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Mild tagging procedures for the structural analysis of glycans

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Abstract

The reductive oxyamination of model glycan structures has been investigated as a mild, alternative tagging procedure to reductive amination using *O*-(4-nitrobenzyl)-hydroxylamine. Oxime formation was quantitative, but the reduction step did not always go to completion. Novel *O*- and *N*-substituted 7-hydroxycoumaryl- and 3-methoxybenzylhydroxylamines were synthesized and shown to couple quantitatively with model saccharides by oxime formation and reductive hydroxyamination, respectively, under very mild, aqueous conditions. The fluorescent derivatives produced show good chromatographic and mass spectrometric properties. Both procedures are suitable for the labeling of carbohydrates and oligosaccharide fragments from glycosaminoglycan structures, such as heparin and heparan sulfate.

Keywords: Glycosaminoglycan (GAG); Oxime formation; Reductive hydroxyamination; 3-Methoxy-benzylhydroxylamine derivatives; 7-Hydroxycoumarylhydroxylamine derivatives; HPLC; Electrospray-ionization mass spectrometry (ESIMS)

1. Introduction

Carbohydrates possess considerable structural diversity, and as such, the structural elucidation of complex carbohydrates presents a challenging problem to the analytical chemist. The investigation of unusual structural sequences involved in biologically active binding–recognition systems is often limited to picomole quantities of material. In order to elucidate the structure of these glycan sequences, it is essential to be able to separate the glycan from any other components present efficiently and to be able to detect the structure with high sensitivity. The hydrophilic nature of carbohydrates, coupled with the fact that they do not possess a suitable chromophore, has meant that they are not readily amenable to separation techniques such as reversed-phase HPLC. Although refractive index and pulsed amperometric detection may be used for detection of native carbohydrates, they are not sufficiently sensitive to detect the low concentrations of glycans found in many biological systems.

Abbreviations: 2,5-An-Man6SO_3, 6-sulfo-2,5-anhydro-D-mannose; 2,5-An-Man, 2,5-anhydro-D-mannose; ESIMS, electrospray-ionization mass spectrometry; GAG, glycosaminoglycan; Glc, D-glucose; GlcNAc, *N*-acetyl-D-glucosamine; GlcNSO_3, 2-sulfamido-2-deoxy-α-D-glucopyranose; HPLC, high-performance liquid chromatography; HS, heparan sulfate; NBHA, *O*-(4-nitrobenzyl)hydroxylamine.

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The glycosaminoglycans (GAGs), heparin and heparan sulfate (HS), are known to play crucial roles in many biological processes.[1–5] HS is a complex sulfated polysaccharide, consisting of chains of up to 400 modified sugar residues. It is present in most multicellular animals and has an ubiquitous distribution, being expressed on the cell surface and in extracellular matrices of most tissues. HS exists as a proteoglycan, and despite considerable progress in sequencing and cloning the core polypeptides of the molecule, only limited structural information has been derived from the structurally diverse carbohydrate portion. Although HS is initially biosynthesized as a simple repeat of a glucuronic acid–*N*-acetylglucosamine disaccharide unit, subsequent modifications such as N-deacetylation, N-resulfation, C-5 epimerization of D-glucuronic acid residues to L-iduronic acid, and various O-sulfations of each of the residues results in an extremely diverse structure. HS molecules generally contain short stretches (4–16 residues) of highly sulfated monosaccharides joined by relatively long stretches of non-sulfated units. Heparin, which is present only in mast cell granules, represents an extreme form of HS where sulfation and epimerization are at their most extensive.

In view of its inherent sensitivity and the potential for the formation of structurally informative fragment ions, mass spectrometry has been investigated as a means of characterization of GAG fragments.[6–13] Because of the presence of multiple sulfate groups, the mass spectrometric analysis of GAG fragments has traditionally been carried out in the negative-ion mode. When mass analyzed as free acids or as ammonium salts, extensive gas-phase desulfation occurs.[11,13] This lowers the overall sensitivity and tends to suppress the formation of structurally informative fragment ions. Although sodium salts are much more stable, the formation of clustered sodiated ions complicates the assignment of the molecular mass.[11,13]

Heparin fragments have been analyzed in the positive-ion mode as complexes with an arginine-rich peptide or protein using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry.[8] The use of a peptide/GAG fragment complex was found to increase sensitivity, obviate the interference from inorganic cations, and reduce desulfation of lower molecular weight oligosaccharides. However, the degree of desulfation was found to increase with increasing numbers of sulfate groups present. Measurement of the molecular weight may be used to establish the number of monosaccharide subunits, sulfate groups, and *N*-acetyl groups present, but it does not provide further structural information such as the location of sulfate groups.

The methods commonly employed for separation of GAG fractions from either nitrous acid or enzymatic cleavage are gel-permeation chromatography (GPC), strong anion-exchange (SAX) chromatography,[14] ion-pairing reversed-phase high-performance liquid chromatography (IP-RP-HPLC),[15] polyacrylamide gel electrophoresis (PAGE)[16–18] and capillary electrophoresis (CE).[19–21] The majority of these techniques requires buffers and/or salt solutions for optimum separation. The anionic nature of GAGs bestows a high affinity for Na^+, K^+, Ca^{2+} and other cationic species, which can complicate the isolation of pure, salt-free compounds and render the samples unstable for long-term storage. These counter-ions also pose a major problem for analysis of GAG oligosaccharides by mass spectrometry as they can adduct to the ionizing molecule in varying proportions, producing multiple molecular ions at different m/z values. The desalting and replacement of these cations with a single counter-ion usually requires dialysis for the larger fragments or exchange with a salt, such as NH_4HCO_3, which is volatile enough for the excess to be removed under vacuum.

A number of procedures have been devised to tag glycans selectively at the reducing terminus with a group that will enhance the sensitivity of detection, many of which also facilitate chromatographic separations. These have been the subject of a recent review by Hase.[22] Reductive amination is the most commonly used method for the introduction of a tag at the reducing terminus of the carbohydrate. It typically requires heating the sample at elevated temperatures and low pH for ex-

tended periods with a large excess of an arylamine reagent in the presence of the reducing agent. Such harsh reaction conditions can lead to the degradation of heat-sensitive carbohydrates and desulfation of GAG fragments. The procedure also suffers from considerable variations in yield, because of the competing direct reduction of the carbohydrate itself, which occurs at the same pH. One of the favored reagents for reductive amination of sugars, 2-aminopyridine, has found particular application in the quantitative analysis by HPLC of sialylated N-linked oligosaccharides from glycoproteins,[23,24] utilizing an improved two-step procedure that overcomes the competition between derivatization and reduction.[25] This still requires high temperatures for both Schiff's base formation (90 °C for 60 min) and reduction (80 °C for 50 min) and was considered unsuitable for sulfated sugars.

Investigations were therefore initiated into developing a procedure for selective derivatization at the reducing terminus under mild conditions that would be suitable, not only for GAG fragments, but also for a range of different sugars. Properties that were required of the tag were that it should (1) quantitatively and selectively couple to the reducing terminus of the carbohydrate to form a stable derivative under mild reaction conditions; (2) be fluorescent in aqueous media; (3) be hydrophobic enough to facilitate reversed-phase HPLC separations; (4) have a low molecular weight so that the chromatographic property of the derivatized oligosaccharide is primarily determined by the structure of the carbohydrate moiety and not that of the tag; and (5) introduce a site that enhances protonation efficiency and increases mass spectrometric sensitivity in the positive-ion mode.

A method that was expected to meet these requirements was reductive oxyamination with *O*-substituted hydroxylamine derivatives. Commercially available *O*-(4-nitrobenzyl)hydroxylamine (NBHA)[26,27] was used to optimize the reaction conditions. Fluorescent *O*-substituted methoxybenzyl- and 7-hydroxycoumaryl derivatives of hydroxylamine were synthesized, and their reaction with model glycans was investigated. Subsequently, the corresponding *N*-substituted hydroxylamines were also prepared and shown to form *N*,*N*-disubstituted hydroxylamines with sugars under extremely mild conditions.

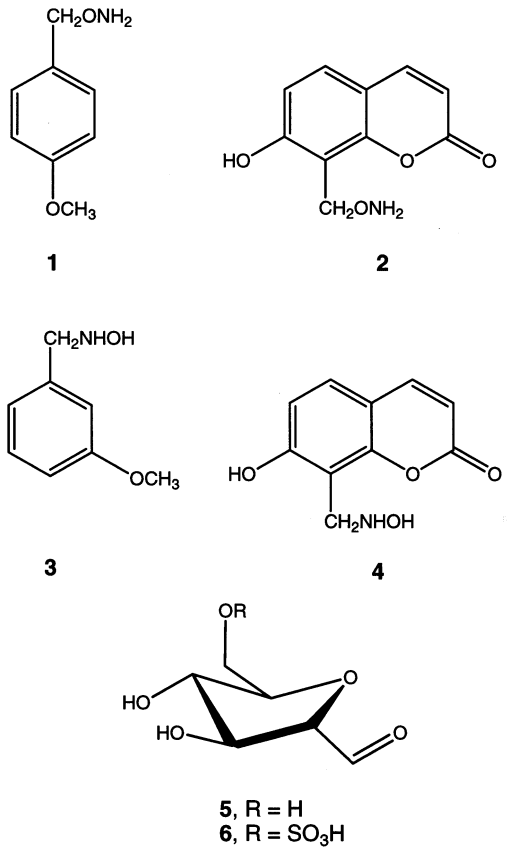
2. Results and discussion

Several *N*- and *O*-substituted hydroxylamine derivatives (**1**–**4**) were synthesized, and their spectroscopic properties together with that of NBHA are shown in Table 1. Reaction conditions for the coupling of these tags to oligosaccharides were optimized using model monosaccharides. D-Glucose (Glc) and *N*-acetyl-D-glucosamine (GlcNAc) were used as model hexoses. Nitrous acid degradation of heparin and heparan sulfate generates products with 2,5-anhydro-D-mannose (**5**, 2,5-An-Man) and 2,5-anhydro-6-*O*-sulfo-D-mannose (**6**, 2,5-An-Man6SO_3) residues at the reducing terminus. These monosaccharides were therefore used as model compounds for the labeling of heparin and heparan sulfate fragments arising from nitrous acid degradation.

The established conditions for oxime formation require heating the reducing oligosaccharide in pyridine with an *O*-substituted hydroxylamine,[26,27] or mixing an aldehyde in water and sodium hydroxide with addition of hydroxylamine.[28] However, alkaline conditions have been shown to cause C-2 epimerization of *N*-sulfo glucosamines[29] and could also lead to alkaline 'peeling', that is the stepwise hydrolysis of residues from the reducing terminus.[30] As pyridine can be difficult to re-

Table 1
Spectroscopic properties of *N*- and *O*-substituted hydroxylamine tags

Compound	UV max (nm)	Fluorescence (nm)
O-(4-Nitrobenzyl)-hydroxylamine (NBHA)	284	
O-(4-Methoxybenzyl)-hydroxylamine (**1**)	207, 275	307
8-Aminooxymethyl-7-hydroxy-coumarin (**2**)	335	460
N-(3-Methoxybenzyl)-hydroxylamine (**3**)	207, 275	307
7-Hydroxy-8-hydroxyamino-methylcoumarin (**4**)	335	460



move, neither of these conditions for oxime formation was suitable for GAG fragment labeling. Instead, we explored the use of aqueous conditions at mildly acidic pH values.

NBHA was introduced by Darvill and co-workers as a reducing terminus tag as it offered improved chromatographic separation for neutral and acidic oligosaccharides, whilst also providing a suitable UV chromophore.[26,27] In order to avoid the use of pyridine and the rather laborious derivatization procedure used by Darvill and co-workers, we investigated the reaction of NBHA with Glc and GlcNAc, using water alone as the solvent. The formation of the oxime at pH values from pH 3 to 6 was monitored by reversed-phase HPLC using a diode array detector. The reaction produced the oxime product in quantitative yields at an optimum value of pH 4, which was used for all subsequent oxime preparations. HPLC analysis did, however, indicate formation of multiple products (Fig. 1(a)). The fact that HPLC separation had effectively desalted the derivatives allowed the separated derivatives to be collected and subjected to electrospray-ionization mass spectrometric (ESIMS) analysis. The mass spectrum of each of the products indicated that they all had the same molecular

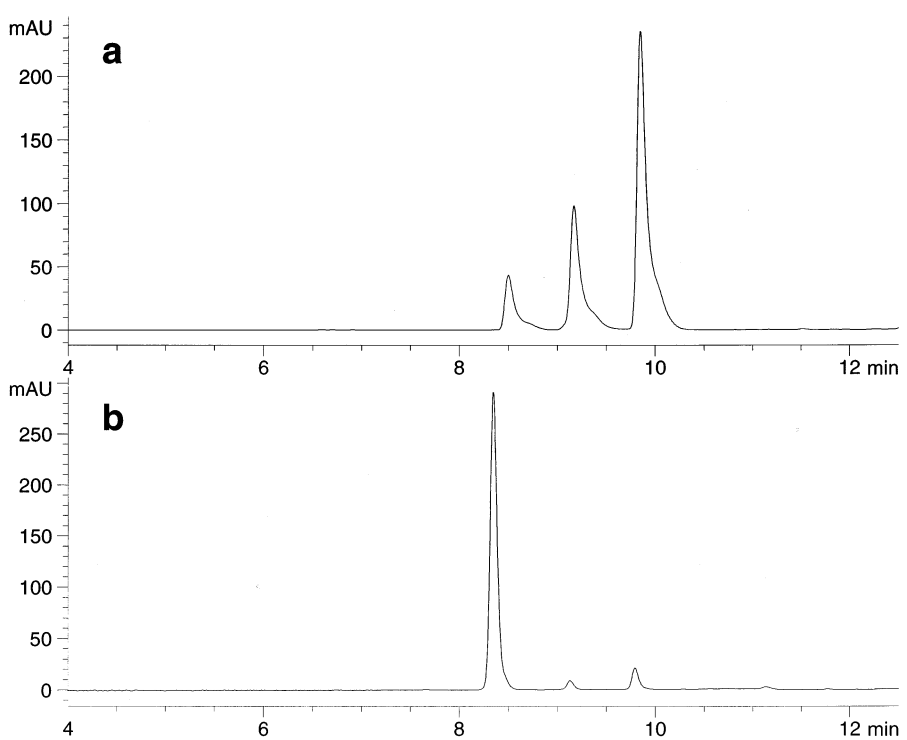
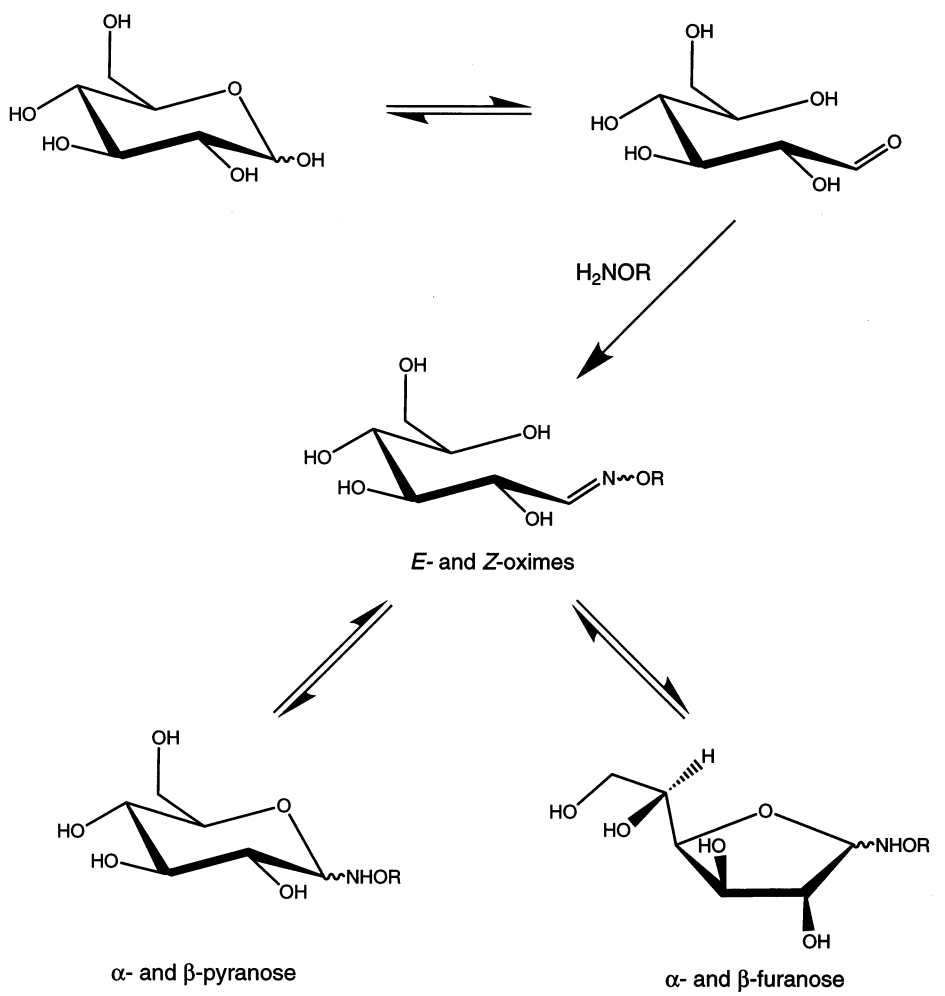


Fig. 1. HPLC chromatograms showing: (a) GlcNAc–NBHA oxime derivative and (b) reduced GlcNAc–NBHA derivative.

weight, which suggested that they were isomeric, namely, the *E*- and *Z*-acyclic oximes, and α- and β-cyclic products (Scheme 1). Isolation of any one of the peaks, followed by reinjection into the HPLC, produced a chromatogram that was similar to that of the original mixture, showing that the products were readily interconvertible. HPLC analysis showed that, at room temperature and pH 4, a quantitative yield of Glc–NBHA and GlcNAc–NBHA derivatives was achieved after 130 and 350 min, respectively. At 70 °C and pH 4 the reaction with Glc and GlcNAc was complete in 30 and 80 min, respectively. In contrast, coupling of NBHA to the exposed aldehyde group of 2,5-An-Man (**5**) and 2,5-An-Man6SO_3 (**6**) was complete at room temperature and pH 4 in under 20 min.

The fact that several oxime products of Glc and GlcNAc appear in the HPLC chromatogram complicates the analysis of unknown structures and reduces the overall sensitivity. Moreover, monitoring of the Glc-oxime derivatives indicated that hydrolysis of the purified oximes collected from HPLC occurs at a rate of about 2% per day during storage below 0 °C in 0.1% TFA. It was therefore decided to reduce the oxime derivative to a single, more stable *N*,*O*-disubstituted hydroxylamine product to improve chromatographic analysis, increase sensitivity, and allow storage over longer periods. The formation of a secondary amine group would also provide a suitable site for protonation during positive ion ESIMS analysis.

Sodium cyanoborohydride ($NaCNBH_3$) was chosen as a reductant as it is a 'soft' reducing agent and has a pH dependency, whereby some selectivity can be gained.[31] Oximes were preformed at pH 4 with excess NBHA, and



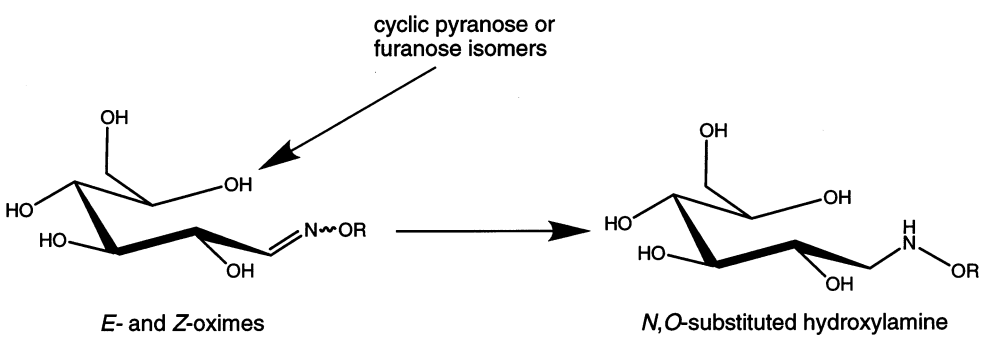
Scheme 1. Formation of various isomeric oxime derivatives with glucose.

the pH was then adjusted to pH 3 before the addition of 2 equiv of $NaCNBH_3$. The reaction was monitored by reversed-phase HPLC. The reduction of the Glc–NBHA and GlcNAc–NBHA oxime derivatives to their respective *N*,*O*-disubstituted hydroxylamine derivative both resulted in a single major product (Fig. 1(b), Scheme 2). Whilst the reduction was slow (~ 5 h at room temperature for Glc–NBHA and 20 h for GlcNAc–NBHA) yields were consistently $> 90\%$, but not quantitative due to the presence of traces of unreduced oxime (Fig. 1(b)). The products were stable for some months when stored at -20 °C. The reason for the slow reduction of these derivatives appears to lie in the sluggish interconversion of the non-reducible cyclic to acyclic isomers (Scheme 1). This was supported by the observation that reduction of the acyclic 2,5-An-Man–NBHA oxime derivative proceeded rapidly and quantitatively. Reduction of the 2,5-An-Man6SO_3–NBHA oxime, however, also did not go to completion, presumably due to the presence of the sulfate group.

Fluorescent reagents are required for chromatographic detection of these derivatives at the sub-picomolar level. Since the UV chromophore NBHA is not fluorescent and none are available commercially, it was necessary to synthesize low-molecular-weight fluorescent reagents. The fluorescent *O*-substituted hydroxylamine tags *O*-(4-methoxybenzyl)-hydroxylamine (**1**) and 8-aminooxymethyl-7-hydroxycoumarin (**2**) were prepared and reacted with the four model glycans (Glc, GlcNAc, **5** and **6**), under the conditions used for NBHA derivatization. As with NBHA, HPLC analysis indicated that **1** and **2** formed multiple isomeric oxime derivatives. The oximes were produced in quantitative yield, under mild reaction conditions (pH 4, rt). Reduction of the oximes resulted in a single major, stable, fluorescent *N*,*O*-disubstituted hydroxylamine product together with variable amounts of unreduced oxime, comparable to the results for the NBHA-oximes. The identities of the oximes and their reduction products were confirmed by ESIMS that revealed molecular ions consistent with the proposed structures.

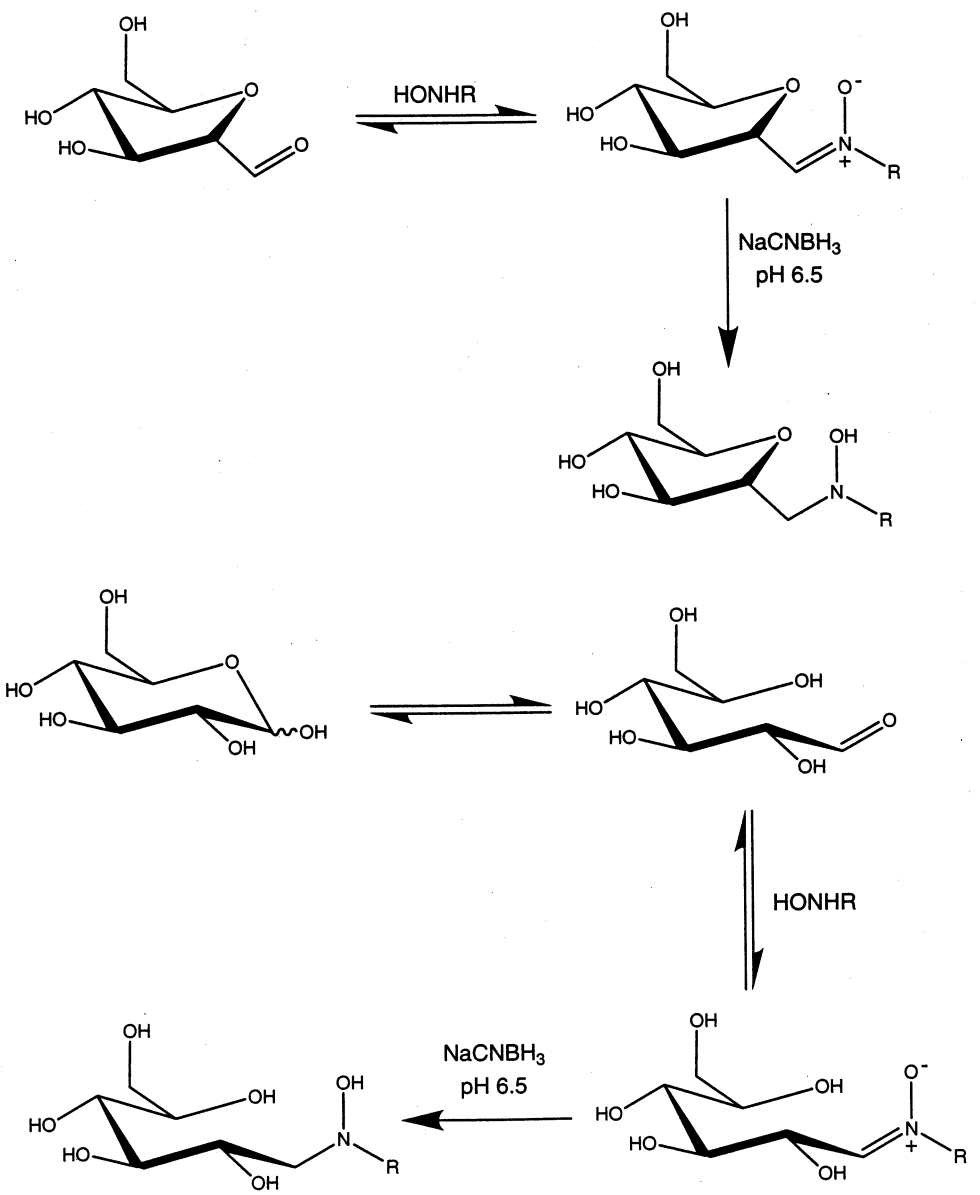
The rationale behind the preparation of the *N*-substituted hydroxylamines as tagging reagents for the sugars resulted from the observation of an anomalous peak in the HPLC trace during studies of the reductive oxyamination reaction. While probing the kinetics of the $NaCNBH_3$ reductive oxyamination of GlcNAc and NBHA, we observed under certain conditions (pH > 4, rt, 10:1 GlcNAc–NBHA) a new product at a much shorter HPLC retention time. This was isolated and identified by ESIMS–MS as a reduced bis-sugar derivative of NBHA with an MH^+ at m/z 579. We postulated that its formation must involve a quaternary ammonium ion intermediate, reducible by $NaCNBH_3$ without the need for the presence of acid. This unexpected result led us to propose that the nitrone product formed between an *N*-substituted hydroxylamine and the aldehyde group of our model sugars, which contains a formal positive charge on the nitrogen atom, should likewise be reduced by $NaCNBH_3$ to the corresponding *N*,*N*-disubstituted hydroxylamine under near neutral pH conditions.

To test this proposal, the two fluorescent *N*-substituted hydroxylamines, *N*-(3-methoxy-



Scheme 2. Reduction of *E*- and *Z*-oximes to a single *N*,*O*-substituted hydroxylamine.

benzyl)-hydroxylamine (**3**) and 7-hydroxy-8-hydroxyaminomethylcoumarin (**4**) were synthesized by initially forming an oxime, via condensation of hydroxylamine with the relevant aryl aldehyde, and then rapidly reducing the oxime with $NaCNBH_3$ at pH 3. The four model glycans (Glc, GlcNAc, **5** and **6**) were reacted with **3** and **4** under aqueous conditions at pH 6.5 and room temperature in the presence of $NaCNBH_3$ in a one-pot reaction to give the corresponding *N*,*N*-disubstituted hydroxylamines in quantitative yield. The reaction was carried out at pH 6.5 as *N*,*N*-disubstituted hydroxylamines have been shown to autoxidize at pH 7 and above,[32,33] whilst below pH 6 competitive direct reduction of the aldose is possible. By performing the reaction on Glc and GlcNAc without added $NaCNBH_3$, the nitrone intermediates (Scheme 3) were shown by HPLC to be present in the aqueous solution at pH 6.5 at surprisingly high equilibrium concentrations of 17 and 12%, respectively. Moreover, the nitrones could be isolated from aqueous solution, and identified by ESIMS. Nitrones are commonly reduced to the corresponding substituted amine with powerful reductants such as palladium–H_2 or lithium aluminum hydride,[34,35]



Scheme 3. Nitrone formation and reduction of 2,5-anhydro-D-mannose and D-glucose using an *N*-substituted hydroxylamine derivative.

but it appears that the nitrone is sufficiently polarized for the milder $NaCNBH_3$ to reduce it. There are potentially several isomeric forms for the intermediate nitrone derivatives analogous to those of the oxime derivatives, namely, acyclic *E*- and Z-nitrones (all model glycans), α- and β-pyranose and α- and β-furanose conformers (Glc and GlcNAc only). The nitrone intermediates in the cyclic forms are non-reducible, and their formation would hinder the nitrone reduction process unless interconversion between cyclic and acyclic contributors is rapid. The Glc–nitrone intermediate is reduced quickly and quantitatively so it is presumably acyclic. The reduction of the GlcNAc–nitrone is slow by comparison even if carried out as a one-pot procedure, possibly because it exists predominantly in one or more of the cyclic conformers. This is supported by the one-pot reductive hydroxyaminations of 2,5-An-Man (**5**) and 2,5-An-Man6SO_3 (**6**) at pH 6.5 and room temperature, which were complete within 30 min (Scheme 3), since the aldehyde group of 2,5-An-Man cannot be tied up in a cyclic structure.

To demonstrate the chromatographic and mass spectrometric properties of the *N*-substituted tags, a dextran hydrolysate and corn syrup were both labeled with **4**, using the reductive hydroxyamination procedure described, and analyzed using reversed-phase HPLC. The chromatogram of the labeled dextran hydrosylate (Fig. 2) indicated a discrete series of labeled glucose polymers, Glc_{1-14+} differing in molecular weight (ESIMS) by 162 mass units. The obvious benefit of reversed-phase HPLC analysis of the labeled product, as opposed to traditional anion-exchange chromatography, is that the fractions which are eluted do not contain buffers such as sodium hydroxide and sodium acetate, and so are immediately amenable to mass spectrometric analysis.

Commercial corn syrup, also labeled with **4**, was subjected to HPLC analysis, and the collected fractions were analyzed by ESIMS. Positive-ion ESIMS analysis of the labeled Glc_n polymers produced protonated molecular ions for each fraction (Fig. 3). The series also exhibited formation of Y-type glycosidic cleavages,[36] with charge retention on the basic *N*,*N*-disubstituted hydroxylamine moiety.

In order to investigate the tagging of heparin and heparan sulfate oligosaccharide fragments which arise from enzymatic cleavage, a monosaccharide that is frequently present at the reducing end of these fragments, 2-deoxy-2-sulfamido-α-D-glucopyranose ($GlcNSO_3$), was subjected to both oxime formation and reductive hydroxyamination with **1** and **3**, respectively. The oxime derivative of $GlcNSO_3$ was formed quantitatively with **1** but consisted of a mixture of isomers. As the reduction of oxime derivatives was earlier shown to not be quantitative for Glc, GlcNAc and 2,5-An-Man6SO_3, reduction of the oxime derivative of $GlcNSO_3$ was not attempted. The *N*,*N*-

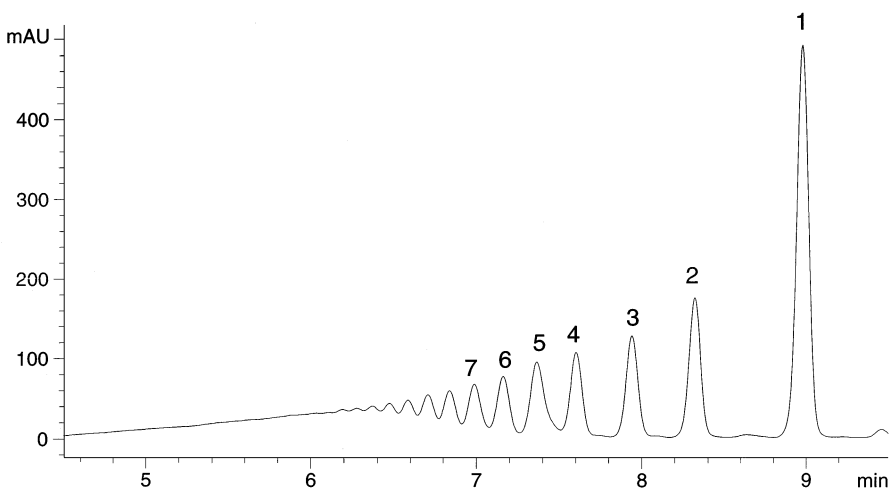


Fig. 2. Reversed-phase HPLC chromatogram of a dextran hydrolysate labeled with **4**.

disubstituted hydroxylamine derivative of $GlcNSO_3$, from reductive hydroxyamination with **3**, although a single compound, was produced in a yield of only 49%. Oxime formation is therefore the preferred method for derivatization of enzymatically cleaved heparin and heparan sulfate fragments some of which contain $GlcNSO_3$ at the reducing terminus. Although the number of isomers present could complicate chromatographic separation of the oximes, this problem may be minimized when dealing with large oligosaccharides when properties of the sulfated oligosaccharide, and not the tag, can dominate the separation characteristics of the derivative. This is especially true when using PAGE gel chromatography, for which compound **2** was specifically prepared.

To summarize, the *N*-substituted hydroxylamines (**3** and **4**) satisfy all of the previously stated ideal properties required for tagging the reducing end of labile sugars. The reductive hydroxyamination proceeds in a one-pot reaction selectively and quantitatively (with the exception of $GlcNSO_3$) to give a single derivative; conditions are extremely mild (aq solution, pH 6.5, rt); the products are fluorescent, have good HPLC properties and ESIMS sensitivity. The *N*,*N*-disubstituted hydroxyamine products autoxidise above pH 7, and unlike the oximes, are therefore not amenable to standard PAGE gel chromatography performed at pH > 8.[32,33] Although **3** and **4** are not as yet commercially available, they can be readily prepared using the procedure outlined in Section 3.

The *O*-substituted hydroxylamines (**1** and **2**) formed oximes quantitatively with all of the model monosaccharides (Glc, GlcNAc, $GlcNSO_3$, **5** and **6**) under mild conditions (aq solution, pH 4, rt), but reduction to the corresponding *N*,*O*-disubstituted oxyamine, although consistently high, was less than quantitative for Glc, GlcNAc and **6**. It is therefore recommended that derivatization should be carried through only as far as the oxime. Formation of multiple isomeric oximes with Glc and GlcNAc could complicate HPLC analysis, but they would be suitable for PAGE gel-chromatography because of their stability at basic pH values. Thus this is the method of choice for the study of N-sulfated oligosaccharides from enzymatic cleavage of heparins.

Application of the above two complementary derivatization procedures to the struc-

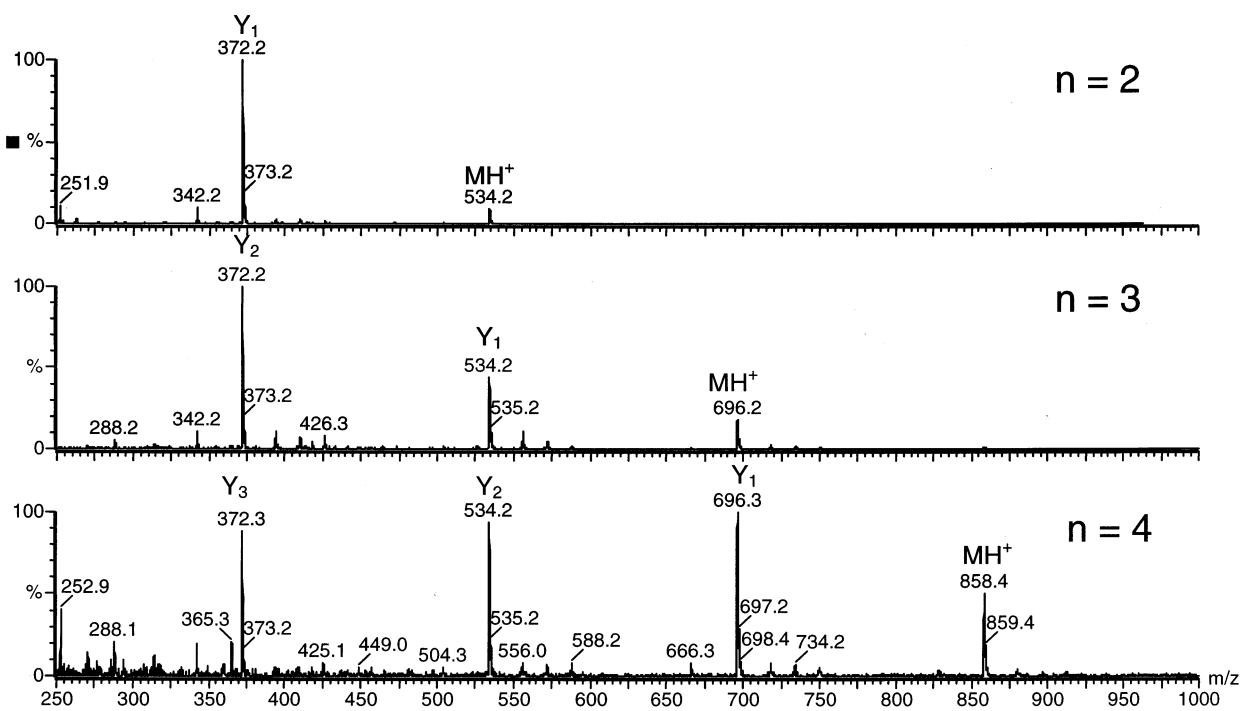


Fig. 3. ESIMS analysis of collected corn syrup fractions (Glc_n) labeled with **4**, where $n = 2$, 3, or 4.

tural analysis of heparins will be the subject of future publications.

3. Experimental

General methods.—Melting points were measured on a Reichert melting point apparatus without correction. ^{1}H NMR spectra were recorded at 500 or 300 MHz with Varian Inova or Gemini spectrometers, respectively. Chemical shifts are given in δ-units (ppm) with reference to either Me_4Si (δ 0) as an internal standard, or to water (δ 4.78) when D_2O was used as the solvent. High-resolution electron-ionization mass spectra (HREIMS) were acquired on a Micromass AutoSpec double-focussing mass spectrometer operating at a resolution of 10,000 with PFK as reference. Electrospray-ionization mass spectra (ESIMS) were acquired using a Micromass Quattro II triple quadrupole mass spectrometer, fitted with an electrospray probe. The solvent composition was 1:1 water–MeCN, and the flow rate into the electrospray probe was typically 10 μL/min. The cone voltage used was in the range 40–90 V.

Reversed-phase HPLC analysis.—Separations were performed using a Hewlett–Packard HP1090M ternary solvent delivery system, fitted with a diode-array detector and a HP1046A programmable fluorescence detector. Alltech Alltima C_{18} columns (5 μm, 250 × 4.6 mm) were used for all analyses. The formation of tagged model glycans was monitored using a binary solvent system consisting of 0.1% aq TFA as solvent A, and 0.1% TFA in MeCN as solvent B. A solvent gradient was used with the concentration of solvent B rising from an initial concentration of 5% to a final concentration of 40% over 20 min. The flow rate was 1 mL/min.

Preparation or purchase of model saccharides.—Glucose, *N*-acetylglucosamine, 2-deoxy-2-sulfamido-α-D-glucopyranose and all other reagents were purchased from Sigma–Aldrich unless stated otherwise. Commercial corn syrup was obtained from a health food store. 2,5-Anhydro-D-mannose and 2,5-anhydro-6-*O*-sulfo-D-mannose were prepared by an adaptation of published procedures[37] as follows. Nitrous acid was perpared by adding $NaNO_2$ (100 μL, 1 M) to citrate buffer at pH 4 (400 μL, 1 M) and allowed to stand at rt for 10 min prior to use. Glucosamine (or glucosamine 6-sulfate) (10 μL, 1 M) dissolved in water (1 mL) was then added to the HNO_2 solution and allowed to stand at rt for 30 min. Excess HNO_2 was eliminated by addition of ammonium sulfamate until evolution of gas ceased. The solution of 2,5-anhydro-D-mannose (or 2,5-anhydro-6-*O*-sulfo-D-mannose) was stored below 0 °C until needed.

Hydrolysis of dextran.—Dextran (100 mg) was dissolved in HCl (1 mL, 1 M) and heated at 100 °C for 2 h. The hydrosylate was lyophilized prior to use.

General procedure for the coupling of model saccharides to O-*substituted hydroxylamine derivatives via an oxime intermediate.*—Saccharides were reacted with a tenfold excess of *O*-substituted hydroxylamine derivative in citrate or acetate buffer (100 mM, pH 4). When the reaction was carried out at rt, HPLC analysis revealed that the oxime was formed in quantitative yield for Glc and GlcNAc after 130 and 350 min, respectively. The reaction with 2,5-An-Man and 2,5-An-Man6SO_3 was complete in less than 20 min. At 70 °C the reaction with Glc and GlcNAc was completed in 30 and 80 min, respectively. The pH was then adjusted to pH 3 using HCl (1 M) before addition of 2 equiv of $NaCNBH_3$. Reduced products were purified by reversed-phase HPLC.

Coupling of saccharides to N-*substituted hydroxylamine derivatives via a nitrone intermediate.*—Saccharides were reacted with a tenfold excess of *N*-substituted hydroxylamine derivative in phosphate buffer (100 mM, pH 6.5), with the typical final concentration of tag in the range of 50–100 mM. $NaCNBH_3$ was then added to a concentration of 50 mM. The reaction with 2,5-An-Man was complete in less than 20 min, whereas the reaction with Glc and GlcNAc was complete in 1 and 4 h, respectively, at rt.

O-*(4-Methoxybenzyl)hydroxylamine* (**1**).—To a stirred solution of 4-methoxybenzyl alcohol (1.28 g, 10 mmol) in THF (50 mL), was added *N*-hydroxyphthalimide (1.63 g, 10 mmol) and triphenylphosphine (2.62 g, 10

mmol) which was stirred at rt until dissolved. Diethyl azodicarboxylate (DEAD) (1.74 mL, 11 mmol) or dipropyl azodicarboxylate (DPAD) (2.17 mL, 11 mmol) was then added slowly to the solution over a period of several min. The color of the solution remained red–yellow once the DEAD or DPAD was in excess. Solvent was removed under vacuum, and the product was dissolved in CH_2Cl_2 and filtered. The product was recrystallized from CH_2Cl_2–hexane several times to give white crystals of *N*-phthaloyl 4-methoxybenzyl-*O*-hydroxylamine (1.95 g, 69%). The *N*-phthaloyl 4-methoxybenzyl-*O*-hydroxylamine (850 mg, 3 mmol) was dissolved in MeOH (60 mL), hydrazine hydrate (0.46 mL, 9.5 mmol) was added, and the solution stirred at rt for 3 h. A pH of 2 was maintained by addition of HCl (2 M). The solution was cooled on ice, and the resultant precipitate was removed by vacuum filtration. Solvent was removed, and the product was partitioned between CH_2Cl_2 and water (adjusted to pH 8 with NH_4HCO_3). Addition of methanolic or ethereal HCl produced the hydrochloride salt of **1** as white crystals. Addition of hexane to the solution produced additional **1** (369 mg, 64%) as white crystals: mp 145–146.5 °C; ^{1}H NMR (D_2O): δ 7.32 (d, 2 H, *J* 8.6 Hz), 6.93 (d, 2 H, *J* 8.6 Hz), 4.89 (s, 2 H); 3.72 (s, 3 H); HREIMS: Anal. Calcd for $C_8H_{11}NO_2$, 153.0790; found: *m*/*z* 153.0794.

8 - Aminooxymethyl - 7 - hydroxycoumarin (**2**).—To stirred TFA (10 mL) on ice was added 7-acetylumbelliferone (1.5 g, 7.4 mmol) and hexamethylenetetramine (1.5 g, 10.7 mmol). The solution was allowed to warm to rt, and then it was refluxed for 8 h. The excess TFA was removed under vacuum, 2 vol of water were then added to the remaining solution, and the mixture was warmed at 60 °C whilst stirring for 30 min. Upon cooling on ice a pale-yellow solid precipitated from solution and was collected by filtration and washed several times with water to give 8-formyl-7-hydroxycoumarin[38] as a pale-yellow solid (757 mg, 54%). Methoxymethyl chloride (1.2 mL, 15.8 mmol) was slowly added to a stirred solution of 8-formyl-7-hydroxycoumarin (400 mg, 2.1 mmol) in THF (20 mL) and triethylamine (2 mL, 14.3 mmol) at 0 °C. The reaction mixture was allowed to warm to rt and was stirred until the reaction was complete ($\sim$2 h). The solvents were then removed under vacuum, and CH_2Cl_2 (10 mL) was added. Ice water (10 g) was added with stirring, and the aqueous layer was extracted with CH_2Cl_2 (2×10 mL). The organic layers were combined and dried ($MgSO_4$), and the solvent was removed under vacuum to give 8-formyl-7-methoxymethyloxy coumarin as a mustard solid (468 mg, 95%). To a stirred solution of 8-formyl-7-methoxymethyloxycoumarin (580 mg, 2.5 mmol) in EtOH (10 mL) and CH_2Cl_2 (10 mL) at rt, was added $NaBH_4$ (500 mg, 13.2 mmol). The solution was stirred for 30 min until the dark-yellow color had faded. Hydrochloric acid (10%) was added sparingly, with stirring, so that only sufficient acid was added to destroy the excess $NaBH_4$ without hydrolyzing the methoxymethyl group. This was achieved by addition of acid until the evolution of hydrogen ceased. The aqueous layer was then extracted twice with CH_2Cl_2. The organic layers were combined and dried ($MgSO_4$), and the solvent was removed under vacuum to give 8-hydroxymethyl-7-methoxymethyloxycoumarin as a pale-yellow oily residue (550 mg, 93%). To a stirred solution of 8-hydroxymethyl-7-methoxymethyloxycoumarin (500 mg, 2.1 mmol) in THF (100 mL), was added *N*-hydroxyphthalimide (350 mg, 2.1 mmol) and triphenylphosphine (550 mg, 2.1 mmol), and the mixture stirred at rt until the reagents were dissolved. DEAD (0.5 mL, 3.2 mmol) was then added slowly to the solution over a period of several min. The color of the solution remained red–yellow once the DEAD was in excess. The reaction mixture was stirred overnight at rt. The solvent was reduced to one-third the volume under vacuum to give a pale-yellow precipitate that was collected via filtration. Recrystallization of the precipitate from EtOH–CH_2Cl_2 gave *N*-phthaloyl - 8 - hydroxylaminemethylene - 7 - methoxymethyloxycoumarin as white crystals (640 mg, 80%). The methoxymethyl protecting group was removed by stirring *N*-phthaloyl 8-hydroxylaminemethylene-7-methoxymethyloxycoumarin (365 mg, 0.96 mmol) in TFA (5 mL) and HCl (5 mL, 10%) at rt until TLC showed that deprotection was complete ($\sim$2

h). Solvent was then removed under vacuum, and the residue was recrystallized from 1:1 MeOH–CH_2Cl_2 to give *N*-phthaloyl 8-hydroxylaminemethylene-7-hydroxycoumarin as an off-white precipitate (285 mg, 88%). To a solution of *N*-phthaloyl 8-hydroxylaminemethylene-7-hydroxycoumarin (60 mg, 0.18 mmol), stirred in 1:1 EtOH–CH_2Cl_2 (20 mL) was added hydrazine hydrate (60 μL, 1.9 mmol). The reaction was stirred at rt until TLC showed that removal of the phthaloyl group was complete (~ 2 h). The solution was then made alkaline (pH 9–10) with $NaHCO_3$ solution (1 M) and partitioned between CH_2Cl_2 and water, making sure that all traces of excess hydrazine were removed. The organic layer was dried ($MgSO_4$) and then reacidified with aq methanolic HCl. The solvent was then gradually removed under vacuum until the hydrochloride salt of 8-aminooxymethyl-7-hydroxycoumarin (**2**) (28 mg, 64%) precipitated out of solution as a white solid: mp 168–171 °C; ^{1}H NMR (D_2O): δ 7.83 (d, 1 H, *J* 10.0 Hz), 7.49 (d, 1 H, *J* 9.0 Hz), 6.85 (d, 1 H, *J* 8.5 Hz); 6.20 (d, 1 H, *J* 10.5 Hz), 5.22 (s, 2 H); HREIMS: Anal. Calcd for $C_{10}H_9NO_4$, 207.0532; found: *m*/*z* 207.0531.

N-*(3-Methoxybenzyl)hydroxylamine* (**3**).—3-Methoxybenzaldehyde (5 mL, 41.1 mmol) was stirred with hydroxylamine hydrochloride (5 g, 72.0 mmol) in EtOH (25 mL), with NaOH (2 M) used to adjust the pH to 4. After stirring for 30 min at rt, the solvent was removed until a white precipitate formed, and the precipitate was collected by vacuum filtration. The oxime was reduced without further purification by stirring in MeOH (25 mL) with $NaCNBH_3$ (1.25 g, 19.9 mmol) whilst maintaining pH 2–3 using HCl (2 M) at rt for 30 min. After removing the solvent under vacuum, the product was back-extracted with CH_2Cl_2 and $NaHCO_3$ solution (1 M, pH 9). Acidification of the dried CH_2Cl_2 portion with ethereal HCl caused some hydrochloride salt of the product to precipitate from solution, but an oil began to form. To avoid oil formation, hexane was used to crystallize out the remaining *N*-(3-methoxybenzyl)hydroxylamine hydrochloride (**3**) (2.33 g, 30%) from solution as a white solid: mp 133–135 °C; ^{1}H NMR (D_2O): δ 7.34 (t, 1 H, *J* 8.7), 7.02 (m, 3 H), 4.32 (s, 2 H); 3.76 (s, 3 H); ^{13}C NMR ($CDCl_3$): δ 159.6, 135.0, 129.6, 121.9, 114.9, 114.0, 56.6, 55.1; HREIMS: Anal. Calcd for $C_8H_{11}NO_2$, 153.0790; found: *m*/*z* 153.0788.

*7-Hydroxy-8-hydroxyaminomethylcoumarin (**4**).*—8-Formyl-7-hydroxycoumarin was formed as described for **2**. Hydroxylamine hydrochloride (400 mg, 5.8 mmol) was stirred with 8-formyl-7-hydroxycoumarin (600 mg, 3.2 mmol) in 1:1 water–THF (20 mL), with the pH adjusted to pH 4 using NaOH (2 M), for 1 h at rt. 7-Hydroxy-8-[(hydroxyimino)-methyl]coumarin (540 mg, 83%) was precipitated by slowly removing volatile solvents under vacuum. 7-Hydroxy-8-[(hydroxyimino)-methyl]coumarin (500 mg, 2.4 mmol) was reduced by stirring in THF (10 mL) with $NaCNBH_3$ (900 mg, 14.3 mmol) whilst maintaining a pH of 2–3 using HCl (2 M) at rt. The solvent was slowly removed under vacuum until the product precipitated as the hydrochloride salt. The process was repeated several times to give 7-hydroxy-8-hydroxyaminomethylcoumarin (**4**) (560 mg, 96%) as a white solid: mp > 310 °C; ^{1}H NMR (MeOH-d_4): δ 7.64 (d, 1 H, *J* 9.5 Hz), 7.22 (d, 1 H, *J* 8.6 Hz), 6.62 (d, 1 H, *J* 8.6 Hz), 5.99 (d, 1 H, *J* 9.5 Hz), 4.16 (s, 2 H); ^{13}C NMR (D_2O): δ 170.95, 166.91, 160.93, 153.33, 138.58, 120.11, 119.37, 118.37, 109.66, 50.64.

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