °C (entries 9, 10a). When the same sample was then cooled down to 21 °C, the ratio dropped to 2.9:1 (entry 10b). A likely explanation for this observation is that the acyclic products are entropically favored because they possess more degrees of conformational freedom than the cyclic forms. Since the entropy term of the Gibbs–Helmholtz equation ($\Delta G = \Delta H \; T\Delta S$) is temperature-dependent, it carries more weight at increased temperature resulting in more acyclic products under these conditions. Consequently, one has to keep in mind that a higher temperature leads not only to a faster reaction but also to a bigger proportion of the entropically favored acyclic products.

Ligation with *O*-ethyl-*N*-methylhydroxylamine

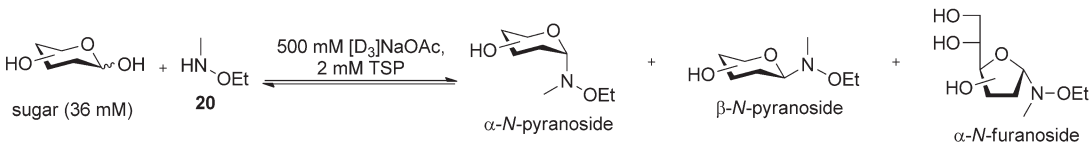
The experiments with primary oxyamines discussed so far revealed that in all cases the acyclic form predominates. Since protein–sugar binding usually relies on the cyclic structure of the sugar moiety, *N*-alkyl-oxyamines are often utilized for sugar ligation reactions because the products can only exist in the cyclic form (Scheme 1). For a better understanding of this reaction we employed the secondary *O*-ethyl-*N*-methylhydroxylamine **20** and reacted it with several sugars (Table 2).

First, we treated glucose with 1.2 equivalents of **20** at pH 3–6 at 21 °C (Table 2, entries 1–5). In all cases, only the β-*N*-pyranoside was formed. We observed the same trend with respect to reaction speed and yield as in the reactions of ethoxyamine **11** with glucose. Yields ranged from 45% at pH 6 to hardly any product formation at pH 3. At the same time, $t_{0.9}$ dropped by a factor of 20 from 673 (pH 6) to 33 h (pH 3). Compared to primary oxyamine **11**, the reaction of secondary oxyamine **20** is significantly slower and gives considerably lower equilibrium yields. As in the experiments with **11**, all further experiments were carried out at pH 5.

Since the reaction times of Glc with **20** were unacceptably high, we elevated the temperature to 39 °C in order to accelerate the reaction. As expected the reaction proceeded faster, and $t_{0.9}$ decreased from 151 to 19.3 h (Table 2, entries 4 and 6). Surprisingly, we observed a remarkably decreased yield from 37% at 21 °C to 29% at 39 °C at the same time. When we increased the amount of **20** to two equivalents, the reaction time decreased ($t_{0.9} = 17.3$ h) and the yield improved to 43% (entry 7a). To verify the influence of the temperature, we heated the same sample to 60 °C, which was accompanied by a decline in equilibrium yield from 43 to 30% (entry 7b). When the same sample was cooled down to 21 °C, the equilibrium yield rose again to 51% (entry 7c). However, equilibration took 20 days at this temperature. These experiments demonstrate that an elevated temperature is only advantageous with respect to the reaction time, whereas reaction yield is significantly reduced. This lowered yield can of course be compensated by using a larger excess of secondary oxyamine.

In experiments with GlcNAc instead of glucose and two equivalents of **20** at 39 °C, the reaction took much longer and the yield was lower (Table 2, entries 7a and 8). Similar to the trend observed with ethoxyamine **11**, the reaction of **20** with 2-deoxyglucose gave the highest reaction rate and the best yield (entry 9). The reactivity of mannose (entry 10) is between that of glucose and 2-deoxyglucose, but in this case we observed three different products. Major products were the α- and β-*N*-pyranosides that were distinguished by their $^1J_{C1-H1}$ coupling constants with the α-anomer displaying a larger coupling constant than the β-anomer.[29] As minor product the *N*-

Table 2. Results of ligation reactions with *O* ethyl *N* methylhydroxylamine **20**.



Entry	Sugar	**20** [equiv]	pH	*T* [°C]	Ratio α /β Pyr/α Fur[a]	$t_{0.5}$ [h][b]	$t_{0.9}$ [h][b]	Equilibrium yield [%]
1	Glc	1.2	3	21	0:1:0			<1
2	Glc	1.2	4	21	0:1:0	9.61 ± 0.41	33.3 ± 1.16	13
3	Glc	1.2	4.5	21	0:1:0	21.6 ± 0.37	72.6 ± 1.00	24
4	Glc	1.2	5	21	0:1:0	43.7 ± 1.63	151 ± 4.36	37
5	Glc	1.2	6	21	0:1:0	207 ± 17.5	673 ± 48.5	45
6	Glc	1.2	5	39	0:1:0	5.52 ± 0.07	19.3 ± 0.20	29
7a	Glc	2	5	39	0:1:0	4.74 ± 0.07	17.3 ± 0.18	43
7b	Glc	2	5	60	0:1:0			30
7c	Glc	2	5	21	0:1:0			51
8	GlcNAc	2	5	39	0:1:0	18.3 ± 0.19	60.4 ± 0.54	36
9	2dGlc	1.2	5	39	0:1:0	0.12 ± 0.01	0.41 ± 0.03	39
10	Man	2	5	39	3.5:1.5:1	1.25 ± 0.04	4.24 ± 0.12	20[c]
11	$(GlcNAc)_2$	2	5	39	0:1:0	21.6 ± 0.27	70.2 ± 0.75	39

[a] α *N* glycopyranoside/β *N* glycopyranoside/α *N* glycofuranoside. [b] Errors determined according to the guide to the expression of uncertainty in measurement.[28] [c] Combined equilibrium yield.

furanoside with assumed α-configuration[17] was obtained. The combined yield for this sugar was with 20% the lowest. Again, the disaccharide *N*,*N*′-diacetylchitobiose (entry 11) gave similar results as GlcNAc. In this case, however, the reaction was slightly slower.

Although the secondary oxyamine **20** resulted in lower yields, these numbers are still higher than the absolute yield for the cyclic products obtained using ethoxyamine **11**. For example, secondary oxyamine **20** gave 43% of β-*N*-pyranoside when reacted with glucose at 39 °C (Table 2, entry 7a). Primary oxyamine **11** on the other hand gave only 17% of β-*N*-pyranoside under the same conditions (Table 1, entry 7a). In the case of mannose, the difference is even more pronounced. Whereas the reaction of **20** gave 12% α- and 5% β-*N*-pyranoside (Table 2, entry 10), the proportion of α-*N*-pyranoside for the reaction of **11** with mannose under the same conditions is only 5% (Table 1, entry 14).

Conclusion

In summary, we have presented a systematic investigation of the ligation reactions of unprotected (reducing) carbohydrates and several oxyamines under equilibrium conditions. Using NMR spectroscopy, we determined both equilibrium yields and $t_{0.9}$ times as a practical measure of reaction rates. We found that stepwise lowering the pH from 6 to 3 led to steadily increasing reaction rates, whereas the yields were decreasing at the same time. This trend was observed for both primary and secondary oxyamines. Carrying out the reaction at pH 5 turned out to be the best compromise between reaction time and yield. Accordingly, all subsequent reactions were carried out at pH 5. Thought of as a means to optimize the reaction performance, we found that an increased temperature is not always advantageous. In the case of primary oxyamines, the acyclic/cyclic product ratio increased at higher temperatures, which was observed for several carbohydrates. For the reaction of glucose with ethoxyamine **11** the ratio shifted from 3.0:1 at 27 °C to 6.8:1 at 60 °C. In the reaction of the secondary oxyamine **20** with glucose, even more strikingly, higher temperatures led to a pronounced decrease of the equilibrium yield from 51% at 21 °C to 30% at 60 °C.

Variation of the carbohydrate resulted in significantly altered reaction rates and product ratios at equilibrium, whereas variation of the oxyamine had only a limited influence. In general, the acyclic ligation products are predominantly formed. In case of Glc and GlcNAc, we observed an acyclic/cyclic ratio of 3:1, which is in line with earlier reports.[7b,8,9] Similar results were obtained with *N*,*N*′-diacetylchitobiose. Interestingly, the application of Man led to a significantly increased ratio of 18:1 and in the case of 2-deoxyglucose, no cyclic product was formed at all. A higher proportion of cyclic ligation product could be obtained employing 2,2,2-trifluoroethoxyamine **19**; however, this was at the expense of the reaction rate. The only way to confine the product portfolio to cyclic compounds is the application of secondary oxyamines. Using *O*-ethyl-*N*-methylhydroxylamine **20**, we could show that under the same reaction conditions $t_{0.9}$ times were drastically larger and yields of glycoconjugates significantly lower compared to primary oxyamine **11**. Nevertheless, these yields of exclusively cyclic products are still higher compared to the proportion of cyclic products obtained with ethoxyamine **11**. Our investigation provides a deeper insight into the equilibria occurring during oxyamine-based ligation reactions of reducing carbohydrates. Since this reaction is gaining increasing interest for the study of the biological roles of carbohydrates, we expect our results to be of value for planning future experiments in this field.

Experimental Section

Deuterated acetate buffer (500 mM) containing 2 mM TSP

To prepare 10 mL buffer, the required ingredients (TSP and CD_3COOD) were dissolved in 8 mL D_2O. Subsequently, the pH was adjusted with NaOD in D_2O to the desired value and the solution diluted to a final volume of 10 mL with D_2O. To determine the pH, a pH-meter that had been calibrated in non-deuterated buffers, was employed. The value pH* displayed by the instrument, was converted to the real pH according to the formula $pH = 0.9291 \times pH^* + 0.421$.[30]

Oxyamine stock solution

Stock solutions (240 mM) of the primary or secondary oxyamines, sometimes bought as free amine or the HCl salt, were prepared in deuterated acetate buffer (500 mM, 2 mM TSP). After dissolution, the pH was readjusted to the desired value with DCl or NaOD in D_2O.

NMR monitoring of the reaction of oxyamines with reducing carbohydrates

The reducing carbohydrate (18 μmol) was dissolved in oxyamine stock solution (90 μL corresponding to 1.2 equiv of oxyamine or 150 μL corresponding to 2 equiv) and deuterated acetate buffer was added to give a total volume of 500 μL. The reaction mixture was transferred into a 5 mm NMR tube and the reaction was followed by ^{1}H NMR spectroscopy. Spectra were recorded with 4 scans and a pulse angle of 30°. A relaxation delay of 20 s was used, corresponding to more than 5 times T1 (cf. Supporting Information). After the equilibrium had been reached, signals of the reaction mixture were assigned by 2D NMR spectroscopy (COSY, HSQC, HMBC, NOESY).

For integration of ^{1}H spectra, only well-separated resonances were used. The integrals were converted to concentrations using the known concentration of added standard (TSP). Combined yields of products were plotted against time and an exponential regression ($y = y_0 + Ae(R_0x)$) using Origin 9.0 was carried out to determine $t_{0.9}$ and $t_{0.5}$ values. Errors were calculated according to the guide to the expression of uncertainty in the measurement (GUM).[28]

Acknowledgements

This work was supported by contract research 'Glykobiologie/Glykomik' of the Baden-Württemberg Stiftung, by the University of Konstanz, and by the Konstanz Research School Chemical Biology (KoRS-CB).

[1] *Essentials of Glycobiology*, 2nd ed. (Eds.: A. Varki, R. D. Cummings, J. D. Esko, H. H. Freeze, P. Stanley, C. R. Bertozzi, G. W. Hart, M. E. Etzler), Cold Spring Harbor Laboratory Press, Plainview, NY, **2009**.

[2] M. Dalziel, M. Crispin, C. N. Scanlan, N. Zitzmann, R. A. Dwek, *Science* **2014**, *343*, 1235681.

[3] K. Larsen, M. B. Thygesen, F. Guillaumie, W. G. T. Willats, K. J. Jensen, *Carbohydr. Res.* **2006**, *341*, 1209 1234.

[4] a) S. L. Ramsay, C. Freeman, P. B. Grace, J. W. Redmond, J. K. MacLeod, *Carbohydr. Res.* **2001**, *333*, 59 71; b) A. Lohse, R. Martins, M. R. Jørgensen, O. Hindsgaul, *Angew. Chem. Int. Ed.* **2006**, *45*, 4167 4172; *Angew. Chem.* **2006**, *118*, 4273 4278.

[5] S. I. Nishimura, K. Niikura, M. Kurogochi, T. Matsushita, M. Fumoto, H. Hinou, R. Kamitani, H. Nakagawa, K. Deguchi, N. Miura, K. Monde, H. Kondo, *Angew. Chem. Int. Ed.* **2005**, *44*, 91 96; *Angew. Chem.* **2005**, *117*, 93 98.

[6] a) M. r. Lee, I. Shin, *Org. Lett.* **2005**, *7*, 4269 4272; b) X. Zhou, J. Zhou, *Biosens. Bioelectron.* **2006**, *21*, 1451 1458.

[7] a) M. Vila Perelló, R. Gutiérrez Gallego, D. Andreu, *ChemBioChem* **2005**, *6*, 1831 1838; b) C. Jiménez Castells, B. de La Torre, D. Andreu, R. Gutiérrez Gallego, *Glycoconjugate J.* **2008**, *25*, 879 887.

[8] a) Y. Liu, T. Feizi, M. A. Campanero Rhodes, R. A. Childs, Y. Zhang, B. Mulloy, P. G. Evans, H. M. I. Osborn, D. Otto, P. R. Crocker, W. Chai, *Chem. Biol.* **2007**, *14*, 847 859; b) E. Cló, O. Blixt, K. J. Jensen, *Eur. J. Org. Chem.* **2010**, *3*, 540 554.

[9] M. B. Thygesen, K. K. Sorensen, E. Clo, K. J. Jensen, *Chem. Commun.* **2009**, 6367 6369.

[10] D. Bini, L. Russo, C. Battocchio, A. Natalello, G. Polzonetti, S. M. Doglia, F. Nicotra, L. Cipolla, *Org. Lett.* **2014**, *16*, 1298 1301.

[11] a) J. P. Mitchell, K. D. Roberts, J. Langley, F. Koentgen, J. N. Lambert, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2785 2788; b) D. Bini, R. Marchetti, L. Russo, A. Molinaro, A. Silipo, L. Cipolla, *Carbohydr. Res.* **2016**, *429*, 23 28.

[12] a) S. E. Cervigni, P. Dumy, M. Mutter, *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 1230 1232; *Angew. Chem.* **1996**, *108*, 1325 1328; b) N. Matsubara, K. Oiwa, T. Hohsaka, R. Sadamoto, K. Niikura, N. Fukuhara, A. Takimoto, H. Kondo, S. I. Nishimura, *Chem. Eur. J.* **2005**, *11*, 6974 6981.

[13] a) J. M. Langenhan, N. R. Peters, I. A. Guzei, F. M. Hoffmann, J. S. Thorson, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 12305 12310; b) J. M. Langenhan, J. M. Engle, L. K. Slevin, L. R. Fay, R. W. Lucker, K. R. Smith, M. M. Endo, *Bioorg. Med. Chem. Lett.* **2008**, *18*, 670 673.

[14] Y. Hatanaka, U. Kempin, P. Jong Jip, *J. Org. Chem.* **2000**, *65*, 5639 5643.

[15] a) Y. Ruff, J. M. Lehn, *Biopolymers* **2008**, *89*, 486 496; b) A. Richard, A. Barras, A. B. Younes, N. Monfilliette Dupont, P. Melnyk, *Bioconjugate Chem.* **2008**, *19*, 1491 1495; c) M. B. Thygesen, J. Sauer, K. J. Jensen, *Chem. Eur. J.* **2009**, *15*, 1649 1660; d) B. E. Benediktsdóttir, K. K. Sørensen, M. B. Thygesen, K. J. Jensen, T. Gudjónsson, Ó. Baldursson, M. Másson, *Carbohydr. Polym.* **2012**, *90*, 1273 1280.

[16] J. Ishida, H. Hinou, K. Naruchi, S. I. Nishimura, *Bioorg. Med. Chem. Lett.* **2014**, *24*, 1197 1200.

[17] J. M. Langenhan, M. M. Endo, J. M. Engle, L. L. Fukumoto, D. R. Rogalsky, L. K. Slevin, L. R. Fay, R. W. Lucker, J. R. Rohlfing, K. R. Smith, A. E. Tjaden, H. M. Werner, *Carbohydr. Res.* **2011**, *346*, 2663 2676.

[18] D. Teze, M. Dion, F. Daligault, V. Tran, C. André Miral, C. Tellier, *Bioorg. Med. Chem. Lett.* **2013**, *23*, 448 451.

[19] O. Bohorov, H. Andersson Sand, J. Hoffmann, O. Blixt, *Glycobiology* **2006**, *16*, 21C 27C.

[20] M. L. Huang, M. Cohen, C. J. Fisher, R. T. Schooley, P. Gagneux, K. Godula, *Chem. Commun.* **2015**, *51*, 5326 5329.

[21] a) F. Peri, P. Dumy, M. Mutter, *Tetrahedron* **1998**, *54*, 12269 12278; b) G. S. Chen, N. L. Pohl, *Org. Lett.* **2008**, *10*, 785 788; c) S. Munneke, J. R. C. Prevost, G. F. Painter, B. L. Stocker, M. S. M. Timmer, *Org. Lett.* **2015**, *17*, 624 627.

[22] S. A. Loskot, J. Zhang, J. M. Langenhan, *J. Org. Chem.* **2013**, *78*, 12189 12193.

[23] M. R. Carrasco, M. J. Nguyen, D. R. Burnell, M. D. MacLaren, S. M. Hengel, *Tetrahedron Lett.* **2002**, *43*, 5727 5729.

[24] a) A. Dirksen, T. M. Hackeng, P. E. Dawson, *Angew. Chem. Int. Ed.* **2006**, *45*, 7581 7584; *Angew. Chem.* **2006**, *118*, 7743 7746; b) M. B. Thygesen, H. Munch, J. Sauer, E. Cló, M. R. Jørgensen, O. Hindsgaul, K. J. Jensen, *J. Org. Chem.* **2010**, *75*, 1752 1755; c) M. Rashidian, M. M. Mahmoodi, R. Shah, J. K. Dozier, C. R. Wagner, M. D. Distefano, *Bioconjugate Chem.* **2013**, *24*, 333 342; d) M. Wendeler, L. Grinberg, X. Wang, P. E. Dawson, M. Baca, *Bioconjugate Chem.* **2014**, *25*, 93 101.

[25] a) Y. Shinohara, H. Sota, M. Gotoh, M. Hasebe, M. Tosu, J. Nakao, Y. Hasegawa, M. Shiga, *Anal. Chem.* **1996**, *68*, 2573 2579; b) C. Leteux, R. A. Childs, W. Chai, M. S. Stoll, H. Kogelberg, T. Feizi, *Glycobiology* **1998**, *8*, 227 236; c) K. Godula, C. R. Bertozzi, *J. Am. Chem. Soc.* **2010**, *132*, 9963 9965.

[26] J. Kalia, R. T. Raines, *Angew. Chem. Int. Ed.* **2008**, *47*, 7523 7526; *Angew. Chem.* **2008**, *120*, 7633 7636.

[27] W. P. Jencks, *Prog. Phys. Org. Chem.* **1964**, *2*, 63 128.

[28] ISO/IEC Guide 98 3:2008 Uncertainty of measurement Part 3: Guide to the expression of uncertainty in measurement (GUM:1995), **2008**.

[29] R. J. Batchelor, D. F. Green, B. D. Johnston, B. O. Patrick, B. M. Pinto, *Carbohydr. Res.* **2001**, *330*, 421 426.

[30] A. Kréżel, W. Bal, *J. Inorg. Biochem.* **2004**, *98*, 161 166.