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Glycomethanethiosulfonates: powerful reagents for protein glycosylation

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Abstract

Twelve novel glycomethanethiosulfonate (glyco-MTS) protein glycosylation reagents have been prepared. Their use in a controlled site-selective glycosylation strategy that combines site-directed mutagenesis with chemical modification allows protein glycosylation with concomitant control of (i) site, (ii) carbohydrate, (iii) anomeric stereochemistry, (iv) sugar to protein spacer arm nature and (v) degree of glycan protection. The ability of these highly selective and yet reactive reagents has been illustrated by the introduction of D-glucosyl and *N*-Ac-D-glucosaminyl residues to both external and hindered internal sites in a model protein — the serine protease enzyme subtilisin *Bacillus lentus* (SBL) — using corresponding gluco-MTS **1** and *N*-Ac-glucosamine-MTS **2**. Molecular modelling studies provide a rationale for the strikingly different effects of these reagents on the properties of the protein despite differing only in the nature of their C-2 substituents.

1. Introduction

The glycosylation of proteins plays a key role in determining their expression, folding,[1] thermal and proteolytic stability[2] and, in the case of enzymes, catalytic activity.[3] Furthermore, the role of glycoproteins as cell surface markers in communication events such as microbial invasion,[4] inflammation[5] and immune responses[6] depends crucially on the correct glycosylation pattern. There is evidence that even very slight alterations in the sugars that decorate the exterior of a protein can cause remarkable changes in these properties. For example, a turn is induced in aqueous solution in a SYSPTSPSYS segment of the C-terminal domain of RNA polymerase II when the threonine side chain is *N*-acetyl-D-glucosaminylated, whereas the corresponding non-glycosylated peptide adopts a randomly-coiled structure.[7] Moreover,

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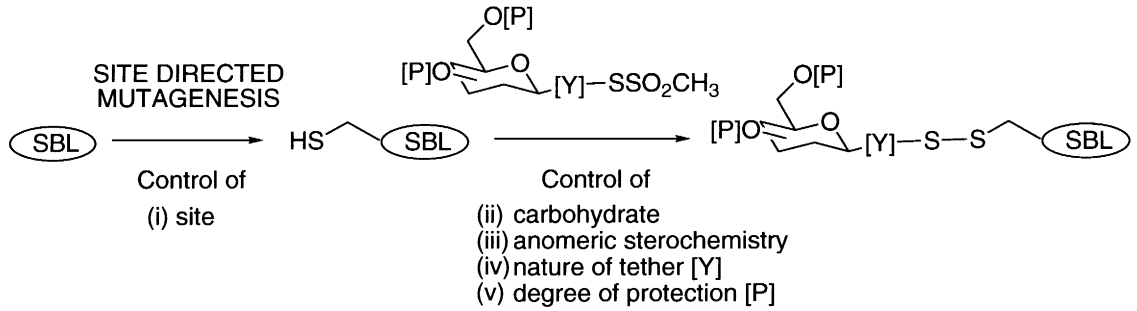
different forms of pancreatic ribonuclease B displayed a fourfold variation in hydrolytic activity although differing only in the nature of the glycans attached to the enzyme.[3]

Despite this striking ability of carbohydrates to influence protein properties, systematic studies of the effects of protein glycosylation have been limited and have required extensive chromatographic separations from natural abundant glycoprotein sources.[3] These difficulties are exacerbated by the natural occurrence of microheterogeneous mixtures of glycoforms:[8] glycoprotein forms that possess the same peptide backbone, but differ in both the nature and site of glycosylation.

Chemical glycosylation provides an alternative method for the preparation of glycosylated proteins.[9] In particular, such methods allow control of the glycan structure and the nature of the sugar–protein bond. However, despite these advantages, existing methods typically generate mixtures through the indiscriminate modification of the wide variety of functionality found in proteins. Advances in the site-specific glycosylation of the inactive protein bovine serum albumin (BSA) have been made.[10] Pioneering work by Flitsch and co-workers established the validity of cysteine modification as a strategy for chemoselective protein glycosylation. Thus, reaction of the α-iodoacetamide of *N*-acetyl-D-glucosamine with oxidized bovine serum albumin (BSA) allowed modification of the single free cysteine that is naturally present.[10a] Later this method was applied by Dwek and co-workers to introduce chitotriose and a heptasaccharide from horseradish peroxidase to BSA.[10b] Boons and co-workers have also described the use of a glycosyl dithiopyridine as another valuable addition to the cysteine-reactive reagents available for protein glycosylation.[10c] However, these methods rely upon modification of an existing cysteine in BSA and, as such, allow no flexibility in the choice of glycosylation site. For full control of glycosylation, both choice of site (site-selectivity) and glycan are needed. This paper describes a combined site-directed mutagenesis and chemical modification approach using well-defined and highly specific glycosylating agents. This method is rapid, efficient and is virtually unlimited in the scope of both the sites and the glycans that may be conjugated. The strategy involves the introduction of cysteine as a chemoselective tag at preselected positions within a given protein and then reaction of its thiol residue with glycomethanethiosulfonate reagents (Scheme 1). Methanethiosulfonate (MTS) reagents react specifically and quantitatively with thiols[11] and allow the controlled formation of neutral disulfide linkages.



Scheme 1. A combined site-directed mutagenesis and chemical modification approach to site-selective glycosylation

This paper reports the detailed syntheses of these powerful reagents (Fig. 1) and their use in the modification of the model protein: the serine protease subtilisin *B. lentus* (SBL). SBL is an ideal protein for evaluating the validity of this strategy as it does not contain a natural cysteine and is not naturally glycosylated. We chose four sites in SBL for glycosylation. S156[12] is a surface-exposed residue that permits the introduction of externally-disposed glycans resembling those found naturally in glycoproteins.[13] Furthermore, we were interested in probing the effect of glycosylation on enzyme activity as part of an ongoing programme to tailor activity, specificity and stability for use in synthesis.

Promising results have indicated the potential of glycoenzymes[14] but due to a lack of site-selectivity in glycosylation no precise structure–activity relationship (SAR) determinations have until now been possible. For this reason, we chose three further sites with internally oriented side chains, one in each of three key substrate binding pockets: N62 in the S_2[15] pocket, S166 in the S_1 pocket and L217 in the S_1' pocket. Moreover, position 62 is occupied by an asparagine residue, and positions 156 and 166 are occupied by serine residues in the wild-type enzyme and, in this respect, are representative of natural *N*-(Asn) and *O*-(Ser/Thr) glycosylation sites, respectively.[16]

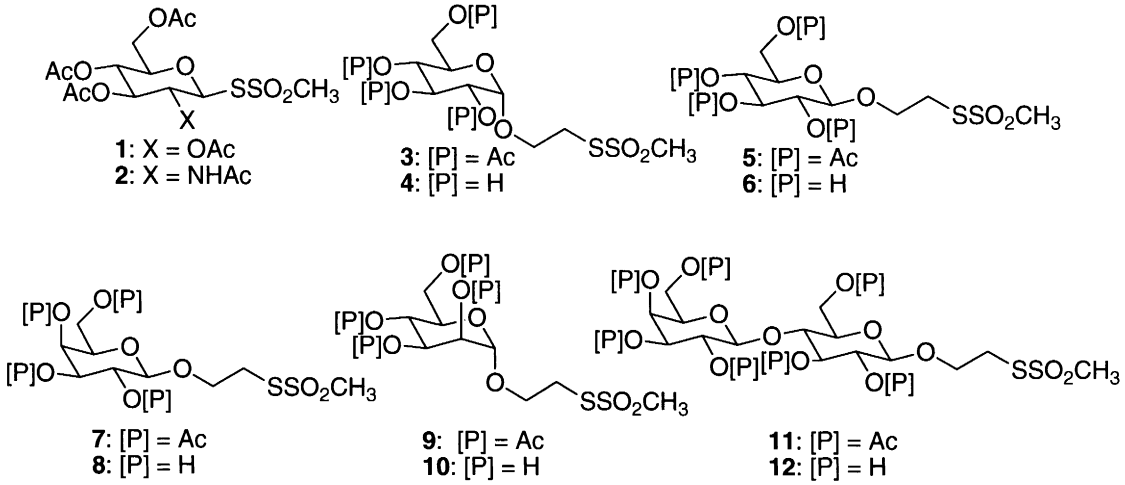


Fig. 1.

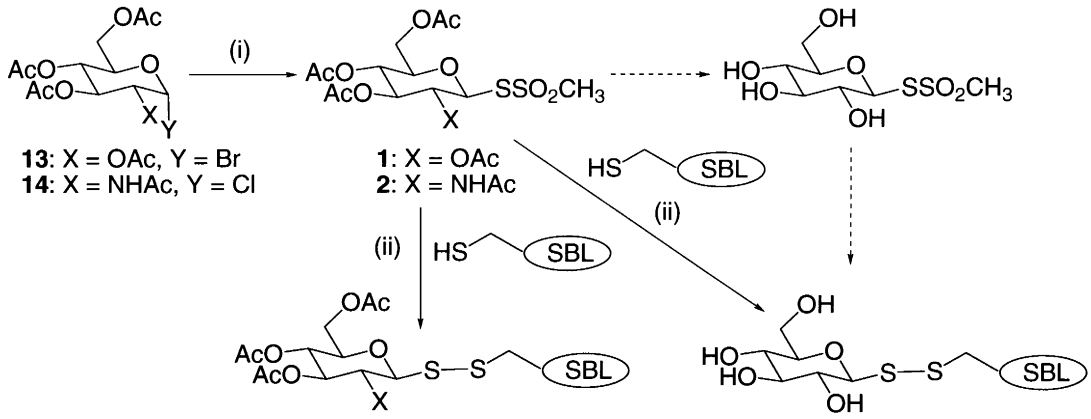
The synthesis of a range of glycomethanethiosulfonates (glyco-MTS) **1**–**12** allows the preparation of glycoproteins with control of (i) site, (ii) parent carbohydrate, (iii) anomeric stereochemistry, (iv) length of the glycan–protein tether and (v) degree of glycan protection (thereby allowing the effects of increased steric bulk and hydrophobicity to be assessed); some of this work has appeared as a preliminary publication.[14h] The determination of valuable glycosylation–activity SARs is illustrated by the glycosylation of SBL with reagents derived from D-glucose **1** and *N*-acetyl-D-glucosamine **2**. Molecular modelling studies are reported which provide a good rationale for the strikingly different effects of these two reagents upon the catalytic activity of SBL.

2. Results and discussion

*2.1. Preparation of directly linked glyco-MTS **1** and **2***

Previous synthetic work has demonstrated[17,18] that, of the wide variety of methods available,[19] direct nucleophilic displacement of a halide group by methanethiosulfonate ion provides an efficient and high yielding method for the preparation of alkyl methanethiosulfonates. Furthermore, the ease with which the anomeric leaving group of peracetylated glycosyl halides may be displaced by certain sulfur nucleophiles[20] prompted the synthesis of **1** and **2** by direct displacement using sodium methanethiosulfonate as a nucleophile (Scheme 2).[21] Thus, brief treatment (20 min) of a solution of sodium methanethiosulfonate in ethanol at 90°C with acetobromo-D-glucose **13** gave the fully protected anomeric β-D-gluco-MTS **1** in 63% yield; prolonged treatment or the use of other solvents gave substantially lower yields. Similarly, **2** was synthesized from acetochloro-*N*-acetyl-D-glucosamine **14**, through displacement

of the anomeric chloride by methanethiosulfonate to give crystalline β-GlcNAc-MTS **2** in a modest 25% yield. In contrast to the synthesis of **1**, highest yields of **2** were obtained without heating and by the use of DMF as a solvent. Deprotection of **1** and **2** was attempted under a variety of conditions,[22] but in all cases resulted either in the hydrolysis of the thioglycosidic bond, cleavage of the anomeric methanethiosulfonate group or failed to cleave the acetate protecting groups. Gratifyingly, careful control of pH and conditions during subsequent protein glycosylation (vide infra) allowed in situ deprotection of **1** and the introduction of partially and fully deprotected D-glucose to our model protein.



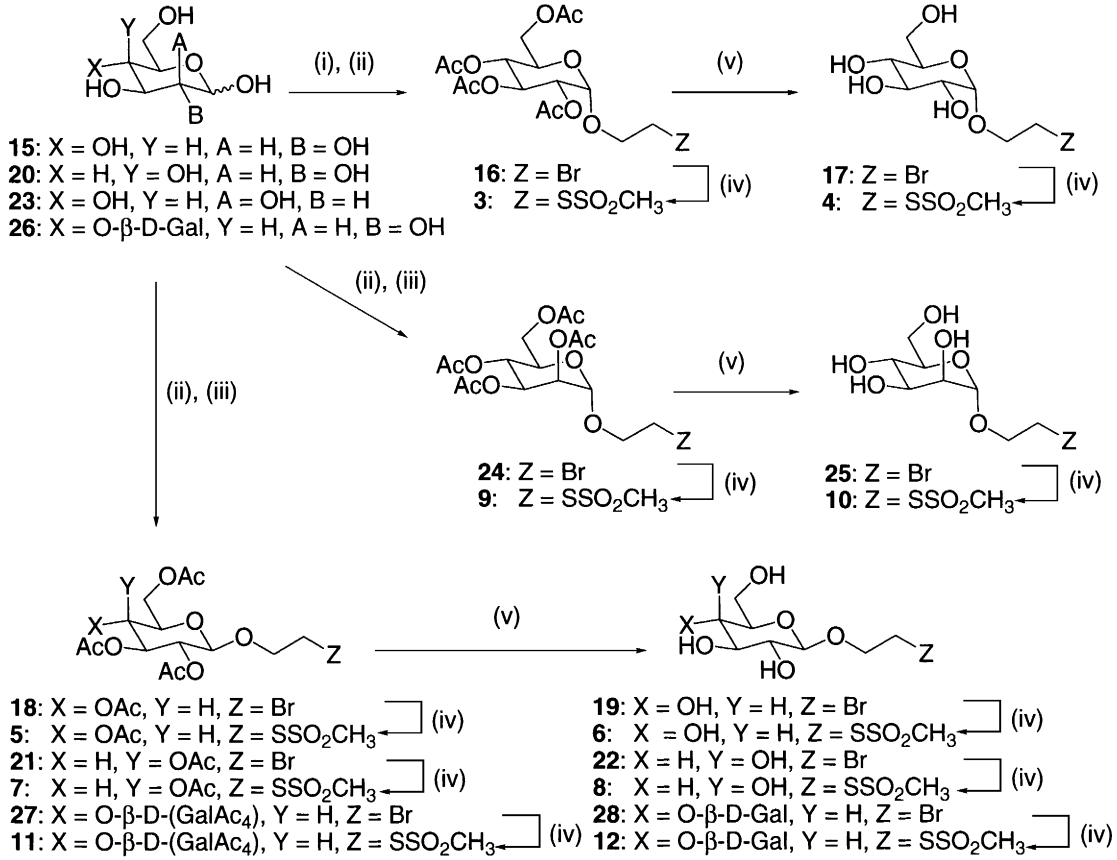
Scheme 2. (i) $NaSSO_2CH_3$, EtOH, 90°C for **1** or DMF, rt for **2**; (ii) acetonitrile, aqueous buffer (various pH; see Section 2.3)

*2.2. Preparation of ethyl tethered α-gluco-, β-gluco, β-galacto-, α-manno- and lacto-MTS **3–12***

For the preparation of the ethyl glyco-MTS **3**–**12**, we chose a strategy that allowed the introduction of the methanethiosulfonate group as a final step and which would therefore avoid the problems encountered in the deprotection of **1** and **2**. The preparation of these reagents in fully protected **3**,**5**,**7**,**9**,**11** and deprotected **4**,**6**,**8**,**10**,**12** forms allowed the effects of increased steric bulk and hydrophobicity to be assessed. Furthermore, the choice of a range of commonly occurring parent carbohydrate structures allowed us to explore the generality of the method. $BF_3 \cdot Et_2O$-catalyzed Fischer glycosidation of D-glucose **15** in 2-bromoethanol afforded a bromoethyl α-D-glucopyanoside, which for ease of purification was isolated as its tetraacetate **16**. Treatment of **16** with $NaSSO_2CH_3$ in DMF at 50°C allowed the preparation in 90% yield of the peracetylated ethyl α-D-gluco-MTS **3** (43% overall yield from **15**). Alternatively, Zemplén deprotection[22a] of bromide **16** using a catalytic amount of NaOMe gave the fully deprotected bromide **17**. Nucleophilic displacement of bromide by methanethiosulfonate ion proceeded smoothly at 50°C to yield the deprotected ethyl α-gluco-MTS **4** in 73% yield (33% overall yield from **15**).

The β-D-gluco-MTS reagents **5** and **6**, epimeric at C-1 to **3** and **4**, respectively, were prepared from the corresponding peracetylated β-bromide **18**.[23] The preparation of **18** took advantage of well-defined methodology utilizing Lewis acid-catalyzed displacement of anomeric acetate groups by alcohols.[24] As a consequence of anchimeric assistance by the neighbouring C-2 acetate group, the β-glucoside **18** is the major product of the treatment of a solution of pentaacetyl glucose and 2-bromoethanol with $BF_3 \cdot Et_2O$. Compound **18** was then elaborated to the corresponding peracetylated, **5**, and deprotected, **6**, ethyl β-gluco-MTS reagents in an essentially identical manner to that used for the epimeric α-gluco-MTS reagents. Thus, using $NaSSO_2CH_3$, **18** gave **5** in 80% yield (48% overall yield from **15**) and, following deprotection, **19**[25] afforded **6** in 74% yield (42% overall yield from **15**).

A parallel route starting from D-galactose **20** allowed the efficient preparation of the β-D-galacto-MTS **7** (55% overall yield from **20**) and **8** (43% overall yield from **20**), epimeric at C-4 to **5** and **6**, respectively, via bromide **21**[23] (Scheme 3). Similarly, ethyl α-D-manno-MTS **9** and **10**, epimeric at C-2 to **3** and **4**, respectively, were prepared using the same acetate displacement methodology**.** Thus, treatment of peracetyl D-mannose with 2-bromoethanol and $BF_3 \cdot$etherate again proceeded with anchimeric assistance by the C-2 acetate and yielded bromoethyl α-mannoside **24** in 64% yield. This key intermediate was elaborated to fully protected ethyl D-manno-MTS **9** (in 57% overall yield from D-mannose **23**) and after deprotection ethyl D-manno-MTS **10** (49% overall yield from **23**).



Scheme 3. (i) $Br(CH_2)_2OH$, $BF_3 \cdot Et_2O$; (ii) Ac_2O, py.; (iii) $Br(CH_2)_2OH$, $BF_3 \cdot Et_2O$, DCM; (iv) $NaSSO_2CH_3$, DMF, 50°C; (v) NaOMe, MeOH

The adaptability of this method to disaccharides was illustrated by the preparation of the peracetylated **11** and fully deprotected **12** disaccharide ethyl lacto-MTS, without cleavage of the interresidue glycosidic bond, in overall yields from lactose **27** of 47% and 32%, respectively.

2.3. Site-selective protein glycosylation

To illustrate the ability of glyco-MTS as protein glycosylation reagents we glycosylated the model protein SBL using β-D-gluco-MTS **1** and *N*-acetyl-β-D-glucosamine MTS **2**. This allowed us to explore the effects of two configurationally identical reagents bearing different substituents at C-2. Thus, **1** and **2** were reacted in aqueous buffer with four cysteine mutants prepared by site-directed mutagenesis:[26] SBL-

N62C, -S156C, -S166C and -L217C. These reactions were rapid (complete within 1 h) and quantitative, as judged by monitoring changes in specific activity and by titration of residual free thiols with Ellman's reagent.[27] The glycosylated proteins were purified by size-exclusion chromatography and dialysis, and their structures were confirmed by rigorous ES-MS analysis (m/z ± 5). The high level of purity indicated by ES-MS was confirmed by non-denaturing gradient polyacrylamide gel electrophoresis (PAGE) and fast protein liquid chromatography (FPLC). In all cases, complete integrity of the site selectivity was retained. The active enzyme concentration of the resulting glycosylated SBL solutions was determined by active site titration with α-toluenesulfonyl fluoride (PMSF) using a fluoride ion-sensitive electrode.[28]

The extent of deacetylation during modification is highly reagent-dependent (Table 1). Modification of L217C with reagent **1** at pH 9.5 was accompanied by complete in situ deacetylation, and the sole product was the fully deprotected glucosylated-SBL, L217C-S-β-Glc. Furthermore, through the variation of pH the level of deacetylation was controlled. Thus, the reaction of L217C with **1** at pH 7.5 and 5.5 yielded products in which the glucosyl residue introduced retained two and three acetate groups, forming L217C-S-β-Glc$(Ac)_2$ and L217C-S-β-Glc$(Ac)_3$, respectively. In contrast, treatment of cysteine mutant L217C with **2** under identical conditions resulted in no concomitant deacetylation during glycosylation and the formation of the fully protected glucosylated-SBL, L217C-S-β-GlcNAc$(Ac)_3$. We have previously attributed this valuable site-dependent deacetylation to a novel intramolecular SBL-catalyzed process.[14h] Due to the similarity in structure of **1** and **2** we may attribute their different reactivities solely to the presence of an NHAc at C-2 in **2** but an OAc group at C-2 in **1** and we speculate that deacylation of **2** is inhibited by the presence of a less labile acetamide rather than acetate group. Glycosylation at sites 62, 156 and 166 with **1** and **2** at pH 9.5 introduced only fully acetylated glucosyl residues.

Table 1
Kinetic parameters of glycosylated enzymes in amide hydrolysis[a]

Glycan Site	Pocket	Glyco MTS	pH	Glycosylation Product	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($s^{-1}mM^{-1}$)
SBL-WT	-	-	-	-	153 ± 4[b]	0.73 ± 0.05[b]	209 ± 15[b]
62	S_2	**1**	9.5	N62C-S-β-Glc$(Ac)_4$	67.9 ± 3.5[c]	0.52 ± 0.07[c]	130.6 ± 18.8[c]
		2	9.5	N62C-S-β-GlcNAc$(Ac)_3$	86.4 ± 0.9	0.62 ± 0.02	140.1 ± 4.4
217	S_1'	**1**	9.5	L217C-S-β-Glc	27.7 ± 0.4[c]	0.79 ± 0.03[c]	35.1 ± 1.4[c]
		1	7.5	L217C-S-β-Glc$(Ac)_2$	44.9 ± 2.0[c]	0.44 ± 0.06[c]	102.0 ± 14.6[c]
		1	5.5	L217C-S-β-Glc$(Ac)_3$	36.3 ± 0.8[c]	0.36 ± 0.03[c]	100.8 ± 8.7[c]
		2	9.5	L217C-S-β-GlcNAc$(Ac)_3$	61.3 ± 1.0	0.87 ± 0.04	70.2 ± 3.1
156	S_1	**1**	9.5	S156C-S-β-Glc$(Ac)_4$	54.8 ± 1.3[c]	0.70 ± 0.04[c]	78.3 ± 4.8[c]
		2	9.5	S156C-S-β-GlcNAc$(Ac)_3$	54.8 ± 1.1	0.66 ± 0.04	82.8 ± 4.9
166	S_1	**1**	9.5	S166C-S-β-Glc$(Ac)_4$	33.8 ± 1.3[c]	0.66 ± 0.06[c]	51.2 ± 5.0[c]
		2	9.5	S166C-S-β-GlcNAc$(Ac)_3$	18.2 ± 0.4	1.34 ± 0.07	13.5 ± 0.7

[a] Michaelis-Menten constants were measured at 25 °C according to the initial rates method in 0.1 M Tris-HCl buffer at pH 8.6, 0.005% Tween 80, 1% DMSO, suc-AAPF-*p*NA as the substrate.

[b] From Ref. 26.

[c] From Ref. 14i.

The effects of glycosylation upon the enzyme SBL were assessed by the determination of kinetic parameters k_{cat} and K_M for the hydrolysis of the amide substrate succinyl-AAPF-*p*-nitroanilide (Suc-AAPF-*p*NA) and the ester substrate succinyl-AAPF-S-benzyl (Suc-AAPF-SBn) at pH 8.6 (Tables 1 and 2, respectively).

In all cases and at all positions, glycosylation lowers SBL's amide hydrolysis activity. The two most dramatic reductions are caused by glycosylations in the S_1' and S_1 pockets. Introduction of

Table 2
Kinetic parameters of glycosylated enzymes in ester hydrolysis[b]

Glycan Site	Pocket	Glyco MTS	pH	Glycosylation Product	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($s^{-1}mM^{-1}$)
SBL-WT	-	-	-	-	1940 ± 180[b]	0.54 ± 0.07[b]	3560 ± 540[b]
62	S_2	**1**	9.5	N62C-S-β-Glc(Ac)$_4$	-	-	2502 ± 19[c]
		2	9.5	N62C-S-β-GlcNAc(Ac)$_3$	1051 ± 63	0.49 ± 0.09	2160 ± 420
217	S_1'	**1**	9.5	L217C-S-β-Glc	-	-	6350 ± 186[c]
		1	7.5	L217C-S-β-Glc(Ac)$_2$	-	-	8776 ± 86[c]
		1	5.5	L217C-S-β-Glc(Ac)$_3$	4428 ± 101[c]	0.15 ± 0.01[c]	29517±2070[c]
		2	9.5	L217C-S-β-GlcNAc(Ac)$_3$	2370 ± 118	0.71 ± 0.08	3318 ± 422
156	S_1	**1**	9.5	S156C-S-β-Glc(Ac)$_4$	-	-	2537 ± 50[c]
		2	9.5	S156C-S-β-GlcNAc(Ac)$_3$	546 ± 34.8[b]	0.52 ± 0.10	1054 ± 207
166	S_1	**1**	9.5	S166C-S-β-Glc(Ac)$_4$	-	-	1356 ± 17[c]
		2	9.5	S166C-S-β-GlcNAc(Ac)$_3$	896 ± 47.5[b]	1.08 ± 0.14[b]	828 ± 115[b]

[a] Kinetic constants determined by method of initial rates in 0.1 M Tris buffer, pH 8.6, 0.005% Tween 80, 1% DMSO with suc-AAPF-SBn as substrate. [S] = 30 μM to 2 mM, [E] = 9.6 x 10^{-11} to 1.1 x 10^{-10} M.

[b] From Ref. 29;

[c] From Ref. 14j.

fully deprotected untethered glucose to site 217 in the S_1' pocket, forming L217C-S-β-Glc, lowered k_{cat}/K_M to sixfold lower than unglycosylated SBL-WT. Interestingly, the results of glycosylation with both acetylated D-glucose or *N*-Ac-D-glucosamine to form L217C-S-β-Glc(Ac)$_2$, L217C-S-β-Glc(Ac)$_3$ or L217C-S-GlcNAc(Ac)$_3$ are far less marked, leading to reductions of no more than threefold in activity, and suggest that capping of the hydrophilic hydroxyl groups reduces this deleterious effect of glycosylation.

The introduction of peracetylated *N*-Ac-D-glucosamine to site 166 in the S_1 pocket to form S166C-S-β-GlcNAc(Ac)$_3$ reduces amide hydrolysis activity 15.5-fold relative to WT. This striking decrease is in contrast to the effect of introducing peracetylated D-glucose to form S166C-S-β-Glc(Ac)$_4$ which causes only a fourfold reduction in activity. Differences between these two glycosylated enzymes can be attributed to changing a single group at C-2 in the sugar introduced. The effect of changing a C-2 -OAc group in S166C-S-β-Glc(Ac)$_4$ to a -NHAc in S166C-S-β-GlcNAc(Ac)$_3$ halves catalytic turnover (k_{cat}) and binding interactions (as judged by K_M), perhaps due to unfavourable interactions of the N–H hydrogen bond donor found only in S166C-S-β-GlcNAc(Ac)$_3$ with the phenyl side-chain of the substrate suc-AAPF-*p*NA.

The two glyco-MTS reagents **1** and **2** had generally opposite effects upon the ester hydrolysis activity of SBL. Whereas **2** gave glycosylated enzymes with lower activities than the unglycosylated enzyme in all cases, excitingly **1** gave three enzymes with better than WT activities. These three enhanced enzymes all resulted from glycosylations at position 217, in the S_1' pocket. Glycosylation with fully deprotected D-glucose to form L217C-S-β-Glc increased k_{cat}/K_M by 1.8-fold. Furthermore, increasing the number of acetate groups present in the introduced glycosyl residue further improved this beneficial effect to 2.4-fold greater than WT for L217C-S-β-Glc(Ac)$_2$ and to a dramatic 8.4-fold greater than WT for L217C-S-β-Glc(Ac)$_3$.[14j] Interestingly, the corresponding effect of introducing a peracetylated *N*-Ac-D-glucosaminyl residue at the same site (217) is almost negligible. The striking differences in behaviour caused by glycosylation at site 217 between **1** and **2** were further investigated through molecular modelling.

2.4. Molecular modelling

The results of molecular mechanics analysis of the structures of L217C-S-β-Glc(Ac)$_3$ and L217C-S-β-GlcNAc(Ac)$_3$ are shown in Fig. 2. Similar overall minimum energy structures emerge as a result of the extensive solvation of the C-3, 4 and 6 substituents of the carbohydrate residues (bulk solvent omitted for clarity). This in turn forces the C-2 substituent (OAc or NHAc) towards the key nucleophilic serine of the catalytic triad of SBL. We have previously suggested[14j] that the carbonyl oxygen of the C-2 acetate of L217C-S-β-Glc(Ac)$_3$ might stabilize a crucial nucleophilic water molecule by acting as a hydrogen bond (1.89 Å) acceptor. This may create a channel of solvent to the active site of SBL, leading to enhanced esterase activity. This is supported by the minimum energy structure shown in Fig. 2, left.

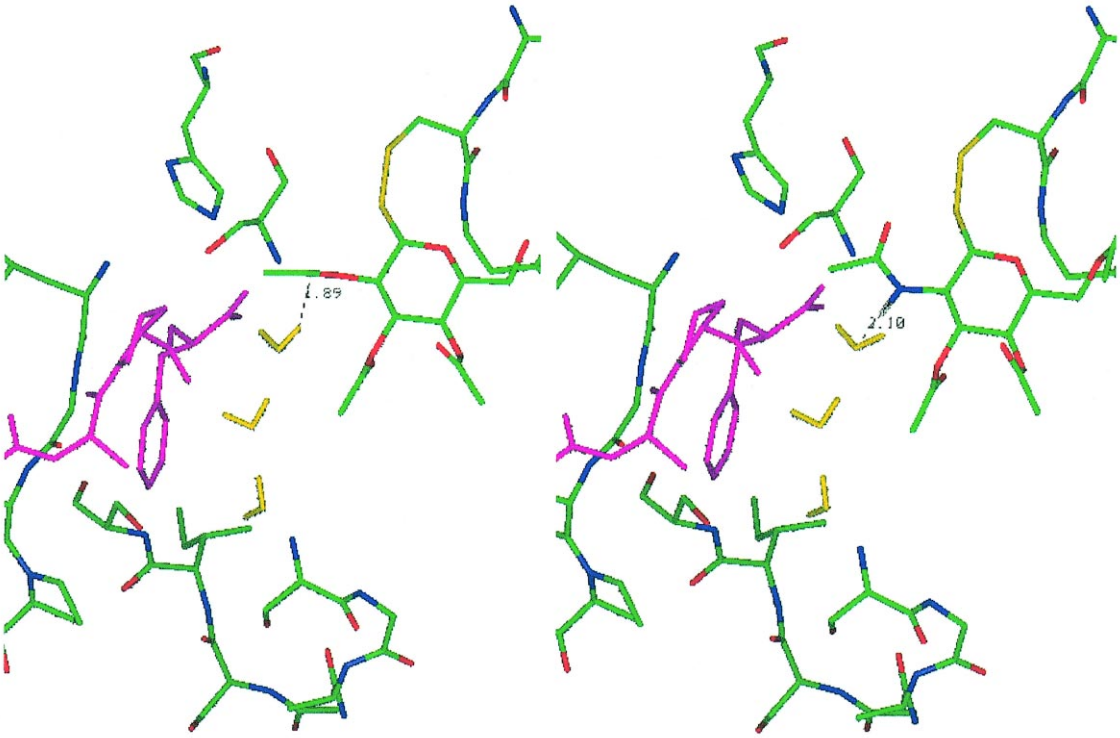


Fig. 2. Molecular mechanics analysis of L217C-S-β-Glc(Ac)$_3$ (left) and L217C-S-β-GlcNAc(Ac)$_3$ (right). The substrate mimic AAPF (purple) is shown binding in the S_1 subsite, whilst glycan occupies the S_1' subsite. These carbohydrate moieties allow a channel of water molecules (yellow) to enter the active site from bulk through hydrogen bonding interactions

In L217C-S-β-GlcNAc(Ac)$_3$ (Fig. 2, right) the mode of bonding and hence the role of the C-2 substituent (here NHAc) is reversed. The N–H of NHAc now acts as a hydrogen bond (2.10 Å) donor to the oxygen atom of an equivalent nucleophilic water. This serves to reduce the nucleophilicity by lowering electron density. There is consequently no enhancement of the esterase activity of L217C-S-β-GlcNAc(Ac)$_3$.

3. Conclusions

With the aim of creating precise structure–activity relationships (SARs) for protein glycosylation which may be of use in the preparation of, for example, novel therapeutics or enhanced catalysts,[30] we have developed a novel chemical glycosylation strategy that uses powerful glyco-MTS reagents. These reagents are readily synthesized and allow a high level of flexibility in the nature of SARs that can be surveyed. For example, experiments on heterogeneous glycoconjugate samples have demonstrated that the nature, and in particular the length, of the spacer arm that is used to conjugate carbohydrates to proteins influences both the efficiency of conjugation[31] and the strength of binding interactions of the resulting conjugate.[32] In order to probe the effect of distance between sugar and protein in a precise manner, we also intend to construct glyco-MTS in which the tether length may be systematically varied. The results of these further syntheses and full details of SARs created using reagents **1**–**12** will be published elsewhere.[14i,j]

4. Experimental

4.1. General experimental

Mutants of subtilisin *B. lentus* (SBL) were generated, and WT and mutant enzymes purified as described previously.[26] These mutants were glycosylated with **1** and **2** and purified as described previously.[14i] The preparation of $NaSSO_2CH_3$ has been described previously.[18] Acetobromo-D-glucose **17**[33] in 73% yield, pentaacetylglucose[34] in 99% yield and pentaacetylmannose[34] in 92% yield, pentaacetylgalactose[34] in 99% yield, octaacetyllactose[35] in 82% yield were prepared according to literature methods. DMF was distilled under N_2 from CaH_2 and stored over molecular sieve under N_2 before use. Dichloromethane, methanol and ethanol were distilled under N_2 from CaH_2, Mg/I_2 and Mg/I_2, respectively, immediately prior to use. $Br(CH_2)_2OH$ was stood over and distilled from CaO under reduced pressure and stored under N_2 prior to use. All other chemicals were used as received from Sigma-Aldrich, Fluorochem or Baker. Thin layer chromatography (TLC) was carried out on aluminium sheets coated with silica gel $60F_{254}$. Plates were developed using an ammonium molybdate dip. Flash chromatography was performed using silica gel (Whatman, 60 Å, 230–400 Mesh). Melting points were determined using an Electrothermal IA9000 series digital or Gallenkamp melting point apparatus and are uncorrected. Optical rotations were measured on an Optical Activity Ltd. AA-10 or Perkin–Elmer 241 polarimeter with a path length of 1 dm. Concentrations are given in g/100 ml. IR spectra were recorded on Bomem MB, Perkin–Elmer Spectrum 1000 or Perkin–Elmer Series 1600 Fourier Transform spectrophotometers. ^1H NMR and ^{13}C NMR spectra were recorded on Varian Gemini 200, Unity 300, Unity 400 or Unity 500 NMR spectrometers at the frequencies indicated. Where indicated, NMR peak assignments were made using COSY or DEPT experiments; all others are subjective. All chemical shifts were referenced to residual solvent as an internal standard; for ^{13}C NMR in D_2O 1,4-dioxan (67.6 ppm) was used. Protein ES-MS data were acquired using a PE SCIEX API III biomolecular mass spectrometer. Mass spectra were acquired using Micromass 70–250S, Micromass Autospec, Micromass ZAB-SE or VG Platform mass spectrometers according to the ionization methods indicated. High Resolution ESMS were performed by the EPSRC Mass Spectrometry Service at Swansea, UK. Microanalyses were performed by the microanalysis service at Durham University, Chemistry Department. Solvents were removed in vacuo.

4.2. 2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl methanethiosulfonate **1**

Acetobromoglucose **13** (1 g, 2.43 mmol) was added to a solution of $NaSSO_2CH_3$ (380 mg, 2.84 mmol) in ethanol (4 ml) at 90°C under N_2. After 20 min the resulting suspension was cooled and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 9:11) and the resulting solid recrystallized from ether to give **1** (674 mg, 63%) as a white solid; mp 151–152°C, melts then decomp. (ether); $[\alpha]_D^{27}=-19.0$ (*c* 1.24, $CHCl_3$); IR (KBr) 1749 (C=O), 1333, 1140 cm^{-1} (S–SO_2); ^1H NMR (400 MHz, $CDCl_3$) δ 2.00, 2.04, 2.06, 2.07 (s×4, 3H×4, Ac×4), 3.44 (s, 3H, CH_3SO_2-), 3.82 (ddd, $J_{4,5}$ 10.1 Hz, $J_{5,6}$ 5.9 Hz, $J_{5,6'}$ 2.2 Hz, 1H, H-5), 4.08 (dd, $J_{5,6}$ 5.9 Hz, $J_{6,6'}$ 12.5 Hz, 1H, H-6), 4.31 (dd, $J_{5,6'}$ 2.2 Hz, $J_{6,6'}$ 12.5 Hz, 1H, H-6′), 5.05 (t, *J* 9.8 Hz, 1H, H-4), 5.07 (dd, $J_{1,2}$ 10.5 Hz, $J_{2,3}$ 9.4 Hz, 1H, H-2), 5.25 (d, $J_{1,2}$ 10.5 Hz, 1H, H-1), 5.29 (t, *J* 9.3 Hz, 1H, H-3); ^{13}C NMR (50 MHz, $CDCl_3$) δ 20.5, 20.7 (*C*H_3COO-×4), 52.8 (CH_3SO_2-), 61.8, 68.0, 68.7, 73.3, 76.6 (C-2, C-3, C-4, C-5, C-6), 86.4 (C-1), 169.3, 169.3, 169.7, 170.1 (CH_3*C*OO-×4); HRMS m/z (EI+): found 443.0636 ($M+H^+$); $C_{15}H_{23}O_{11}S_2$ requires 443.0682.

4.3. 2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl methanethiosulfonate **2**

2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride 3,4,6-triacetate **14** (2 g, 5.722 mmol) was added to a solution of $NaSSO_2CH_3$ (900 mg, 6.72 mmol) in DMF (35 ml) under N_2 and stirred for 72 h. The reaction course was followed by TLC (EtOAc:hexane, 70:30) which showed the consumption of starting material (R_f 0.2) and the formation a major product (R_f 0.4). The solvent was removed and the residue purified by flash chromatography (EtOAc:hexane, 70:30) to give an off-white powder. The powder was recrystallized (EtOAc/hexane) to give **2** as a white crystalline powder (0.584 g, 25%); mp 139–140°C; $[\alpha]_D^{20}=-33.0$ (*c* 0.06, $CHCl_3$); IR (KBr) 3400 (br, N–H), 1748 (C=O), 1657 (amide I), 1542 (amide II), 1311, 1139 cm^{-1} (S–SO_2); ^1H NMR (300 MHz, CD_3CN) δ 1.84, 1.96, 1.99, 2.01 (s×4, 3H×4, Ac×4), 3.46 (s, 3H, SSO_2CH_3), 3.9–3.96 (ddd, $J_{5,6}$ 2.4 Hz, $J_{5,6'}$ 6.2 Hz, $J_{6,6'}$ 10.0 Hz, 1H, H-5), 4.05–4.15 (m, 2H), 4.20 (dd, $J_{5,6}$ 2.4 Hz, $J_{6,6'}$ 12.4, 1H, H-6), 5.00 (pt, *J* 9.8 Hz, 1H, H-2), 5.28 (pt, *J* 9.8 Hz, 1H, H-3), 5.40 (d, $J_{1,2}$ 10.8 Hz, 1H, H-1), 6.6 (d, *J* 10 Hz, 1H, N*H*Ac); ^{13}C NMR (50.3 MHz, CD_3CN) δ 20.1, 20.2 (*C*H_3COO-×3), 22.2 (*C*H_3CONH-), 51.8 (SSO_2CH_3), 52.2, 62.4, 68.6, 72.8, 76.2 (C-2,3,4,5,6), 87.62 (C-1), 169.8, 170.4, 170.4, 170.6 (CH_3*C*OO×3, CH_3*C*ONH); m/z (ES): 464 (14, $M+Na^+$), 352 (100%, oxazoline+Na^+). Found: C, 41.22; H, 5.43; N, 2.90%; $C_{15}H_{23}O_{10}NS_2$ requires: C, 40.81; H, 5.25; N, 3.17%.

4.4. 2-(2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl)ethyl methanethiosulfonate **3**

$BF_3\cdot Et_2O$ (145 μL, 1.1 mmol) was added dropwise to a suspension of D-glucose **15** (1.45 g, 8.1 mmol) in $Br(CH_2)_2OH$ (19 ml) under N_2 and the resulting mixture heated to 105°C. After 8 h the resulting solution was cooled and the solvent removed. The residue was dissolved in Ac_2O:pyridine (2:3 v/v, 16 ml) under N_2. After a further 24 h the reaction solvent was removed and the residue purified by repeated flash chromatography (EtOAc then EtOAc:hexane, 3:7) to give 2-bromoethyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside **16** (1.76 g, 48%) as a colourless oil that crystallized on standing to give a white solid; mp 86–88°C; $[\alpha]^{25}_D=+130.6$ (*c* 0.21, $CHCl_3$); IR (film) 1749 cm^{-1} (C=O); ^1H NMR (400 MHz, $CDCl_3$) δ 2.01, 2.03, 2.07, 2.09 (s×4, 3H×4, Ac×4), 3.51 (t, *J* 5.9 Hz, 2H, -CH_2Br), 3.83 (dt, J_d 11.6 Hz, J_t 5.8 Hz, 1H, -OC*H*H′-), 3.96 (dt, J_d 11.6 Hz, J_t 5.8 Hz, 1H, -OCH*H*′-), 4.10 (dd, $J_{5,6}$ 2.2 Hz, $J_{6,6'}$ 12.0 Hz, 1H, H-6), 4.14 (ddd, $J_{4,5}$ 10.2 Hz, $J_{5,6}$ 2.2 Hz, $J_{5,6'}$ 4.4 Hz, 1H, H-5), 4.24 (dd, $J_{5,6'}$ 4.4 Hz, $J_{6,6'}$ 12.0 Hz, 1H, H-6′), 4.84 (dd, $J_{1,2}$ 3.8 Hz, $J_{2,3}$ 10.3 Hz, 1H, H-2), 5.05 (t, *J* 9.7 Hz, 1H, H-4), 5.14 (d, $J_{1,2}$ 3.8

Hz, 1H, H-1), 5.49 (dd, $J_{2,3}$ 10.3 Hz, $J_{3,4}$ 9.5 Hz, 1H, H-3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.6, 20.7 (CH_3COO-×4), 29.9 (-CH_2Br), 61.9, 67.8, 68.5, 68.8, 70.0, 70.8 (-OCH_2-, C-2, C-3, C-4, C-5, C-6), 96.0 (C-1), 169.6, 170.0, 170.2, 170.6 (CH_3COO-×4); HRMS m/z (FAB+): found 477.0381 (M+Na^+); $C_{16}H_{23}O_{10}BrNa$ requires 477.0372. $NaSSO_2CH_3$ (75 mg, 0.56 mmol) was added to a solution of **16** (190 mg, 0.42 mmol) in DMF (6 ml) under N_2 and warmed to 50°C. After 21 h the solution was cooled and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:1) to give **3** (183 mg, 90%) as a colourless oil; $[\alpha]_D^{27}$=+92.1 (*c* 0.39, $CHCl_3$); IR (film) 1748 (C=O), 1322, 1134 cm^{-1} (S–SO_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.01, 2.03, 2.07, 2.09 (s×4, 3H×4, Ac×4), 3.41 (t, *J* 5.7 Hz, 2H, -CH_2S-), 3.41 (s, 3H, CH_3SO_2-), 3.75 (dt, J_d 10.8 Hz, J_t 5.7 Hz, 1H, -OC*H*H′-), 3.99–4.06 (m, 2H, H-5, -OCH*H*′-), 4.09 (dd, $J_{5,6}$ 2.4 Hz, $J_{6,6'}$ 12.6 Hz, 1H, H-6), 4.25 (dd, $J_{5,6'}$ 4.6 Hz, $J_{6,6'}$ 12.6 Hz, 1H, H-6′), 4.87 (dd, $J_{1,2}$ 3.9 Hz, $J_{2,3}$ 10.3 Hz, 1H, H-2), 5.06 (t, *J* 9.8 Hz, 1H, H-4), 5.12 (d, $J_{1,2}$ 3.9 Hz, 1H, H-1), 5.43 (t, *J* 9.8 Hz, 1H, H-3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.6, 20.6, 20.7, 20.7 (CH_3COO×4), 36.0, (-CH_2S-), 50.8 (CH_3SO_2-), 61.8 (-OCH_2-), 67.0, 67.8, 68.3, 69.8, 70.7 (C-2, C-3, C-4, C-5, C-6), 96.0 (C-1), 169.5, 170.0, 170.6 (CH_3COO-×4); HRMS m/z (FAB+): found 487.0946 (M+H^+); $C_{17}H_{27}O_{12}S_2$ requires 487.0944.

*4.5. 2-(α-D-Glucopyranosyl)ethyl methanethiosulfonate **4***

A solution of NaOMe (0.1 M, 0.3 ml) was added to a suspension of **16** (300 mg, 0.66 mmol) in MeOH (3 ml) under N_2 and stirred vigorously. After 6 h the resulting solution was passed through a Dowex 50W(H^+) plug (2×1 cm, eluant MeOH) and the solvent removed to give 2-bromoethyl α-D-glucopyranoside bromide[36] **17** (178 mg, 94%) as a white solid. $NaSSO_2CH_3$ (100 mg, 0.75 mmol) was added to a solution of **4b** (178 mg, 0.62 mmol) in DMF (7 ml) under N_2 and warmed to 50°C. After 25 h the solution was cooled and the solvent removed. The residue was purified by flash chromatography (MeOH:EtOAc, 1:9) to give **4** (144 mg, 73%) as a hygroscopic foam; $[\alpha]_D^{27}$=+109.9 (*c* 1.11, H_2O); IR (film) 3423 (OH), 1309, 1128 cm^{-1} (S–SO_2); ^{1}H NMR (500 MHz, D_2O, COSY) δ 3.16 (t, *J* 9.5 Hz, 1H, H-4), 3.28 (t, *J* 5.9 Hz, 2H, -CH_2S-), 3.30 (s, 3H, CH_3SO_2-), 3.31 (dd, $J_{1,2}$ 3.8 Hz, $J_{2,3}$ 9.9 Hz, 1H, H-2), 3.44 (t, *J* 9.5 Hz, 1H, H-3), 3.47–3.53 (m, 2H, H-6, H-6′), 3.58–3.61 (m, 1H, H-5), 3.62 (dt, J_t 5.4 Hz, J_d 10.8 Hz, 1H, -OC*H*H′-), 3.79 (dt, J_t 6.3 Hz, J_d 10.8 Hz, 1H, -OCH*H*′), 4.72 (d, $J_{1,2}$ 3.8 Hz, 1H, H-1); ^{13}C NMR (100 MHz, D_2O) δ 36.7 (-CH_2S-), 50.7 (CH_3SO_2-), 61.5 (-OCH_2-), 67.2, 70.5, 72.3, 73.2, 74.0 (C-2, C-3, C-4, C-5, C-6), 99.4 (C-1); HRMS m/z (FAB+): found 319.0517 (M+H^+); $C_9H_{19}O_8S_2$ requires 319.0521.

*4.6. 2-(2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl)ethyl methanethiosulfonate **5***

$BF_3 \cdot Et_2O$ (3.3 ml, 26.0 mmol) was added dropwise over the course of 15 min to a solution of 1,2,3,4,6-penta-*O*-acetyl-α,β-D-glucose (2 g, 5.1 mmol) and $Br(CH_2)_2OH$ (0.45 ml, 6.3 mmol) in CH_2Cl_2 (9 ml) at 0°C under N_2. After 1.5 h the solution was warmed to room temperature. After 20 h the reaction solution was added to ice water (15 ml) and extracted with CH_2Cl_2 (15 ml×3). These extracts were combined, washed with water (15 ml), satd $NaHCO_3$ (aq. 15 ml), water (15 ml), dried ($MgSO_4$), filtered and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:3) to give 2-bromoethyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside **18** (1.42 g, 61%) as a white solid; mp 118–120°C (EtOAc/*iso*-octane) [lit.,[23] mp 117.3°C (EtOH)]; $[\alpha]_D^{27}$=−11.9 (*c* 1.65, $CHCl_3$) [lit.,[37] $[\alpha]_D^{20}$=−12.3 (*c* 0.2, $CHCl_3$)]; ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.00, 2.02, 2.07, 2.09 (s×4, 3H×4, Ac×4), 3.42–3.51 (m, 2H), 3.67–3.87 (m, 2H), 4.10–4.31 (m, 3H), 4.57 (d, $J_{1,2}$ 8 Hz, 1H, H-1), 4.97–5.27 (m, 3H). $NaSSO_2CH_3$ (260 mg, 1.94 mmol) was added to a solution of **18** (640 mg, 1.41 mmol) in

DMF (18 ml) under N_2 and warmed to 50°C. After 25 h the solution was cooled and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:1) and the resulting solid recrystallized from EtOAc/hexane to give **5** (544 mg, 80%) as a white solid; mp 115–116°C (EtOAc/hexane); $[\alpha]_D^{27}$=+5.4 (*c* 1.06, $CHCl_3$); IR (KBr) 1758, 1741 (C=O), 1314, 1133 cm^{-1} (S–SO_2); ^{1}H NMR (500 MHz, $CDCl_3$, COSY) δ 1.99, 2.02, 2.06, 2.08 (s×4, 3H×4, Ac×4), 3.30–3.38 (m, 2H, -CH_2S-), 3.34 (s, 3H, CH_3SO_2-), 3.70 (ddd, $J_{4,5}$ 9.9 Hz, $J_{5,6}$ 2.2 Hz, $J_{5,6'}$ 4.6 Hz, 1H, H-5), 3.83 (ddd, *J* 5.6 Hz, *J* 7.4 Hz, *J* 10.5 Hz, 1H, -OC*H*H′-), 4.13–4.18 (m, 2H, H-6, -OCH*H*′-), 4.24 (dd, $J_{5,6'}$ 4.6 Hz, $J_{6,6'}$ 12.4 Hz, 1H, H-6′), 4.55 (d, $J_{1,2}$ 8.1 Hz, 1H, H-1), 4.98 (dd, $J_{1,2}$ 8.1 Hz, $J_{2,3}$ 9.7 Hz, 1H, H-2), 5.07 (t, *J* 9.9 Hz, 1H, H-4), 5.19 (t, *J* 9.6 Hz, 1H, H-3); ^{13}C NMR (125 MHz, $CDCl_3$, DEPT) δ 20.5, 20.7 (q×2, *C*H_3COO-×4), 36.0 (t, -CH_2S-), 50.6 (q, CH_3SO_2-), 61.6 (t, -OCH_2-), 68.1, 70.8, 71.9, 72.5 (d×4, C-2, C-3, C-4, C-5), 68.4 (t, C-6), 100.8 (d, C-1), 169.3, 170.0, 170.5 (s×3, CH_3*C*OO-×4); HRMS m/z (FAB+): found 487.0940 ($M+H^+$); $C_{17}H_{27}O_{12}S_2$ requires 487.0944.

*4.7. 2-(β-D-Glucopyranosyl)ethyl methanethiosulfonate **6***

A solution of NaOMe (0.1 M, 0.3 ml) was added to a suspension of **18** (300 mg, 0.66 mmol) in MeOH (3 ml) under N_2 and stirred vigorously. After 4 h the resulting solution was passed through a Dowex 50W(H^+) plug (2×1 cm, eluant MeOH) and the solvent removed to give 2-bromoethyl β-D-glucopyranoside **19** (176 mg, 93%) as a white solid that was used directly in the next step. A sample was recrystallized from EtOH/EtOAc to give a colourless, crystalline solid; mp 74–78°C (EtOH/EtOAc) [lit.,[37] mp 74–75°C (EtOH/EtOAc)]; $[\alpha]_D^{26}$=−22.4 (*c* 1.63, H_2O) [lit.,[37] $[\alpha]_D^{19}$=−26.1 (*c* 3.0, H_2O)]; ^{1}H NMR (400 MHz, CD_3OD) δ 3.30 (t, *J* 8.4 Hz, 1H, H-2), 3.39–3.49 (m, 3H), 3.64–3.80 (m, 3H), 3.97 (br d, $J_{6,6'}$ 11.7 Hz, 1H, H-6′), 4.02 (dt, J_t 6.5 Hz, J_d 11.3 Hz, 1H, -OC*H*H′-), 4.23 (dt, J_t 6.5 Hz, J_d 11.3 Hz, 1H, -OCH*H*′-), 4.44 (d, $J_{1,2}$ 7.9 Hz, 1H, H-1). $NaSSO_2CH_3$ (100 mg, 0.75 mmol) was added to a solution of **19** (176 mg, 0.61 mmol) in DMF (7 ml) under N_2 and warmed to 50°C. After 15 h the solution was cooled and the solvent removed. The residue was purified by flash chromatography (MeOH:EtOAc, 1:9) to give **6** (144 mg, 74%) as a hygroscopic foam; $[\alpha]_D^{27}$=−15.8 (*c* 0.88, H_2O); IR (KBr) 3400 (OH), 1310, 1131 cm^{-1} (S–SO_2); ^{1}H NMR (500 MHz, D_2O, COSY) δ 3.07 (dd, $J_{1,2}$ 8.1 Hz, $J_{2,3}$ 9.4 Hz, 1H, H-2), 3.16 (dd, $J_{3,4}$ 9.0 Hz, $J_{4,5}$ 9.8 Hz, 1H, H-4), 3.24 (ddd, $J_{4,5}$ 9.8 Hz, $J_{5,6}$ 6.0 Hz, $J_{5,6'}$ 2.3 Hz, 1H, H-5), 3.27 (t, *J* 9.0 Hz, 1H, H-3), 3.30–3.33 (m, 2H, -CH_2S-), 3.34 (s, 3H, CH_3SO_2-), 3.50 (dd, $J_{5,6}$ 6.0 Hz, $J_{6,6'}$ 12.4 Hz, 1H, H-6), 3.69 (dd, $J_{5,6'}$ 2.3 Hz, $J_{6,6'}$ 12.4 Hz, 1H, H-6′), 3.81 (dt, J_t 5.8 Hz, J_d 11.5 Hz, 1H, -OC*H*H′-), 4.00 (dt, J_t 5.7 Hz, J_d 11.4 Hz, 1H, -OCH*H*′-), 4.30 (d, $J_{1,2}$ 8.1 Hz, 1H, H-1); ^{13}C NMR (50 MHz, D_2O) δ 36.9 (-CH_2S-), 51.0 (CH_3SO_2-), 62.0 (-OCH_2-), 69.5, 70.9, 74.3, 76.7, 77.3 (C-2, C-3, C-4, C-5, C-6), 103.7 (C-1); HRMS m/z (FAB+): found 341.0351 ($M+Na^+$); $C_9H_{18}O_8S_2Na$ requires 341.0341.

*4.8. 2-(2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl)ethyl methanethiosulfonate **7***

$BF_3 \cdot Et_2O$ (8.5 ml, 67.0 mmol) was added dropwise to a solution of 1,2,3,4,6-penta-*O*-acetyl-α,β-D-galactose (5.1 g, 13.1 mmol) and $Br(CH_2)_2OH$ (1.15 ml, 16.2 mmol) in CH_2Cl_2 (24 ml) at 0°C under N_2. After 1 h the solution was warmed to room temperature. After 24 h the reaction solution was added to ice water (20 ml) and extracted with CH_2Cl_2 (30 ml×3). These extracts were combined, washed with water (20 ml), satd $NaHCO_3$ (aq., 20 ml), water (20 ml), dried ($MgSO_4$), filtered and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:3) to give 2-bromoethyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside **21** (4.01 g, 67%) as a white solid; mp 116–117°C (EtOAc/hexane) [lit.,[23] 111°C; lit.,[39] 114–116°C (EtOAc/light pet. ether)]; $[\alpha]_D^{27}$=−3.8 (*c* 0.81, $CHCl_3$) [lit.,[39] $[\alpha]_D^{23}$=−5 (*c*

1.4, $CDCl_3$)]; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.98, 2.05, 2.08, 2.15 (s×4, 3H×4, Ac×4), 3.43–3.50 (m, 2H), 3.75–3.95 (m, 2H), 4.12–4.24 (m, 3H), 4.53 (d, $J_{1,2}$ 8 Hz, 1H, H-1), 5.02 (dd, $J_{2,3}$ 11 Hz, $J_{3,4}$ 3 Hz, 1H, H-3), 5.23 (dd, $J_{1,2}$ 8 Hz, $J_{2,3}$ 11 Hz, 1H, H-2), 5.40 (br d, $J_{3,4}$ 3 Hz, 1H, H-4). $NaSSO_2CH_3$ (85 mg, 0.63 mmol) was added to a solution of **21** (223 mg, 0.49 mmol) in DMF (6 ml) under N_2 and warmed to 55°C. After 30 h the solution was cooled and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:1) to give **7** (198 mg, 83%) as a white foam; $[\alpha]_D^{27}$=+9.1 (*c* 1.41, $CHCl_3$); IR (film) 1747 (C=O), 1320, 1133 cm^{-1} (S–SO_2); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.98, 2.05, 2.09, 2.15 (s×4, 3H×4, Ac×4), 3.35 (s, 3H, CH_3SO_2-), 3.35–3.38 (m, 2H, -CH_2S-), 3.84 (ddd, *J* 6.1 Hz, *J* 7.1 Hz, *J* 10.5 Hz, 1H, -OC*H*H′-), 3.92 (td, $J_{4,5}$ 1.1 Hz, J_t 6.6 Hz, 1H, H-5), 4.10–4.21 (m, 3H, H-6, H-6′, -OCH*H*′-), 4.52 (d, $J_{1,2}$ 8.0 Hz, 1H, H-1), 5.01 (dd, $J_{2,3}$ 10.3 Hz, $J_{3,4}$ 3.5 Hz, 1H, H-3), 5.20 (dd, $J_{1,2}$ 8.0 Hz, $J_{2,3}$ 10.3 Hz, 1H, H-2), 5.40 (dd, $J_{3,4}$ 3.5 Hz, $J_{4,5}$ 1.1 Hz, 1H, H-4); ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.6, 20.7, 20.8 (CH_3COO-×4), 36.1, (-CH_2S-), 50.6 (CH_3SO_2-), 61.2 (-OCH_2-), 67.0, 68.3, 68.5, 70.8, 71.0 (C-2, C-3, C-4, C-5, C-6), 101.3 (C-1), 169.5, 170.0, 170.1, 170.4 (CH_3COO-×4); HRMS m/z (FAB+): found 487.0936 (M+H^+); $C_{17}H_{27}O_{12}S_2$ requires 487.0944.

*4.9. 2-(β-D-Galactopyranosyl)ethyl methanethiosulfonate **8***

A solution of NaOMe (0.104 M, 0.8 ml) was added to a solution of **21** (778 mg, 1.71 mmol) in MeOH (10 ml) under N_2. After 4 h the reaction solution was passed through a Dowex 50W(H+) plug (3×1 cm, eluant MeOH) and the solvent removed to give 2-bromoethyl β-D-galactopyranoside[38] **22** (450 mg, 92%) as a white solid which was used directly in the next step. $NaSSO_2CH_3$ (180 mg, 1.34 mmol) was added to a solution of **22** (290 mg, 1.01 mmol) in DMF (12 ml) under N_2 and warmed to 50°C. After 15 h the solution was cooled and the solvent removed. The residue was purified by flash chromatography (MeOH:EtOAc, 1:9) to give **8** (229 mg, 71%) as a white foam; $[\alpha]_D^{27}$=+2.9 (*c* 0.58, H_2O); IR (film) 3358 cm^{-1} (br, *O*–H), 1306, 1120 cm^{-1} (S–SO_2); ^{1}H NMR (500 MHz, D_2O, COSY) δ 3.29–3.33 (m, 2H, -CH_2S-), 3.30 (dd, $J_{1,2}$ 7.7 Hz, $J_{2,3}$ 10.0 Hz, 1H, H-2), 3.35 (s, 3H, CH_3SO_2-), 3.43 (dd, $J_{2,3}$ 10.0 Hz, $J_{3,4}$ 3.6 Hz, 1H, H-3), 3.48 (ddd, $J_{4,5}$ 0.9 Hz, $J_{5,6}$ 4.3 Hz, $J_{5,6'}$ 7.9 Hz, 1H, H-5), 3.52 (dd, $J_{5,6}$ 4.3 Hz, $J_{6,6'}$ 11.7 Hz, 1H, H-6), 3.57 (dd, $J_{5,6'}$ 7.9 Hz, $J_{6,6'}$ 11.7 Hz, 1H, H-6′), 3.70 (dd, $J_{3,4}$ 3.6 Hz, $J_{4,5}$ 0.9 Hz, 1H, H-4), 3.80 (dt, J_d 11.2 Hz, J_t 6.1 Hz, 1H, -OC*H*H′-), 4.01 (dt, J_d 11.4 Hz, J_t 5.8 Hz, 1H, -OCH*H*′-), 4.24 (d, $J_{1,2}$ 7.7 Hz, 1H, H-1); ^{13}C NMR (100 MHz, D_2O) δ 36.7 (-CH_2S-), 50.8 (CH_3SO_2-), 61.9 (-OCH_2-), 69.2, 69.6, 71.7, 73.7, 76.2 (C-2, C-3, C-4, C-5, C-6), 104.0 (C-1); HRMS m/z (FAB+): found 319.0523 (M+H^+); $C_9H_{19}O_8S_2$ requires 319.0521.

*4.10. 2-(2,3,4,6-Tetra-O-acetyl-α-D-mannopyranosyl)ethyl methanethiosulfonate **9***

$BF_3 \cdot Et_2O$ (7.7 ml, 60.7 mmol) was added dropwise over the course of 15 min to a solution of 1,2,3,4,6-penta-*O*-acetyl-α,β-D-mannose (4.7 g, 12.1 mmol) and $Br(CH_2)_2OH$ (1.05 ml, 14.8 mmol) in CH_2Cl_2 (22 ml) at 0°C under N_2. After 1 h the solution was warmed to room temperature. After 25 h the reaction solution was added to ice water (20 ml) and extracted with CH_2Cl_2 (20 ml×2). These extracts were combined, washed with water (20 ml), satd $NaHCO_3$ (aq. 20 ml), water (20 ml), dried ($MgSO_4$), filtered and the solvent removed. The residue was crystallized from EtOAc/*iso*-octane to give 2-bromoethyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside **24** (3.52 g, 64%). Purification of the resulting mother liquor by flash chromatography (EtOAc:hexane, 1:3) gave further **24** (320 mg, 6%; 70% in total) as a white highly crystalline solid; mp 121–123°C [lit.,[39] 118–119°C (EtOAc/*iso*-octane)]; $[\alpha]_D^{28}$=+48.3 (*c* 1.31, $CHCl_3$) [lit.,[39] $[\alpha]_D^{23}$=+45 (*c* 0.6, $CDCl_3$)]; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.99, 2.05, 2.10, 2.16 (s×4, 3H×4, Ac×4), 3.52 (t, *J* 6 Hz, 2H, -CH_2Br), 3.82–4.04 (m, 2H, -OCH_2-), 4.09–4.16 (m, 1H, H-5),

4.13 (dd, $J_{5,6}$ 2 Hz, $J_{6,6'}$ 12 Hz, 1H, H-6) 4.28 (dd, $J_{5,6'}$ 6 Hz, $J_{6,6'}$ 12 Hz, 1H, H-6′), 4.87 (br s, 1H, H-1), 5.22–5.40 (m, 3H, H-2, H-3, H-4). $NaSSO_2CH_3$ (230 mg, 1.72 mmol) was added to a solution of **24** (600 mg, 1.32 mmol) in DMF (17 ml) under N_2 and warmed to 55°C. After 20 h the solution was cooled and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 9:11) and the resulting solid recrystallized from Et_2O/hexane to give **9** (566 mg, 88%) as a white solid; mp 128–129°C (Et_2O/hexane); $[\alpha]_D^{27}$=+53.2 (*c* 0.92, $CHCl_3$); IR (KBr) 1739 (C=O), 1325, 1129 cm^{-1} (S–SO_2); ^1H NMR (500 MHz, $CDCl_3$, COSY) δ 1.97, 2.04, 2.09, 2.14 (s×4, 3H×4, Ac×4), 3.37–3.40 (m, 2H, -CH_2S-), 3.38 (s, 3H, CH_3SO_2-), 3.79 (dt, J_d 10.5 Hz, J_t 5.8 Hz, 1H, -OC*H*H′-), 3.98–4.03 (m, 2H, -OCH*H′*-, H-5), 4.09 (dd, $J_{5,6}$ 2.5 Hz, $J_{6,6'}$ 12.5 Hz, 1H, H-6), 4.26 (dd, $J_{5,6'}$ 5.6 Hz, $J_{6,6'}$ 12.5 Hz, 1H, H-6′), 4.85 (d, $J_{1,2}$ 0.7 Hz, 1H, H-1), 5.23–5.29 (m, 3H, H-2, H-3, H-4); ^{13}C NMR (50 MHz, $CDCl_3$) δ 20.6, 20.7, 20.8 (*C*H_3COO-×4), 35.7, (-CH_2S-), 50.8 (CH_3SO_2-), 62.5 (-OCH_2-), 66.0, 66.8, 69.0, 69.2, 69.3 (C-2, C-3, C-4, C-5, C-6), 97.7 (C-1), 169.7, 169.9, 170.0, 170.6 (CH_3*C*OO-×4); HRMS m/z (FAB+): found 487.0954 ($M+H^+$); $C_{17}H_{27}O_{12}S_2$ requires 487.0944.

*4.11. 2-(α-D-Mannopyranosyl)ethyl methanethiosulfonate **10***

A solution of NaOMe (0.143 M, 0.7 ml) was added to a suspension of **24** (1 g, 2.2 mmol) in MeOH (10 ml) under N_2. After 3 h the resulting solution was passed through a Dowex 50W(H^+) plug (2×1 cm, eluant MeOH) and the solvent removed. The residue was purified by flash chromatography (MeOH:EtOAc, 2:25) to give 2-bromoethyl α-D-mannopyranoside[40] **25** (606 mg, 96%) as a white foam; $[\alpha]_D^{26}$=+50.7 (*c* 0.91, H_2O); IR (KBr) 3417 cm^{-1} (OH); ^1H NMR (500 MHz, D_2O, COSY) δ 3.38–3.44 (m, 3H, H-4, -CH_2Br), 3.50–3.55 (m, 2H, H-5, H-6), 3.60 (dd, $J_{2,3}$ 3.5 Hz, $J_{3,4}$ 9.7 Hz, 1H, H-3), 3.66 (dd, $J_{5,6'}$ 4.6 Hz, $J_{6,6'}$ 11.2 Hz, 1H, H-6′), 3.68 (ddd, *J* 4.6 Hz, *J* 5.4 Hz, *J* 11.7 Hz, 1H, -OC*H*H′-), 3.76 (dd, $J_{1,2}$ 1.8 Hz, $J_{2,3}$ 3.5 Hz, 1H, H-2), 3.81 (ddd, *J* 5.1 Hz, *J* 6.5 Hz, *J* 11.7, 1H, -OCH*H′*-), 4.71 (d, $J_{1,2}$ 1.8 Hz, 1H, H-1); ^{13}C NMR (100 MHz, D_2O) δ 32.1 (-CH_2Br), 61.7 (-OCH_2-), 67.5, 68.4, 70.7, 71.3, 73.8 (C-2, C-3, C-4, C-5, C-6), 100.5 (C-1); HRMS m/z (FAB+): found 308.9985 ($M+Na^+$); $C_8H_{15}O_6{}^{79}BrNa$ requires 308.9950. $NaSSO_2CH_3$ (150 mg, 1.12 mmol) was added to a solution of **25** (245 mg, 0.85 mmol) in DMF (10 ml) under N_2 and warmed to 50°C. After 16 h the solution was cooled and the solvent removed. The residue was purified by flash chromatography (MeOH:EtOAc, 1:9) to give **10** (217 mg, 80%) as a hygroscopic foam; $[\alpha]_D^{29}$=+58.0 (*c* 1.34, H_2O); IR (KBr) 3441 (OH), 1314, 1132 cm^{-1} (S–SO_2); ^1H NMR (500 MHz, D_2O) δ 3.31 (t, *J* 5.8 Hz, 2H, -CH_2S-), 3.35 (s, 3H, CH_3SO_2-), 3.45 (t, *J* 9.6 Hz, 1H, H-4), 3.49 (ddd, $J_{4,5}$ 9.8 Hz, $J_{5,6}$ 5.8 Hz, $J_{5,6'}$ 1.9 Hz, 1H, H-5), 3.55 (dd, $J_{5,6}$ 5.8 Hz, $J_{6,6'}$ 12.1 Hz, 1H, H-6), 3.60 (dd, $J_{2,3}$ 3.4 Hz, $J_{3,4}$ 9.0 Hz, 1H, H-3), 3.66 (dt, J_d 10.7 Hz, J_t 5.7 Hz, 1H, -OC*H*H′-), 3.69 (dd, $J_{5,6'}$ 1.9, $J_{6,6'}$ 12.1 Hz, 1H, H-6′), 3.77 (dd, $J_{1,2}$ 1.6 Hz, $J_{2,3}$ 3.4 Hz, 1H, H-2), 3.83 (dt, J_d 11.0 Hz, J_t 5.9 Hz, 1H, -OCH*H′*-), 4.72 (d, $J_{1,2}$ 1.6 Hz, 1H, H-1); ^{13}C NMR (125 MHz, D_2O) δ 36.7 (-CH_2S-), 50.7 (CH_3SO_2-), 61.9 (-OCH_2-), 66.7, 67.7, 70.9, 71.5, 74.0 (C-2, C-3, C-4, C-5, C-6), 100.8 (C-1); HRMS m/z (FAB+): found 319.0528 ($M+H^+$); $C_9H_{19}O_8S_2$ requires 319.0521.

*4.12. 2-(2,3,6-Tri-O-acetyl-4-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-β-D-glucopyranosyl)-ethyl methanethiosulfonate **11***

$BF_3 \cdot Et_2O$ (4.0 ml, 31.5 mmol) was added dropwise to a solution of 1,2,3,6-tetra-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-glucopyranoside (5 g, 7.4 mmol) and $Br(CH_2)_2OH$ (0.65 ml, 9.2 mmol) in CH_2Cl_2 (15 ml) at 0°C under N_2. After 1 h the solution was warmed to room temperature. After 20 h the reaction solution was added to ice water (15 ml) and extracted with CH_2Cl_2 (20 ml×2). These extracts were combined, washed with water (20 ml), satd $NaHCO_3$ (aq.

20 ml), water (20 ml), dried ($MgSO_4$), filtered and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:1) to give 2-bromoethyl 2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-glucopyranoside **27** (2.94 g, 53%) as a white foam; $[\alpha]_D^{27}=-7.8$ (*c* 1.28, $CHCl_3$) [lit.,[39] $[\alpha]_D^{23}=-11$ (*c* 1.3, $CHCl_3$)]; ^{1}H NMR (500 MHz, $CDCl_3$, COSY) δ 1.94, 2.02, 2.02 (s×3, 3H×3, Ac×3), 2.04 (s, 6H, Ac×2), 2.10, 2.13 (s×2, 3H×2, Ac×2), 3.38–3.46 (m, 2H, -CH_2Br), 3.59 (ddd, $J_{5',6'''}$ 2.2 Hz, *J* 4.9 Hz, *J* 9.9 Hz, 1H, H-5′), 3.75–3.80 (m, 2H, H-4′, -OC*H*H′-), 3.85 (td, $J_{4,5}$ 1.1 Hz, J_t 6.9 Hz, 1H, H-5), 4.03–4.12 (m, 4H, H-6, H-6′, H-6′′, -OCH*H*′-), 4.45 (d, $J_{1,2}$ 7.8 Hz, 1H, H-1), 4.48 (dd, $J_{5',6'''}$ 2.2 Hz, $J_{6'',6'''}$ 12.1 Hz, 1H, H-6′′′), 4.50 (d, $J_{1',2'}$ 7.9 Hz, 1H, H-1′), 4.89 (dd, $J_{1',2'}$ 7.9 Hz, $J_{2',3'}$ 9.6 Hz, 1H, H-2′), 4.92 (dd, $J_{2,3}$ 10.5 Hz, $J_{3,4}$ 3.4 Hz, 1H, H-3), 5.08 (dd, $J_{1,2}$ 7.8 Hz, $J_{2,3}$ 10.5 Hz, 1H, H-2), 5.18 (t, *J* 9.6 Hz, 1H, H-3′), 5.32 (dd, $J_{3,4}$ 3.4 Hz, $J_{4,5}$ 1.1 Hz, 1H, H-4). $NaSSO_2CH_3$ (87 mg, 0.65 mmol) was added to a solution of **27** (357 mg, 0.48 mmol) in DMF (6 ml) under N_2 and warmed to 50°C. After 22 h the solution was cooled and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 11:9) to give **11** (327 mg, 88%) as a white foam; $[\alpha]_D^{27}=-3.7$ (*c* 1.0, $CHCl_3$); IR (KBr) 1751 (C=O), 1323, 1134 cm^{-1} (S–SO_2); ^{1}H NMR (500 MHz, $CDCl_3$, COSY) δ 1.94, 2.02, 2.02, 2.04, 2.04, 2.11, 2.13 (s×7, 3H×7, Ac×7), 3.29–3.40 (m, 2H, -CH_2S-), 3.32 (s, 3H, CH_3SO_2-), 3.59 (ddd, $J_{4',5'}$ 9.9 Hz, $J_{5',6''}$ 4.9 Hz, $J_{5',6'''}$ 2.2 Hz, 1H, H-5′), 3.77 (t, *J* 9.5 Hz, 1H, H-4′), 3.79–3.86 (m, 2H, H-5, -OC*H*H′-), 4.03–4.13 (m, 4H, H-6, H-6′, H-6′′, -OCH*H*′-), 4.46 (d, $J_{1,2}$ 7.8 Hz, 1H, H-1), 4.50 (d, $J_{1',2'}$ 8.0 Hz, 1H, H-1′), 4.52 (dd, $J_{5',6'''}$ 2.2 Hz, $J_{6'',6'''}$ 11.9 Hz, 1H, H-6′′′), 4.87 (dd, $J_{1',2'}$ 8.0 Hz, $J_{2',3'}$ 9.6 Hz, 1H, H-2′), 4.93 (dd, $J_{2,3}$ 10.5 Hz, $J_{3,4}$ 3.5 Hz, 1H, H-3), 5.08 (dd, $J_{1,2}$ 7.8 Hz, $J_{2,3}$ 10.5 Hz, 1H, H-2), 5.17 (t, *J* 9.3Hz, 1H, H-3′), 5.32 (dd, $J_{3,4}$ 3.5 Hz, $J_{4,5}$ 1.0 Hz, 1H, H-4); ^{13}C NMR (125 MHz, $CDCl_3$, DEPT) δ 20.5, 20.7, 20.8, 20.9 (q×4, *C*H_3COO-×7), 36.0 (t, -CH_2S-), 50.6 (q, CH_3SO_2-), 60.7, 61.6, 68.5 (t×3, -OCH_2-, C-6, C-6′), 66.5, 69.0, 70.6, 70.9, 71.3, 72.6, 72.8, 76.0 (d×8, C-2, C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′), 100.7, 101.1 (d×2, C-1, C-1′), 169.1, 169.7, 169.7, 170.1, 170.2, 170.3, 170.4 (s×7, CH_3*C*OO-×7); HRMS m/z (FAB+): found 775.1793 ($M+H^+$); $C_{29}H_{43}O_{20}S_2$ requires 775.1789.

4.13. 2-(4-O-β-D-Galactopyranosyl-β-D-glucopyranosyl)ethyl methanethiosulfonate ***12***

A solution of NaOMe (0.1 M, 0.6 ml) was added to a solution of **27** (877 mg, 1.18 mmol) in MeOH (6 ml) under N_2. After 3 h the reaction solution was passed through a Dowex 50W(H^+) plug (4×1 cm, eluant MeOH) and the solvent removed to give 2-bromoethyl 4-*O*-β-D-galactopyranosyl-β-D-glucopyranoside **28** (476 mg, 90%) as a white foam which was used directly in the next step. $NaSSO_2CH_3$ (185 mg, 1.38 mmol) was added to a solution of **28** (476 mg, 1.06 mmol) in DMF (24 ml) under N_2 and warmed to 50°C. After 21 h the solution was cooled and the solvent removed. The residue was purified by flash chromatography ($CHCl_3$:MeOH:AcOH:H_2O, 60:30:3:5) to give **12** (346 mg, 68%) as a hygroscopic foam; $[\alpha]_D^{28}=+1.5$ (*c* 1.66, H_2O); IR (KBr) 3416 (br, OH), 1311, 1131 cm^{-1} (S–SO_2); ^{1}H NMR (400 MHz, D_2O, COSY) δ 3.10–3.13 (m, 1H, H-2), 3.30 (t, *J* 6.0 Hz, 2H -CH_2S-), 3.31 (dd, $J_{1',2'}$ 7.8 Hz, $J_{2',3'}$ 10.3 Hz, 1H, H-2′), 3.34 (s, 3H, CH_3SO_2-), 3.38–3.54 (m, 5H), 3.44 (dd, $J_{2',3'}$ 10.3 Hz, $J_{3',4'}$ 3.3 Hz, 1H, H-3′), 3.57 (dd, *J* 8.4 Hz, *J* 11.4 Hz, 1H), 3.59 (dd, *J* 4.9 Hz, *J* 7.3 Hz, 1H), 3.70 (br d, $J_{3',4'}$ 3.3 Hz, 1H, H-4′), 3.77 (dd, *J* 1.0 Hz, *J* 11.5 Hz, 1H), 3.79–3.83 (m, 1H, -OC*H*H′-), 3.97–4.02 (m, 1H, -OCH*H*′-), 4.22 (d, $J_{1',2'}$ 7.8 Hz, 1H, H-1′), 4.33 (d, $J_{1,2}$ 7.8 Hz, 1H, H-1); ^{13}C NMR (125 MHz, D_2O) δ 36.7 (-CH_2S-), 50.8 (CH_3SO_2-), 61.0, 62.1, 69.4, 69.6, 71.9, 73.5, 73.7, 75.3, 75.9, 76.4, 79.3 (-OCH_2-, C-2, C-3, C-4, C-5, C-6, C-2′, C-3′, C-4′, C-5′, C-6′), 103.3, 103.9 (C-1, C-1′); HRMS m/z (FAB+): found 503.0886 ($M+Na^+$); $C_{15}H_{28}O_{13}S_2Na$ requires 503.0869.

4.14. Protein glycosylation

Cysteine mutants of SBL were glycosylated according to previously published methods[14i] in the appropriate buffer: 70 mM CHES, 2 mM $CaCl_2$ (pH 9.5); 70 mM HEPES, 2 mM $CaCl_2$ (pH 7.5); or 70 mM MES, 2 mM $CaCl_2$ (pH 5.5) before quenching in 5 mM MES, 1 mM $CaCl_2$ (pH 6.5 or 5.5). The free thiol content of all CMMs was determined spectrophotometrically by titration with Ellman's reagent[27] in phosphate buffer (0.25 M, pH 8.0). In all cases no free thiol was detected. Modified enzymes were analyzed by nondenaturing gradient (8–25%) gels at pH 4.2, run towards the cathode, on the Pharmacia Phast-system and appeared as a single band. Prior to ES-MS glycosylated enzymes were analyzed by FPLC (BioRad, Biologic System) on a Source 15 RPC matrix (17-0727-20 from Pharmacia) with 5% acetonitrile, 0.01% TFA as the running buffer and eluted with 80% acetonitrile, 0.01% TFA in a one step gradient. MS m/z (ES-MS): N62C-S-β-GlcNAc$(Ac)_3$ calculated 27048, found 27050; L217C-S-β-GlcNAc$(Ac)_3$ calculated 27049, found 27045; S156C-S-β-GlcNAc$(Ac)_3$ calculated 27075, found 27080; S166C-S-β-GlcNAc$(Ac)_3$ calculated 27075, found 27077.

4.15. Enzyme kinetics

Kinetic constants were determined in duplicate by the method of initial rates in 0.1 M Tris buffer, pH 8.6, 0.005% Tween 80, 1% DMSO. [S]=0.125 mM to 3 mM, [E]=1.5×10^{-8} M to 9.0×10^{-8} M for amidase kinetics. [S]=0.015 mM to 3 mM, [E]=3.5×10^{-10} M to 2.3×10^{-9} M for esterase kinetics. The kinetic parameters of esterase activity were determined indirectly following the release of thiobenzyl alcohol from the substrate succinyl-Ala-Ala-Pro-Phe-SBn (suc-AAPF-SBn) with Ellman's reagent.[27]

4.16. Molecular modelling

The X-ray structure of subtilisin *B. lentus* with the peptide inhibitor AAPF bound (Brookhaven database entry 1JEA) was used as the starting point for calculations on wild type and CMMs. The enzyme set-up was performed with Insight II, version 2.3.0.[41] To create initial coordinates for the minimization, hydrogens were added at the pH (8.6) used for kinetic measurements. This protonated all Lys and Arg residues and the N-terminus and deprotonated all Glu and Asp residues and the C-terminal carboxyl group. The protonated form of His 64 was used in all calculations. The model system was solvated with a 5 Å layer of water molecules. The total number of water molecules in the system was 1143. The overall charge of the enzyme–inhibitor complex resulting from this set-up was +4 for the WT enzyme. Energy simulations were performed with the Discover program, version 2.9.5 on a Silicon Graphics Indigo computer, using the consistent valence force field (CVFF) function. A non-bonded cut-off distance of 18 Å with a switching distance of 2 Å was employed. The non-bonded pair list was updated every 20 cycles and a dielectric constant of 1 was used in all calculations. The WT enzyme was minimized in stages, with initially only the water molecules being allowed to move, followed by water molecules and the amino acid side chains, and then finally the entire enzyme. The mutated and chemically modified enzymes were generated by modifying the relevant amino acid using the Builder module of Insight. These structures were then minimized in a similar manner. Initially the side-chain of the mutated residue and the water molecules were minimized. Then all side-chains and the water molecules were minimized while the backbones of the residues were constrained, then all of the atoms were minimized. The AAPF inhibitor was free to move throughout all stages of the minimization. Each stage of energy minimization was conducted by means of the method of steepest descents without Morse or cross terms until the derivative of energy with respect to structural perturbation was less than 5.0 kcal/Å; then the method of

conjugate gradients, without Morse or cross terms until the derivative of energy with respect to structural perturbation was less than 1.0 kcal/Å; and finally the method of conjugate gradients with Morse and cross terms until the final derivative of energy with respect to structural perturbation was less than 0.1 kcal/Å.

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References

1. Helenius, A. *Mol. Biol. Cell* **1994**, *5*, 253–265.
2. Opdenakker, G.; Rudd, P. M.; Ponting, C. P.; Dwek, R. A. *FASEB J.* **1993**, *7*, 1330–1337.
3. Rudd, P. M.; Joao, H. C.; Coghill, E.; Fiten, P.; Saunders, M. R.; Opdenakker, G.; Dwek, R. A. *Biochemistry* **1994**, *33*, 17–22.
4. Sharon, N.; Lis, H. *Essays in Biochem.* **1995**, *30*, 59–75.
5. (a) Lasky, L. A. *Annu. Rev. Biochem.* **1995**, *64*, 113–139. (b) Weis, W. I.; Drickamer, K. *Annu. Rev. Biochem.* **1996**, *65*, 441–473.
6. (a) Varki, A. *Glycobiology* **1993**, *3*, 97–130. (b) Dwek, R. A. *Chem. Rev.* **1996**, *96*, 683–720.
7. Simanek, E. E.; Huang, D.-H.; Pasternack, L.; Machajewski, T. D.; Seitz, O.; Millar, D. S.; Dyson, H. J.; Wong, C.-H. *J. Am. Chem. Soc.* **1998**, *120*, 11567–11575.
8. Rademacher, T. W.; Parekh, R. B.; Dwek, R. A. *Annu. Rev. Biochem.* **1988**, *57*, 785–838.
9. For reviews, see: (a) Stowell, C. P.; Lee, Y. C. *Adv. Carbohydr. Chem. Biochem.* **1980**, *37*, 225–281. (b) *Neoglycoconjugates: Preparation and Applications*; Lee, Y. C.; Lee, R. T., Eds.; Academic: London, 1994. (c) Abelson, J. N.; Simon., M. I., Eds. *Methods Enzymol.* **1994**, *242*. (d) Lee, Y. C.; Lee, R. T., Eds. *ibid.* **1994**, *247*. (e) Bill, R. M.; Flitsch, S. F. *Chem. Biol.* **1996**, *3*, 145–149. (f) Marcaurelle, L. A.; Bertozzi, C. R. *Chem. Eur. J.* **1999**, *5*, 1384–1390. (g) Davis, B. G. *J. Chem. Soc., Perkin Trans. 1* **1999**, 3215–3237.
10. (a) Davis, N. J.; Flitsch, S. L. *Tetrahedron Lett.* **1991**, *32*, 6793–6796. (b) Wong, S. Y. C.; Guile, G. R.; Dwek, R. A.; Arsequell, G. *Biochem. J.* **1994**, *300*, 843–850. (c) Macindoe, W. M.; van Oijen, A. H.; Boons, G.-J. *J. Chem. Soc., Chem. Commun.* **1998**, 847–848.
11. Wynn, R.; Richards, F. M. *Methods Enzymol.* **1995**, *201*, 351–356.
12. Standard one-letter amino acid codes are used in this paper, e.g. S=Ser=Serine and L=Leu=Leucine.
13. *Molecular Glycobiology*; Fukuda, M.; Hindsgaul, O., Eds.; Oxford University Press: Oxford, 1994.
14. (a) Maekawa, K.; Liener, I. E. *Arch. Biochem. Biophys.* **1960**, *91*, 108. (b) Longo, M. A.; Combes, D. *FEBS Lett.* **1995**, *375*, 63–66. (c) Longo, M. A.; Combes, D. *J. Mol. Catal. B* **1997**, *2*, 281–289. (d) Hill, T. G.; Wang, P.; Huston, M. E.; Wartchow, C. A.; Oehler, L. M.; Smith, M. B.; Bednarski, M. D.; Callstrom, M. R. *Tetrahedron Lett.* **1991**, *32*, 6823–6826. (e) Wang, P.; Hill, T. G.; Wartchow, C. A.; Huston, M. E.; Oehler, L. M.; Smith, M. B.; Bednarski, M. D.; Callstrom, M. R. *J. Am. Chem. Soc.* **1992**, *114*, 378–380. (f) Wartchow, C. A.; Wang, P.; Bednarski, M. D.; Callstrom, M. R. *J. Org. Chem.* **1992**, *60*, 2216–2226. (g) Wang, P.; Hill, T. G.; Bednarski, M. D.; Callstrom, M. R. *Tetrahedron Lett.* **1991**, *32*, 6827–6830. (h) Davis, B. G.; Lloyd, R. C.; Jones, J. B. *J. Org. Chem.* **1998**, *63*, 9614–9615. (i) Davis, B. G.; Lloyd, R. C.; Jones, J. B., submitted for publication, **1999**. (j) Lloyd, R. C.; Davis, B. G.; Jones, J. B., submitted for publication, **1999**. (k) Baek, W. O.; Vijayalakshmi, M. A. *Biochim. Biophys. Acta* **1997**, *1336*, 394–402.
15. Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157–162.
16. *Molecular Glycobiology*; Fukuda, M.; Hindsgaul, O., Eds.; Oxford University Press: Oxford, 1994.
17. Berglund, P.; DeSantis, G., Stabile, M. R.; Shang, X.; Gold, M.; Bott, R. R.; Graycar, T. P.; Lau, T. H.; Mitchinson, C.; Jones, J. B. *J. Am. Chem. Soc.* **1997**, *119*, 5265–5266.
18. Kenyon, G. L.; Bruice, T. W. *Methods Enzymol.* **1977**, *47*, 407–430.
19. (a) Douglass, I. B.; Farah, B. S. *J. Org. Chem.* **1959**, *24*, 973–975. (b) Levitt, L. S.; Levitt, B. W. *J. Org. Chem.* **1972**, *37*, 332–334. (c) Weidner, J. P.; Block, S. S. *J. Med. Chem.* **1972**, *15*, 564–567. (d) Palumbo, G.; Caputo, R. *Synthesis* **1981**,

888–890. (e) Block, E.; Zhao, S.-H. *J. Org. Chem.* **1992**, *57*, 5815–5817. (f) Billard, T.; Langlois, B. R.; Large, S.; Anker, D.; Roidot, N.; Roure, P. *J. Org. Chem.* **1996**, *61*, 7545–7550.
20. (a) Fischer, E.; Delbrück, K. *Chem. Ber.* **1909**, *42*, 1476–1482. (b) Schneider, W.; Gille, R.; Eisfeld, K. *Chem. Ber.* **1928**, *61*, 1244–1259. (c) Horton, D. *Methods Carbohydr. Chem.* **1963**, *2*, 433–437. (d) Cerny, M.; Stanek, J.; Pacak, J. *Monatsch. Chem.* **1963**, *94*, 290–294. (e) Apparu, M.; Blanc-Muesser, M.; Defaye, J.; Driguéz, H. *Can. J. Chem.* **1981**, *59*, 314–320.
21. Initial approaches to untethered glyco-MTS reagents similar in type to **1**,**2** were based upon Danishefsky's glycal methodology: Halcomb, R. L.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1989**, *111*, 6661–6666. Tris-TBS glucal was prepared according to the method of Lesimple, P.; Beau, J.-M.; Jaurand, G.; Sinäy, P. *Tetrahedron Lett*. **1986**, *27*, 6201–6204, oxidized to tris-TBS protected 1,2-anhydroglucose using dimethyldioxirane and then reacted with a solution of butylammonium methanethiosulfonate in dichloromethane that had been prepared by ion pair exchange extraction. However, under a variety of conditions and in contrast to the behaviour of other sulfur nucleophiles [see: (a) Gordon, D. M.; Danishefsky, S. J. *Carbohydr. Res.* **1990**, *206*, 361–366. (b) Berkowitz, D. B.; Danishefsky, S. J.; Schultz, G. K. *J. Am. Chem. Soc.* **1992**, *114*, 4518–4529], methanethiosulfonate ion failed to open the epoxide moiety of tris-TBS protected 1,2-anhydro-D-glucose.
22. (a) Zemplén, G.; Kunz, A. *Ber. Dtsch. Chem. Ges.* **1923**, *56*, 1705–1710. (b) Plattner, J. J.; Gless, R. D.; Rappoport, H. *J. Am. Chem. Soc.* **1972**, *94*, 8613–8615. (c) Mori, K.; Sasaki, M. *Tetrahedron Lett.* **1979**, *20*, 1329–1332. (d) Herzig, J.; Nudelman, A. *Carbohydr. Res.* **1986**, *153*, 162–167. (e) Herzig, J.; Nudelman, A.; Gottlieb, H. E.; Fischer, B. *J. Org. Chem.* **1986**, *51*, 727–730. (f) Vekemans, J. A. J. M.; Franken, G. A. M.; Chittenden, G. J. F.; Godefroi, E. F. *Tetrahedron Lett.* **1987**, *28*, 2299–2300. (g) Cinget, F.; Schmidt, R. R. *Synlett* **1993**, 168–170.
23. Coles, H. W.; Dodds, M. L.; Bergeim, F. H. *J. Am. Chem. Soc.* **1938**, *60*, 1020–1022.
24. Dahmén, J.; Frejd, T.; Grönberg, G.; Lave, T.; Magnusson, G.; Noori, G. *Carbohydr. Res.* **1983**, *116*, 303–307.
25. Helferich, B.; Lutzmann, H. *Justus Liebigs Ann. Chem.* **1939**, *541*, 1–16.
26. Stabile, M. R.; Lai, W. G.; DeSantis, G.; Gold, M.; Jones, J. B.; Mitchinson, C.; Bott, R. R.; Graycar, T. P.; Liu, C.-C. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2501–2512.
27. Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherstone, R. M. *Biochem. Pharmacol.* **1961**, *7*, 88–95.
28. Hsia, C. Y.; Ganshaw, G.; Paech, C.; Murray, C. J. *Anal. Biochem.* **1996**, *242*, 221–227.
29. Plettner, E.; DeSantis, G.; Stabile, M. R.; Jones, J. B. *J. Am. Chem. Soc.* **1999**, *121*, 4977–4981.
30. Davis, B. G.; Jones, J. B. *Synlett* **1999**, 1495–1507.
31. Ragupathi, G.; Koganty, R. R.; Qiu, D. X.; Lloyd, K. O.; Livingston, P. O. *Glycoconjugate J.* **1998**, *15*, 217–221.
32. (a) Leteux, C.; Stoll, M. S.; Childs, R. A.; Chai, W.; Vorozhaikina, M.; Feizi, T. *J. Immunol. Methods* **1999**, *227*, 109–119. (b) Leteux, C.; Childs, R. A.; Chai, W. G.; Stoll, M. S.; Kogelberg, H.; Feizi, T. *Glycobiology* **1998**, *8*, 227–236. (c) Kichler, A.; Schuber, F. *Glycoconjugate J.* **1995**, *12*, 275–281. (d) Kretzschmar, G.; Sprengard, U.; Kunz, H.; Bartnik, E.; Schmidt, W. *Tetrahedron* **1995**, *51*, 13015–13030.
33. Prepared from D-glucose according to Scheurer, P. G.; Smith, F. *J. Am. Chem. Soc.* **1954**, *76*, 3224.
34. Prepared from the corresponding parent carbohydrates according to the method of Verley, A.; Bölsing, F. *Ber. Dtsch. Chem. Ges.* **1901**, *34*, 3354–3358, and purified by flash chromatography.
35. Prepared from lactose according to the method of Hudson, C. S.; Johnson, J. M. *J. Am. Chem. Soc.* **1915**, *37*, 1270–1275, and purified by flash chromatography.
36. The synthesis of **4b** as an intermediate has been described previously. In our hands this method gave only a poor yield of product. Nagai, K.; Honda, A.; Kiho, T.; Ukai, S.; Tsuchiya, T. *Carbohydr. Res.* **1989**, *190*, 165–180.
37. Helferich, B.; Lutzmann, H. *Justus Liebigs Ann. Chem.* **1939**, *541*, 1–16.
38. The synthesis of unstable **4e** has been described previously. Dahmén, J.; Frejd, T.; Magnusson, G.; Noori, G.; Carlstroem, A. S. *Carbohydr. Res.* **1984**, *125*, 237–245.
39. Dahmén, J.; Frejd, T.; Grönberg, G.; Lave, T.; Magnusson, G.; Noori, G. *Carbohydr. Res.* **1983**, *116*, 303–307.
40. The use of **4d** as a reactant has been described previously, although no details of preparation or characterization were given. Nilsson, K. G. I. U.S. Patent 4 918 009, 1986.
41. Biosym Technologies, Inc. San Diego, CA, USA.