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One-Pot Synthesis of Unprotected Anomeric Glycosyl Thiols in Water for Glycan Ligation Reactions with Highly Functionalized Sugars

*Sebastian Köhling, Matthias P. Exner, Saba Nojoumi, Jürgen Schiller, Nediljko Budisa, and Jörg Rademann**

Abstract: *Chemical synthesis of oligosaccharide conjugates is essential for studying the functional relevance of carbohydrates, and this task would be facilitated considerably if reliable methods for the anomeric ligation of unprotected sugars in water were available. Here, a method for the preparation of anomeric glycosyl thiols from complex unprotected mono-, di-, and oligosaccharides is presented. By exploiting the neighboring-group effect of the 2-acetamido-group, 1,2-oxazolines are generated and converted into 1-glycosyl thioesters through treatment with 1-thioacids. The unprotected anomeric glycosyl thiolates released in situ were conjugated to Michael acceptors, aliphatic halogenides, and aziridines to furnish versatile glycoconjugates. Conjugation of amino acids and proteins was accomplished using the thiol–ene reaction with terminal olefins. This method gives efficient access to anomeric glycosyl thiols and thiolates, which enables anomeric ligations of complex unprotected glycans in water.*

Many biological functions of eukaryotic cells are mediated by complex oligosaccharides attached to proteins or lipids,[1] since interactions between cells and with the surrounding extracellular matrix are governed by carbohydrate–protein binding.[2] Therefore, synthetic access to glycoconjugates is crucial for studying and understanding carbohydrate-dependent biological processes. In classical carbohydrate chemistry, glycoconjugates are mostly prepared through chemical glycosylation reactions,[3] which require the use of fully protected and highly reactive sugar donors and are thus not compatible with the presence of water. As a result, chemical reactions that allow the synthesis of glycoconjugates without protecting groups and under aqueous or physiological conditions are in high demand.

Several protecting-group-free approaches to carbohydrate conjugates have been applied so far. Enzymatic conjugations are very useful, however, the available biocatalysts and accessible structures are limited.[4] Considering purely chemical methods, glycosyl amines can be formed without protecting groups with ammonium bicarbonate in water,[5] however, their stability is limited strictly to basic conditions. Anomeric glycosyl azides are significantly more stable and are readily accessible from unprotected sugars, as demonstrated by Shoda and co-workers.[6] Glycosyl azides have been used successfully in copper-assisted dipolar coupling reactions to furnish triazol-containing glycoconjugates,[7] although the glycosyl triazolyl linkage can be hydrolyzed by acids such as trifluoroacetic acid (TFA), a standard reagent in the synthesis of (glyco)peptides.[8] In contrast, thioglycosides are stable under both basic and acidic aqueous conditions. They are thus useful linker entities in bioconjugates and for the solid-phase synthesis of oligosaccharides.[9] In addition, they can be applied as glycosyl donors through activation under oxidative or alkylating conditions.[10] During our recent studies on the conjugation of glycosaminoglycans (GAG),[8] the oligosaccharides of extracellular matrices, we realized that glycosyl thiols are required in order to enable more flexible ligation reactions and to obtain stable glycoconjugates.

Glycosyl thiols have been obtained from unprotected glucose and hydrogen sulfide in anhydrous hydrogen fluoride, although in low yield and without stereocontrol.[11] Lawesson's reagent has also been used for the thionation of simple unprotected sugars,[12] however, considerable heating is required, leading to anomeric mixtures in several examples. Lawesson's reagent is also not selective for aldehydes, but thionates all carbonyl functionalities, including carboxylic acids, amides, and esters, which are present in the majority of bioactive glycans.[13] Since a broadly applicable and useful method for the preparation of 1-glycosyl thiols from fully unprotected mono- and oligosaccharides with acetamido, carboxylate, hydroxy, and acetal functionalities has not been available so far, it was our goal to develop such a method, using unprotected 2-acetamido-2-desoxy-D-glucose (**1**) as a model compound (Scheme 1). Recently, aryl and alkyl thioglycosides, but not glycosyl thiols, were prepared from simple unprotected sugars by using the specific anomeric alkylation reagent 2-chloro-1,3-dimethylimidazolium chloride (DMC, **2**) and a base.[14,15]

With the aim of producing 1-glycosyl thiols, we investigated these conditions with **1** and sulfur nucleophiles, including sodium hydrogen sulfide, sodium sulfide, thiocarboxylates, thiourea, benzyl-thiols, trityl thiol, 2-cyanoethyl thiol, and *tert*-butylthiol, but we did not obtain the desired products. Instead, the sulfur nucleophiles reacted directly with **2**, furnishing 2-mercapto imidazolinium salts or the cyclic

[*] M. Sc. S. Köhling, Prof. Dr. J. Rademann
Medicinal Chemistry, Institut für Pharmazie, Freie Universität Berlin
Königin-Luise-Strasse 2+4, 14195 Berlin (Germany)
E-mail: joerg.rademann@fu-berlin.de
Homepage: http://www.fu-berlin.de/agrademann
M. Sc. S. Köhling, Dr. J. Schiller, Prof. Dr. J. Rademann
Institut für Biophysik, Universität Leipzig
Härtelstrasse 16–18, 04107 Leipzig (Germany)
Dr. M. P. Exner, S. Nojoumi, Prof. Dr. N. Budisa
Institut für Chemie, Technische Universität Berlin
Müller-Breslau-Strasse 10, 10623 Berlin (Germany)
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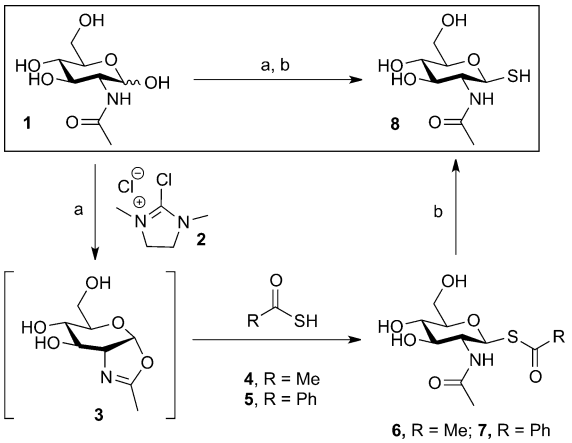


Scheme 1. Protection-group-free conversion of saccharides into glycosyl thiols. Reaction conditions: a) NEt_3, D_2O/MeCN 2:1, 0 °C→RT, 90 min; thioacid **4** or **5** in MeCN, 5 min; b) NaOMe, MeOH, Dowex H^+-resin. DMC = 2-chloro-1,3-dimethyl-imidazolium chloride.

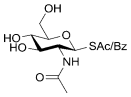
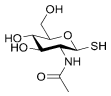
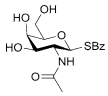
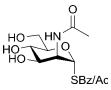
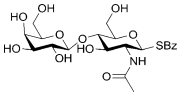
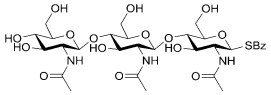
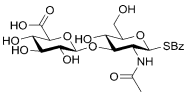
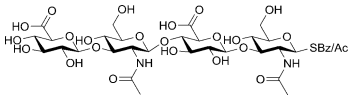
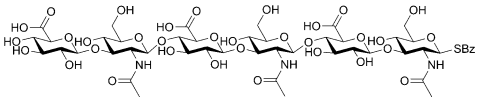
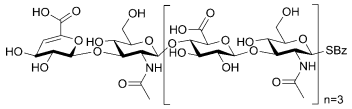
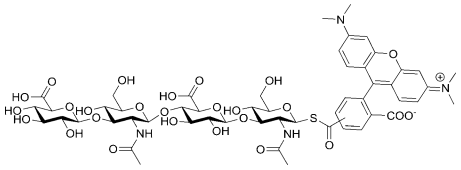
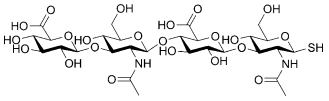
2-thio-urea.[16] Nucleophilic attack on **2** could be avoided by activating **1** with DMC and triethylamine, which furnished oxazoline **3**. While **3** was completely stable toward thiol nucleophiles under basic conditions, we found that it reacts smoothly with thioacetic acid **4** or thiobenzoic acid **5** to stereoselectively furnish the 2-acetamido-glucosyl 1-β-thioesters **6** and **7**. The conditions for these reactions were carefully optimized for both thioacids with respect to the ratio and concentration of reagents and the solvent. Full conversion of the monosaccharide *N*-acetylglucosamine **1** into the stable thioesters **6** or **7** was obtained for a thioacid/DMC/base molar ratio of 2:1:3 in the case of thiobenzoic acid **5** (90% yield of isolated product, vs. 75% for a ratio of 3:1:3) and 6:1:3 in the case of thioacetic acid **4**. A 2:1 mixture of deuterium oxide and acetonitrile was used as the solvent, thus exploiting the reduced hydrolysis rate of DMC in deuterated water[17] and ensuring the solubility of thiobenzoic acid. Our experiments, however, did not indicate a significant difference compared to water/acetonitrile mixtures (86 vs. 90% for **7**). Incorporation of the thioacetyl or thiobenzoyl residues enabled the purification of products **6** and **7** in high yields of 86 and 90%, respectively, by using flash chromatography or HPLC (Table 1, entry 1 and 2). Thioesters **6** and **7** were both converted into the β-2-acetamido-2-deoxy-D-glucopyranosyl thiol **8** quantitatively upon treatment with either sodium methoxide or sodium hydroxide as a base, followed by neutralization (Table 1, entry 3). The odorless anomeric glycosyl thiol **8** was stable for 24 h in aqueous basic solution and did not show mutarotation caused by ring opening of the hemithioacetal function. In contrast, a solution of **8** in water was slightly acidic and **8** degraded slowly, with only 55% of the initial 1-β-thiolaldose remaining after one day (see the Supporting Information).

The scope of the novel reaction was investigated with a selection of mono- and oligosaccharides (Table 1). The method worked smoothly with three *N*-acetyl-D-hexosamines, yielding exclusively the 1,2-*trans* thioglycoside esters.

N-acetyl-galactosamine (entry 4) delivered the β-anomeric S-benzoylthioester **9**. In contrast, *N*-acetyl-mannosamine (entries 5,6) reacted to give the α-anomeric thioesters **10** and **11** owing to its axial 2-acetamide. *N*-acetyl-D-lactosamine, that is, β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranose, was investigated as a disaccharide (entry 7) and *N*,*N*′,*N*″-tri-*N*-acetylchitotriose, a partial structure of the polysaccharide chitin, was selected as a trisaccharide (entry 8). Both compounds furnished the pure thioesters **12** and **13**, respectively, with yields of isolated product in the same range as for monosaccharides (90 and 65%). Glycosaminoglycans (GAG) are especially interesting to the glycobiology community since these polysaccharides form the extracellular matrix (ECM) of higher organisms, which is responsible for many biological functions through carbohydrate–protein interactions.[2a,18] Partial structures of hyaluronic acid (HA), the major GAG in the extracellular matrix, were generated through a chemoenzymatic approach using bovine testis hyaluronidase, according to a recently disclosed method.[8] They comprise the disaccharide, β-D-glucopyranuronyl-(1→3)-D-2-acetamido-2-deoxy-glucopyranose (HA-2 **33**, entry 9), the respective tetrasaccharide (HA-4, entries 10, 11, and 14), and the hexasaccharide (HA-6 **34**, entry 12). The HA-octasaccharide **35** was obtained using a bacterial hyaluronidase from *Streptococcus pyogenes* and was dehydrated at the terminal glucuronic acid residue to give a double bond in the 4-position (ΔHA-8 **35**, entry 13). The partial structures of HA were converted in to the pure β-thioesters **14**, **15**, **18**, and **19** by using thiobenzoic acid, while in the reaction sequence of HA-4 with thioacetic acid, traces of a diacetylated thioester byproduct were observed, which reduced the yield of isolated **16** to 56%. For labeling of HA-4 with a fluorophore, the thioacid of 5,6-carboxytetramethylrhodamine (TAMRA) was prepared through acidic cleavage of the TAMRA triphenylmethyl thioester (**36**, see the Supporting Information) and employed in the anomeric ligation reaction to provide compound **19**. In order to demonstrate that the anomeric thioesters of oligosaccharides can be converted stereospecifically into the corresponding anomeric thiols, the benzoyl thioester of HA-4 tetrasaccharide **15** was cleaved to the anomeric 1-β-thio-glycosides **20** in 97% yield of isolated product. Next, anomeric thiols of mono- and oligosaccharides were investigated in anomeric ligation reactions (Scheme 2). Glycosyl-1-thiolates of **8** and **20** were generated in situ and reacted with acrylonitrile to furnish the Michael addition products **21** and **22** in 73 and 95% yield, respectively.

For attachment to surfaces and incorporation into artificial extracellular matrices, glycans with a spacer carrying a reactive nucleophile are required. Glycans containing a mercapto-terminated spacer (**23**–**25**) were obtained through alkylation of glycosyl-1-thiolate **8** and **20** using the S-trityl-protected mercaptopolyethylene glycol bromide **29**. Formation of the disulfides of **8** and **20** could be prevented by using a catalytic amount of tris-(2-carboxyethyl)-phosphine (TCEP). Removal of the S-trityl group in **23** furnished the free thiol **24** when using trifluoroacetic acid (TFA) in dichloromethane and triethylsilane as a trityl scavenger. The tetrahyaluronyl thiol **20** was also ligated with a C2 spacer containing a primary amine functionality by employing a ring-opening/alkylation reaction of aziridine. Aziridine was generated in situ from 2-chloroethylamine and sodium hydroxide

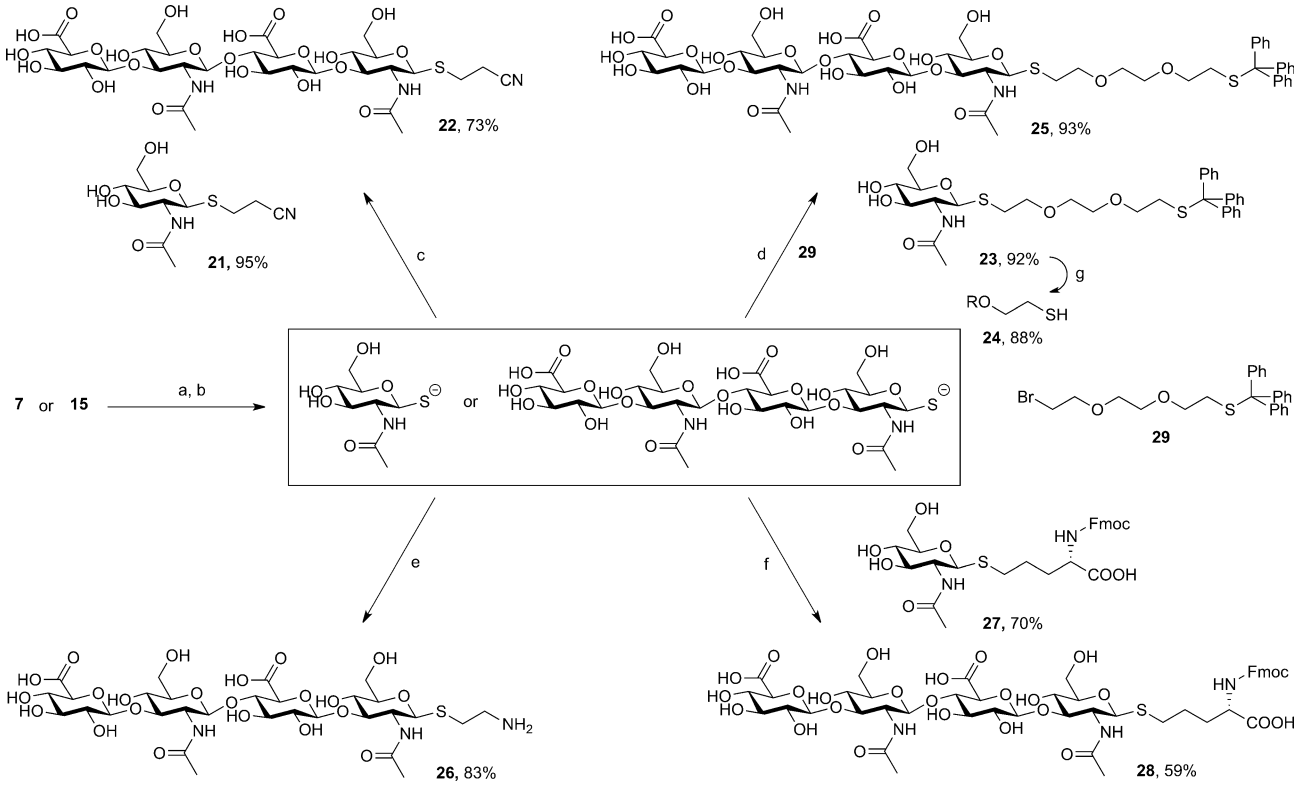
Table 1: Anomeric glycosyl-1-thioacetates, 1-thiobenzoates, and 1-thiols from various mono-, di-, tri-, tetra-, hexa-, and octasaccharides.

Entry	Mono/Oligo-saccharides	Product	Compound number	Yield [%]
1	GlcNAc **1**		**6** (Ac)	86
2			**7** (Bz)	90
3	GlcNAc-SBz **7**		**8**	99
4	GalNAc		**9**	60
5	ManNAc		**10** (Bz)	80
6			**11** (Ac)	58
7	LacNAc		**12**	90
8	$(GlcNAc)_3$		**13**	65
9	HA-2 **33**		**14**	79
10	HA-4		**15** (Bz)	74
11			**16** (Ac)	56
12	HA-6 **34**		**17**	63
13	ΔHA-8 **35**		**18**	60
14	HA-4		**19**	35
15	HA-4-SBz **15**		**20**	97

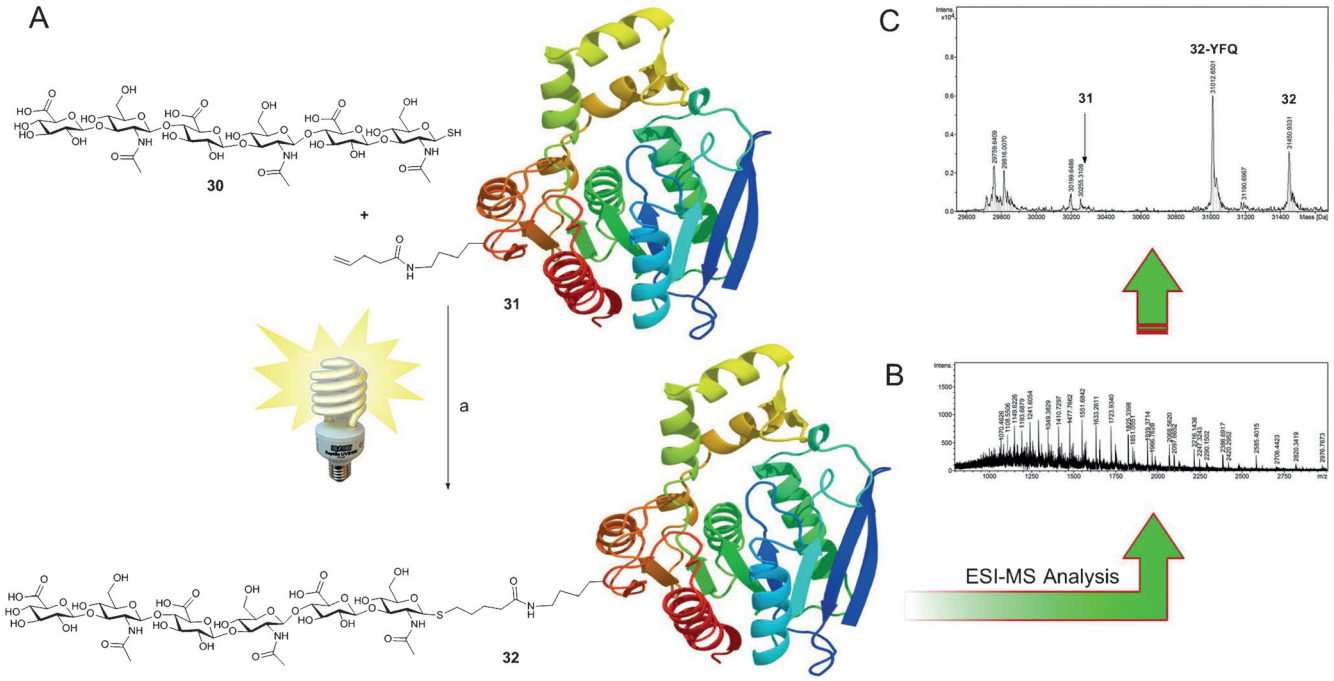
as a base. The three-membered aziridine ring was opened by the thiol nucleophile **20** to provide product **26** in 83% yield. Finally, thiol–ene reactions of the glycosyl thiols were investigated. Commercially available *N*-Fmoc-L-allyl-glycine was employed as a starting material containing the required terminal olefin. Irradiation of the photoinitiator 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone generated thiol radicals that added smoothly to the olefin to yield the S-glycosylated amino acids **27** and **28**, ready for used in solid-phase peptide synthesis, in 70 and 59% yield, respectively, after isolation.

For the direct attachment of the 1-thio-hexa-hyaluronane **30** (HA-6-SH) to a protein, the engineered thermostable lipase TTL from *Thermoanaerobacter thermohydrosulfuricus*, which contains an N-pentenoyl lysine residue in position 221, was used.[21] The non-canonical amino acid N-pentenoyl lysine was incorporated into the protein using stop-codon suppression. Upon applying thiol–ene conditions, quantitative incorporation of the hyaluronan hexasaccharide was accomplished, as indicated by HPLC-MS of the protein (Scheme 3).

In summary, we have demonstrated a novel, high-yielding and highly practical access to unprotected glycosyl thioesters and glycosyl thiols from complex mono- and oligosaccharides. The method involves the activation of unprotected 2-acetamido sugars to anomeric 1,2-oxazolines. Smooth opening of the



Scheme 2. Anomeric ligation reactions with glycosyl-1-thiolates: a) NaOMe [for (c + d)]; b) NaOH [for (e)]; c) acrylonitrile, MeOH; d) TCEP cat., MeOH, 50 °C; e) 2-chloroethylamine hydrochloride, TCEP cat.; f) Dowex-H^+, Fmoc-L-allyl-glycine, cat. 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone in MeOH or MeOH/H_2O 4:1, *hν*; g) CH_2Cl_2/TFA/TES 10:10:1, 5 min. TCEP = tris(2-carboxyethyl)phosphine, TFA = trifluoroacetic acid, TES = triethylsilane.



Scheme 3. A) Protein conjugation of TTL221-PentK with hyaluronan hexasaccharide thiol **30**. Reaction conditions: a) Vazzo44 in 0.25 M PBS, pH 6.0, *hν*,[19] 4 h. B, C) Mass spectrometry analysis before and after deconvolution. Calculated mass of product TTL221-PentK-S-HA-6 **32**: 31 451 Da, found mass: 31450.9 Da (calculated mass of TTL221-PentK **31**: 30 280 Da, see the Supporting Information). The peak at 31 013 Da (−438 Da) belongs to TTL221-PentK-S-HA-6 **32** minus the C-terminally cleaved tripeptide Tyr-Phe-Gln. Vazzo44 = 2,2′-Azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride.[20]

oxazolines formed at the reducing end of mono- and oligosaccharides was affected with thioacids. The obtained anomeric thioesters were formed stereospecifically and could be purified, analyzed, and stored. For further reactions, the anomeric thioesters were converted in situ into anomeric glycosyl thiols, which are efficient reagents in anomeric ligation reactions to provide spacer-extended glycosyl thiols and glycosylamines, S-glycosidic amino acids, and S-glycoproteins. This novel method will considerably facilitate access to stable glycoconjugates and glycan- or GAG-based biomaterials, thereby contributing to the investigation and exploitation of the biological functions and functional potential of glycans.

Acknowledgments

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Keywords: bioorthogonal conjugation · carbohydrate chemistry · glycobiology · glycosyl thiols · oligosaccharides

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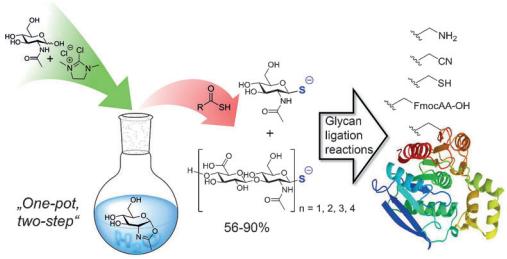
Carbohydrate Chemistry

S. Köhling, M. P. Exner, S. Nojoumi, J. Schiller, N. Budisa, J. Rademann* ——— ■■■■–■■■■

One-Pot Synthesis of Unprotected Anomeric Glycosyl Thiols in Water for Glycan Ligation Reactions with Highly Functionalized Sugars



Sweet dreams are made of these: A direct access to anomeric glycosyl thiols from unprotected mono- and oligosaccharides was developed. Sugar oxazolines were opened smoothly with thioacids to form odorless and storable glycosyl thioesters as precursors for 1-thiol-sugars, thiolates and, further functionalized glycosides, including those with amino and thiol spacers, S-glycosidic amino acids, and S-linked glycoproteins.