DMT-MM mediated functionalisation of the non-reducing end of glycosaminoglycans†

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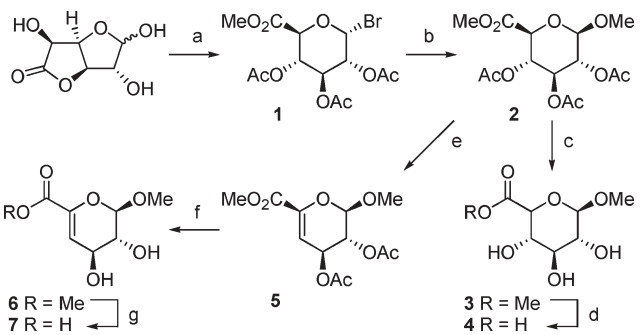
Efficient functionalisation of the non-reducing end of uronic acid derivatives and glycosaminoglycan-derived disaccharides using peptide coupling has been achieved, mediated by the water-soluble agent DMT-MM.

Glycosaminoglycans (GAGs) are linear polysaccharides found almost ubiquitously on animal cell surfaces and within extracellular matrices.[1] They constitute an important class of macromolecules that are implicated in both the structural organization of extracellular matrices, and how cells interact with them, as well as in the regulation of the biological activity of morphogens, growth factors, cytokines, chemokines and enzymes.[2] Their precursors (alternating uronic acid and hexosamine residues) are extensively modified (uronate epimerisation, N-deacetylation, N- and O-sulfation) creating heterogeneous structures. Many of the functions of GAGs are mediated through their interactions with proteins. These occur *via* contacts between the negatively charged groups of GAGs and positively charged amino acid side chains.[3] As a consequence, oligosaccharides in protein-GAG complexes do not occupy hydrophobic pockets, but sit on the protein surface with only a few intermolecular contacts,[4] making NMR structure determination of these complexes extremely challenging.

At present, an atomic level of detail for protein–heparin complexes [where heparin is used as a model for the more-heterogeneous heparan sulfate (HS)] is provided by a limited number of crystal structures and NMR studies,[5] whilst the experimental conformations of free GAGs in solution have been exclusively obtained by NMR.[6] No X-ray crystal structures are available for HS-protein complexes, and the effects of reduced sulfation and GlcA/IdoA isomerism, compared to heparin, on the conformation of the HS chain are poorly understood. In addition, models of GAG–protein complexes are emerging that involve much longer chains and/or their interactions spanning several protein subunits;[7] these await experimental verification. The attachment of paramagnetic moieties to either the reducing, or non-reducing end of GAGs, and observation of the effect of this on complexed protein atoms by NMR, should allow distances between the two molecules to be inferred and the binding position of the GAG oligosaccharide on a protein surface to be determined. Furthermore, whilst FRET and TR-FRET have been widely applied to the field of protein–protein interactions,[8] and the study of DNA and RNA folding;[9] to date, it has not been possible to apply these techniques to GAGs due to the lack of suitable methodology for the selective introduction of a fluorophore at the non-reducing end of the GAG. Thus the development of a robust methodology for the functionalisation of the non-reducing end of GAGs is clearly of high priority if more detailed conformational and binding studies of this important class of biomolecules are to be pursued.

In order to develop methodology for the functionalisation of the non-reducing end of GAGs, we first required a rapid route to the synthesis of simple monosaccharide analogues (Scheme 1). Glycosyl bromide **1** is readily synthesised in three steps from commercially available D-glucurono-6,3-lactone.[10] This was converted to the corresponding methyl glycoside **2** in high yield. Glycoside **2** was treated to a simple two-stage deprotection to give saturated uronic acid **4**.[11] To produce an appropriate model for the terminal $\Delta^{4,5}$-unsaturated uronic acid which results from the lyase-mediated cleavage of GAGs, DBU elimination was effected (70%),[12] followed by the corresponding two-stage deprotection to give unsaturated uronic acid **7**.

Using the uronic acid models **4** and **7**, we then screened an array of commonly used peptide coupling reagents (EDAC/HOBt, pyBOP, *etc.*) under a range of experimental conditions in order to determine whether any could be used as a means of functionalising the non-reducing end of GAGs. In order to achieve maximum compatibility with more complex GAG samples, we set out to



Scheme 1 *Reagents and conditions*: (a) ref. 10; (b) MeOH, Ag_2O, 3 Å mol. sieves (76%); (c) NaOMe (cat.), MeOH (94%); (d) NaOH, MeOH–H_2O (1 : 1) (quant.); (e) DBU, CH_2Cl_2 (75%); (f) NaOMe (cat.), MeOH (74%); (g) NaOH, EtOH–H_2O (1 : 1) (quant.).

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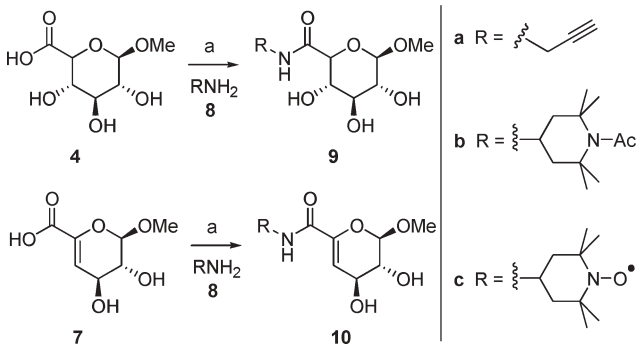
† Electronic supplementary information (ESI) available: Procedures and spectroscopic data for preparation of uronic acid models **4** and **7**. Coupling protocols and spectroscopic data for compounds **9**, **10** and **12**. See DOI: 10.1039/b617038b



Scheme 2 *Reagents and conditions*: (a) amine **8** (1.2 eq.), DMT-MM (1.5 eq.), MeOH–H_2O (9 : 1), RT, 5–14 h.

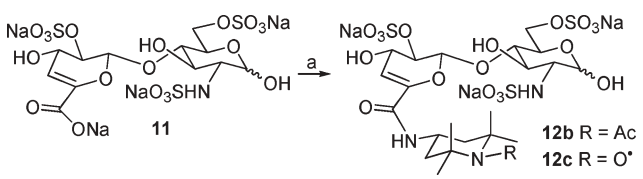
achieve these coupling reactions using either water, or methanol, as the reaction medium. We were unsuccessful in our attempts until we employed the water-soluble agent DMT-MM.[13] Although it has not previously been used in the formation of glycosyl amides from uronic acids, DMT-MM has been used for the condensation of glucosamine with sodium acetate; a reaction with reversed functional polarity (glycosyl amine coupling with functional acid) to that which we hoped to achieve, but a notable precedent nevertheless.[14] In the presence of DMT-MM a number of amines were coupled to the saturated and unsaturated uronic acid models (Scheme 2). Thus propargyl amine (**8a**) could be coupled in excellent yield (94 and 81%, Table 1) to the uronic acid models, opening up the potential for bioorthogonal Cu(I) catalysed Huisgen 1,3-dipolar cycloaddition reactions to a range of azido functionalised tags.[15]‡ Similarly, the DMT-MM mediated coupling reactions of 4-amino TEMPO (**8c**) and its *N*-acetyl analogue (**8b**) have been demonstrated to be highly efficient (77–88%) with uronic acid **4** and the $\Delta^{4,5}$-unsaturated uronic acid **7**.

Finally, we have investigated the DMT-MM mediated coupling of the sodium salt of the unsaturated uronic acid residue of a lyase-cleaved, fully sulfated, heparin-derived disaccharide **11**§ with amines **8b** and **8c** (Scheme 3). In these reactions, the use of a larger excess of the coupling agent DMT-MM led to increased

Table 1 Yields of DMT-MM coupled products

Amine	Uronic acid	Reaction time/h	Product	Yield (%)
8a	**4**	14	**9a**	94
8b	**4**	14	**9b**	88
8a	**7**	5	**10a**	81
8b	**7**	6	**10b**	85
8c	**7**	6	**10c**	77
8b	**11**	18	**12b**[a]	74[b]
8c	**11**	18	**12c**[a]	68[b]

[a] Isolated as a 78 : 22 α : β anomeric mixture. [b] Conversion by HPLC.



Scheme 3 *Reagents and conditions*: (a) amine **8** (1.2 eq.), DMT-MM (3 eq.), MeOH–H_2O (2 : 1), RT for 4 h then −20 °C for 14 h.

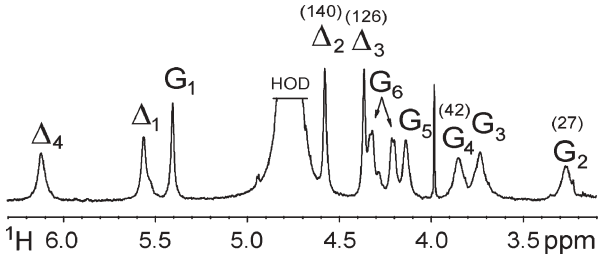


Fig. 1 600 MHz ^{1}H spectrum of **12c**, the TEMPO derivative of a fully sulfated heparin-derived disaccharide. G-glucosamine, Δ-unsaturated uronic acid residue. The numbers in parentheses indicate the non-selective T_1 relaxation times [in ms] of selected protons.

conversion, thus facilitating subsequent purification of the coupled products **12b** and **12c** by SAX-HPLC.

A 600 MHz ^{1}H NMR spectrum of **12c** is presented in Fig. 1. A significant broadening of resonances is apparent, indicating successful incorporation of the spin-label. The structure of **12c** (Fig. 2) modelled using the AMBER force field with explicit H_2O as solvent, is stable in unrestrained MD runs. The non-selective ^{1}H T_1 relaxation times of **12c** correlate well with the electron–proton distances seen in the AMBER model. This experimental result validates our model, in which the dihedral glycosidic angles are within 15° of those observed in the unmodified disaccharide in solution and solid state.[4,6] Attachment of the TEMPO moiety therefore has not significantly changed the conformation of the disaccharide moiety.

It may be thought that the presence of a terminal reporter group (approximately equivalent in size to a monosaccharide unit) on an oligosaccharide might interfere with, and distort, protein interactions. However, GAGs bind primarily to protein surfaces, and not within constrained pockets, a consequence of their natural occurrence as large, highly-charged polymers which inevitably extend either side of any specific, internal, protein-binding oligosaccharide segment. Indeed several X-ray crystal structures involving different GAG–protein complexes have successfully exploited heparin oligosaccharides of 10–14 monosaccharides in length,[5b,16,17] even though protein contacts only occurred within internal segments spanning 6–9 monosaccharides. Similarly, the

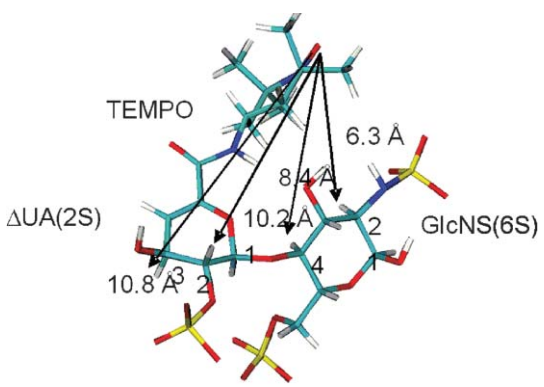


Fig. 2 The lowest energy structure for **12c** obtained using the AMBER force field with explicit H_2O as solvent. The dihedral angles between the two carbohydrate rings are within 15° of those observed for this disaccharide in solution or X-ray structures.[4,6] Radical–proton distances are given in Å.

smallest heparin oligosaccharide with affinity for HGF/SF by surface plasmon resonance was shown to be a tetrasaccharide.[18] The same heparin tetrasaccharide, after tagging with a terminal fluorophore (2-aminoacridone), retained its HGF/SF-binding ability by gel mobility shift assay.[19] Hence we anticipate that in solution phase GAG–protein complexes, the paramagnetic relaxation enhancement induced by the electron radical in **12c** on *protein* protons will lead to generation of important distance restraints.

In conclusion, a new, highly versatile and promising approach to the labelling of the non-reducing end of GAGs has been demonstrated. We are currently investigating the application of this methodology to conformational studies of GAGs using FRET and NMR.

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Notes and references

‡ *Experimental procedure* for the DMT-MM mediated coupling: 50 mg of uronic acid **4** or **7** was dissolved in methanol–water (9 : 1, 5 ml) together with the appropriate amine (1.2 eq.) and the mixture was stirred at room temperature for 10 min. DMT-MM (1.5 eq.) was then added and the reaction stirred at room temperature until complete (5–14 h). The solvent was removed under reduced pressure and the residue co-evaporated with absolute ethanol. Flash chromatography (CH_2Cl_2–MeOH, 15 : 1) afforded the corresponding amide.
§ Purchased from Dextra Laboratories Ltd.

1 R. J. Linhardt and T. Toida, *Acc. Chem. Res.*, 2004, **37**, 431–438.
2 M. Bernfield, M. Gotte, P. W. Park, O. Reizes, M. L. Fitzgerald, J. Lincecum and M. Zako, *Annu. Rev. Biochem.*, 1999, **68**, 729–777.
3 I. Capila and R. J. Linhardt, *Angew. Chem., Int. Ed.*, 2002, **41**, 390–412.
4 S. Faham, R. E. Hileman, J. R. Fromm, R. J. Linhardt and D. C. Rees, *Science*, 1996, **271**, 1116–1120.
5 (*a*) B. Mulloy and R. J. Linhardt, *Curr. Opin. Struct. Biol.*, 2001, **11**, 623–628; (*b*) D. Lietha, D. Y. Chirgadze, B. Mulloy, T. L. Blundell and E. Gherardi, *EMBO J.*, 2001, **20**, 5543–5555; (*c*) H. J. Zhou, J. R. Casas-Finet, R. H. Coats, J. D. Kaufman, S. J. Stahl, P. T. Wingfield, J. S. Rubin, D. P. Bottaro and R. A. Byrd, *Biochemistry*, 1999, **38**, 14793–14802; (*d*) H. J. Zhou, M. J. Mazzulla, J. D. Kaufman, S. J. Stahl, P. T. Wingfield, J. S. Rubin, D. P. Bottaro and R. A. Byrd, *Structure*, 1998, **6**, 109–116.
6 D. Mikhailov, R. J. Linhardt and K. H. Mayo, *Biochem. J.*, 1997, **328**, 51–61; D. Mikhailov, K. H. Mayo, I. R. Vlahov, T. Toida, A. Pervin and R. J. Linhardt, *Biochem. J.*, 1996, **318**, 93–102; B. Mulloy, M. J. Forster, C. Jones and D. B. Davies, *Biochem. J.*, 1993, **293**, 849–858.
7 D. Spillmann, D. Witt and U. Lindahl, *J. Biol. Chem.*, 1998, **273**, 15487–15493.
8 H. Wallrabe and A. Periasamy, *Curr. Opin. Biotechnol.*, 2005, **16**, 19–27.
9 J.-F. Lemay, J. C. Penedo, R. Tremblay, D. M. J. Lilley and D. A. Lafontaine, *Chem. Biol.*, 2006, **13**, 857–868; L. Ying, J. J. Green, H. Li, D. Klenerman and S. Balasubramanian, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 14629–14634.
10 G. N. Bollenback, J. W. Long, D. G. Benjamin and J. A. Lindquist, *J. Am. Chem. Soc.*, 1955, **77**, 3310–3315.
11 S. Oscarson and P. Svahnberg, *J. Chem. Soc., Perkin Trans. 1*, 2001, 873–879.
12 H. G. Bazin, I. Capila and R. J. Linhardt, *Carbohydr. Res.*, 1998, **309**, 135–144.
13 M. Kunishima, C. Kawachi, J. Morita, K. Terao, F. Iwasaki and S. Tani, *Tetrahedron*, 1999, **55**, 13159–13170.
14 M. Kunishima, C. Kawachi, K. Hioki, K. Terao and S. Tani, *Tetrahedron*, 2001, **57**, 1551–1558; B. D. Maxwell and J. C. Bronstein, *J. Labelled Compd. Radiopharm.*, 2005, **48**, 1049–1054.
15 J. A. Prescher and C. R. Bertozzi, *Nat. Chem. Biol.*, 2005, **1**, 13–21; P. F. van Swieten, M. A. Leeuwenburgh, B. M. Kessler and H. S. Overkleeft, *Org. Biomol. Chem.*, 2005, **3**, 20–27.
16 J. Schlessinger, A. N. Plotnikov, O. A. Ibrahimi, A. V. Eliseenkova, B. K. Yeh, A. Yayon, R. J. Linhardt and M. Mohammadi, *Mol. Cell*, 2000, **6**, 743–750.
17 D. Lietha, D. Y. Chirgadze, B. Mulloy, T. L. Blundell and E. Gherardi, *EMBO J.*, 2001, **20**, 5543–5555.
18 M. Delehedde, M. Lyon, R. Vidyasagar, T. J. McDonnell and D. G. Fernig, *J. Biol. Chem.*, 2002, **277**, 12456–12462.
19 M. Lyon, J. A. Deakin, D. Lietha, E. Gherardi and J. T. Gallagher, *J. Biol. Chem.*, 2004, **279**, 43560–43567.

