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EPR STUDIES OF SPIN TRAP ADDUCTS OF CARBOHYDRATE RADICALS: RECOGNITION OF ADDUCT ISOMERISM AND CHIRALITY

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The nitrogen substituents of glycosamines (amides, sulfonamides and amines) react with hypochlorite (HOCl) to form long-lived *N*-chloro derivatives (chloramides, *N*-chlorosulfonamides and chloramines) that are decomposed to nitrogen-centred radicals (amidyl, sulfonamidyl and aminyl radicals) via one-electron reduction. These radicals rearrange via intramolecular hydrogen abstraction to yield carbon-centred radicals that form persistent adducts with the spin traps 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and/or 2-methyl-2-nitrosopropane (MNP). The EPR spectra of some of these adducts are unexpectedly complex, with this shown to be due to isomerism or chirality effects. Thus isomeric adducts formed via trapping of C-2 carbon-centred radicals located on reducing residues inter-convert via mutarotation of the C-1 hemiacetal function as evidenced by time-dependent changes in adduct concentrations. Structurally analogous C-2 carbon-centred radicals formed on non-reducing residues do not form inter-convertible adduct isomers. The MNP adducts of methylene radicals formed on the glycosidic groups of *N*-acetylglucosamine α- and β-methyl glycosides (*t*Bu-NO$^{\bullet}$-CH$_2$-O-sugar) give EPR signals with non-equivalent β-hydrogen hyperfine splittings. Observation of a difference in the magnetic environment of these hydrogens (i.e. chiral recognition) is ascribed primarily to interactions between the nitroxide function and the (chiral) sugar moiety that restrict the rotation of the nitroxide α-C-N bond.

INTRODUCTION

Spin trapping with nitrone and nitroso spin traps has been widely employed to facilitate the observation of transient radicals in chemical and biological systems via the formation of persistent nitroxide radicals (spin trap adducts) that can be examined by conventional EPR spectroscopy (Davies & Timmins, 2000; DeGray & Mason, 1994; Janzen, 1990; Timmins & Davies, 1998). A substantial body of data has been accumulated concerning the EPR spectra of nitroxide spin trap adducts and structurally related nitroxides prepared by alternative chemical syntheses. In EPR spin trapping studies, this pre-existing data is valuable in assisting in the interpretation of EPR spectra of novel radical adducts and identifying the parent radical addend. Relatively little data exists for spin trap adducts of carbohydrate-derived radicals (e.g. Flitter & Mason, 1990; Hawkins & Davies, 1996; Rees, Hawkins & Davies, 2003; Triolet, Thiery, Agnel, Battesti, Raffi & Vincent, 1990, 1992) though extensive EPR studies of simpler hydroxyalkyl nitroxides have been conducted (Davies & Timmins, 2000; DeGray & Mason, 1994; Haire, Kotake & Janzen, 1987; Janzen, 1990; Janzen & Lopp, 1972; Kotake, Kuwata & Janzen, 1979; Madden & Taniguchi, 1993; Taniguchi & Madden, 1999; Timmins & Davies, 1998).

Glycosamines are carbohydrates that bear nitrogen substituents; these are important components of a range of biological polymers such as glycosaminoglycans and proteoglycans, which occur on cell-surfaces and in the extracellular matrix. HOCl, a product of activated phagocytes, reacts with the amino substituents of glycosamines (amides, sulfonamides and amines) to yield long-lived *N*-chloro derivatives (chloramides, *N*-chlorosulfonamides and chloramines) (Hawkins & Davies, 1996; Rees *et al.*, 2003; Rees, Hawkins & Davies, 2004, Rees, Pattison & Davies, unpublished data). One-electron reduction of these *N*-chloro derivatives generates the corresponding nitrogen-centred radicals (amidyl, sulfonamidyl and aminyl radicals), which undergo rapid rearrangement via intramolecular hydrogen atom abstraction reactions to yield carbon-centred radicals (Hawkins & Davies, 1996; Rees *et al.*, 2003). These carbon-centred radicals form persistent adducts with both the nitrone spin trap DMPO and the nitroso spin trap MNP. Here, the EPR spectra of these adducts, which are often highly complex, are discussed in detail with a particular focus on the spectroscopic recognition of adduct isomerism or chirality.

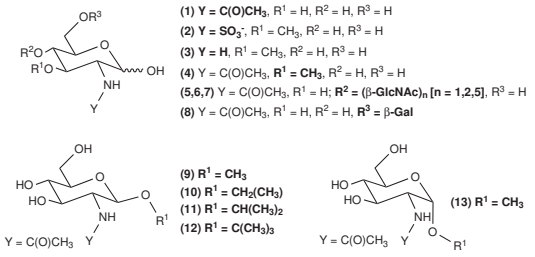

Fig. 1. Glycosamine derivatives. (1) *N-acetylglucosamine*, 2-acetamido-2-deoxy-D-glucopyranose; (2) *glucosamine-N-sulfate*, 2-sulfamino-2-deoxy-D-glucopyranose; (3) *glucosamine*, 2-amino-2-deoxy-D-glucopyranose; (4) 2-acetamido-2-deoxy-3-*O*-methyl-D-glucopyranose; (5) *di-N-acetylchitobiose*, β-D-Glc*p*NAc-(1→4)-D-Glc*p*NAc; (6) *tri-N-acetylchitobiose*, [β-D-Glc*p*NAc-(1→4)-]$_2$D-Glc*p*NAc; (7) *hexa-N-acetylchitohexaose*, [β-D-Glc*p*NAc-(1→4)-]$_5$D-Glc*p*NAc; (8) *N-acetylallolactosamine*, β-D-Gal*p*-(1→6)-D-Glc*p*NAc; (9) *N-acetylglucosamine β-methyl glycoside*, methyl 2-acetamido-2-deoxy-β-D-glucopyranoside; (10) *N-acetylglucosamine β-ethyl glycoside*, ethyl 2-acetamido-2-deoxy-β-D-glucopyranoside; (11) *N-acetylglucosamine β-iso-propyl glycoside*, *iso*-propyl 2-acetamido-2-deoxy-β-D-glucopyranoside; (12) *N-acetylglucosamine β-tert-butyl glycoside*, *tert*-butyl 2-acetamido-2-deoxy-β-D-glucopyranoside; (13) *N-acetylglucosamine α-methyl glycoside*, methyl 2-acetamido-2-deoxy-α-D-glucopyranoside.

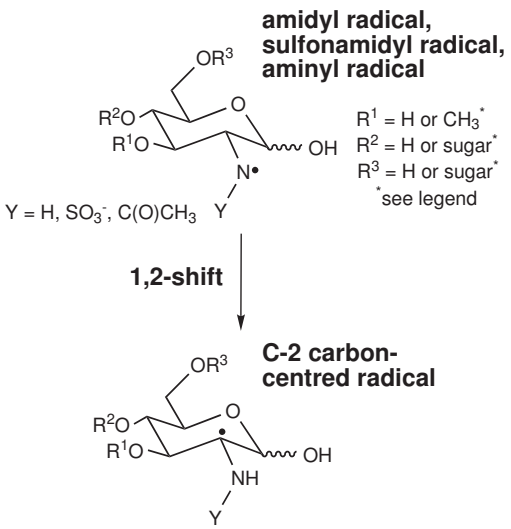

Fig. 2. Formation of C-2 carbon-centred radicals on reducing glycosamine residues via intramolecular 1,2-hydrogen shifts to initial nitrogen-centred radicals ((Rees *et al.*, 2003), Rees and Davies, unpublished data). Adducts of C-2 carbon-centred radicals formed via these reactions were detected with *N*-acetylglucosamine, glucosamine-*N*-sulfate, glucosamine, 2-acetamido-2-deoxy-3-*O*-methyl-D-glucopyranose, di-*N*-acetylchitobiose,tri-*N*-acetylchitotriose, hexa-*N*-acetylchitohexaose and *N*-acetylallolactosamine.

MATERIALS AND METHODS

Materials

Solutions were prepared using water filtered through a four-stage Milli Q system. pH control was achieved using 0.1 M phosphate buffer, pH 7.4, treated with washed Chelex resin (Bio-Rad, Hercules, CA, USA) to remove contaminating trace metal ions. D-Glucosamine hydrochloride was from Sigma (St. Louis, MO, USA). Glucosamine-*N*-sulfate was from Dextra Laboratories (Reading, UK). *N*-Acetyl-D-glucosamine, methyl 2-deoxy-2-acetamido-*β*-D-glucopyranoside, methyl 2-deoxy-2-acetamido-*α*-D-glucopyranoside and 2-acetamido-2-deoxy-3-*O*-methyl-D-glucopyranoside were from Toronto Research Chemicals (North York, Ontario, Canada). D-[2-^{13}C]-Glucosamine hydrochloride, *N*-acetyl-D-[2-^{13}C]-glucosamine and methyl 2-deoxy-2-acetamido-*β*-D-[2-^{13}C]-glucopyranoside were from Omicron Biochemicals (South Bend, Indiana, USA). Ethyl 2-deoxy-2-acetamido-*β*-D-glucopyranoside, *iso*-propyl 2-deoxy-2-acetamido-*β*-D-glucopyranoside and *tert*-butyl 2-deoxy-2-acetamido-*β*-D-glucopyranoside were from CMS Chemicals (Abingdon, Oxfordshire, UK). *N*-Acetyl allolactosamine, di-*N*-acetylchitobiose, tri-*N*-acetylchitotriose and hexa-*N*-acetylchitohexaose were from Seikagaku (Tokyo, Japan). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO; ICN, Seven Hills, NSW, Australia) was purified before use by treatment with activated charcoal. Stock solutions of 2-methyl-2-nitroso-propane (MNP) were made up in CH_3CN and diluted into the final reaction mixture such that the final concentration of CH_3CN was $\leq$ 10% v/v. HOCl solutions were prepared by dilution of a concentrated stock (0.5 M in 0.1 M NaOH) into 0.1 M, pH 7.4, phosphate buffer, with the HOCl concentration determined spectrophotometrically at pH 12 using ε_{292nm} 350 M^{-1} cm^{-1} (Morris, 1966). All other chemicals were of analytical grade. Elimination of O_2 from reaction solutions was achieved by the use of nitrogen-saturated solutions enclosed in pre-sparged HPLC vials sealed with Teflon septa. Liquid transfers were achieved by use of gas-tight syringes.

Electron Paramagnetic Resonance (EPR) spectroscopy

EPR spectra were recorded at ca. 22°C using a Bruker EMX X-band spectrometer with 100kHz

Table. 1. MNP adducts of some glycosamine-derived carbon-centred radicals. Hyperfine splitting parameters reproduced from (Rees et al., 2003) with the exception of adducts derived from glucosamine-*N*-sulfate.

Parent radical/s	**MNP adduct/s**	**Hyperfine splitting (mT)[1]**			
		***a*(N, NO)**	***a*(H)**	***a*(N)**	***a*(^{13}C)**
C-2 carbon-centred radical, *N*-acetyl-[2-^{13}C]-glucosamine	***adduct 1***	1.56	0.10 (γ)	0.26 (β)	0.48 (α)
	adduct 2	1.56	-	0.26 (β)	0.48 (α)
	adduct 3	1.54	0.09 (γ)	0.30 (β)	0.30 (α)
C-2 carbon-centred radical, glucosamine-*N*-sulfate	***adduct 1***	1.52	0.05 (γ)	0.18 (β)	-
	adduct 2	1.53	-	0.18 (β)	-
	adduct 3	1.50	0.08 (γ)	0.22 (β)	-
C-2 carbon-centred radical, 2-acetamido-2-deoxy-3-*O*-methyl-D-glucosamine	***adduct 1***	1.56	0.08 (γ)	0.24 (β)	-
	adduct 2	1.56	-	0.24 (β)	-
	adduct 3	1.54	0.11 (γ)	0.31 (β)	-
C-2 carbon-centred radical, reducing residue of di-*N*-acetylchitobiose, tri-*N*-acetylchitotriose and hexa-*N*-acetylchitohexaose	***adduct 1***	1.55	0.10 (γ)	0.24 (β)	-
	adduct 2	1.55	-	0.24 (β)	-
	adduct 3	1.54	0.08 (γ)	0.21 (β)	-
C-2 carbon-centred radical, reducing residue of *N*-acetylallolactosamine	***adduct 1***	1.54	0.10 (γ)	0.26 (β)	-
	adduct 2	1.54	-	0.26 (β)	-
	adduct 3	1.53	0.13 (γ)	0.31 (β)	-
C-2 carbon-centred radical, *N*-acetyl-[2-^{13}C]-glucosamine β-methyl glycoside	***(no isomers)***	1.54	0.13 (γ)	0.25 (β)	0.48 (α)
C-2 carbon-centred radical, *N*-acetylglucosamine α-methyl glycoside	***(no isomers)***	1.54	0.12 (γ)	0.26 (β)	-
C-2 carbon-centred radical, *N*-acetylglucosamine β-ethyl glycoside	***(no isomers)***	1.54	0.12 (γ)	0.25 (β)	-
C-2 carbon-centred radical, *N*-acetylglucosamine β-*tert*-butyl glycoside	***adduct 1***	1.57	0.13 (γ)	0.23 (β)	-
	adduct 2	1.56	0.19 (γ)	0.23 (β)	-
$^{\bullet}$CH$_2$-O-sugar radical, *N*-acetylglucosamine β-methyl glycoside	***(no isomers)***	1.45	0.53 (β), 0.39 (β)	-	-
$^{\bullet}$CH$_2$-O-sugar radical, *N*-acetylglucosamine α-methyl glycoside	***adduct 1***	1.46	0.60 (β), 0.40 (β)	-	-
$^{\bullet}$CH(CH$_3$)-O-sugar radical, *N*-acetylglucosamine β-ethyl glycoside	***adduct 1***	1.48	0.14 (β)	-	-
	adduct 2	1.51	0.13 (β)	-	-
$^{\bullet}$C(CH$_3$)$_2$-O-sugar radical, *N*-acetylglucosamine β-*iso*-propyl glycoside	***adduct 1***	1.60	-	-	-
	adduct 2	1.62	-	-	-
$^{\bullet}$CH$_2$CH(CH$_3$)-O-sugar radical, *N*-acetylglucosamine β-*iso*-propyl glycoside	***(no isomers)***	1.72	0.71 (β), 0.71 (β)	-	-

[1] Hyperfine coupling constants ± 0.01 mT

modulation and a cylindrical ER4103TM cavity. Samples were contained in a flattened, aqueous sample cell (WG-813-SQ; Wilmad, Buena, NJ, USA). These experiments were performed on sam-

Table. 2 DMPO adducts of some glycosamine-derived carbon-centred radicals. Hyperfine splitting parameters reproduced from (Rees *et al*., 2003) with the exception of adducts derived from glucosamine-*N*-sulfate and glucosamine.

Parent radical/s	**DMPO adduct/s**	**Hyperfine splitting (mT)[1]**		
		***a*(N, NO)**	***a*(β-H)**	***a*(β-^{13}C)**
C-2 carbon-centred radical, *N*-acetyl-[2-^{13}C]-glucosamine	***adduct 1***	1.56	2.29	0.74
	adduct 2	1.55	2.12	0.82
C-2 carbon-centred radical, glucosamine-*N*-sulfate	***adduct 1***	1.61	2.33	-
	adduct 2	1.61	2.14	-
	adduct 3	1.54	2.31	-
C-2 carbon-centred radical, [2-^{13}C]-glucosamine	***adduct 1***	1.56	2.52	0.66
	adduct 2	1.56	2.34	0.69
	adduct 3	1.54	2.25	0.80
$^{\bullet}$CH$_2$-O-sugar radical, *N*-acetyl-[2-^{13}C]-glucosamine β-methyl glycoside	***adduct 1***	1.62	2.27	-
	adduct 2	1.56	2.25	-
$^{\bullet}$CH$_2$-O-sugar radical, *N*-acetylglucosamine α-methyl glycoside	***adduct 1***	1.59	2.25	-
	adduct 2	1.52	2.25	-
$^{\bullet}$CH(CH$_3$)-O-sugar radical, *N*-acetylglucosamine β-ethyl glycoside	***adduct 1***	1.65	2.25	-
	adduct 2	1.57	2.22	-

[1] Hyperfine coupling constants ± 0.01 mT

ples where all the HOCl was consumed in order to avoid direct reaction of HOCl with DMPO; the absence of HOCl in such solutions was established spectrophotometrically. Hyperfine couplings were measured directly from the field scan and confirmed by computer simulation using the program WINSIM (available at http://EPR.niehs.nih.gov). Correlation coefficients between simulated and experimental spectra were > 0.95. Typical EPR spectrometer settings were: gain, 1×10^5; modulation amplitude 0.02 mT; time constant, 0.16 s; scan time, 84 s; resolution 1024 points; centre field, 384 mT; field scan 6.5 – 10 mT; power, 25 mW; frequency, 9.76 GHz; with 1 – 8 scans averaged.

RESULTS AND DISCUSSION

N-chloro derivatives of glycosamines (Fig. 1) were prepared by reaction of the parent materials with HOCl at pH 7.4; this resulted in concentrations of the *N*-chloro species of ≥ ca. 230 μM ((Rees et al., 2003; Rees et al., 2004), Rees, Pattison and Davies, unpublished data). These *N*-chloro derivatives were subsequently decomposed to nitrogen-centred radicals by reaction with Cu^+ (327 μM) [generated *in situ* by reaction of Cu^{2+} (454 μM) with Ti^{3+} (327 μM)] at ca. 22°C under anoxic conditions in the presence of MNP (17 – 20.4 mM) or DMPO (83 – 102 mM). Recording of EPR spectra was initiated 1–2 min after addition of the metalions and sequential spectra were recorded for periods of up to 60 min. The hyperfine coupling constants of substrate-derived radical adduct signals observed in these experiments are summarised in Tables 1 and 2. The intensity of these signals was greatest in the first spectra acquired consistent with rapid decomposition of the *N*-chloro derivatives by Cu^+ (Rees *et al.*, 2003, Rees, Pattison & Davies, unpublished data).

The EPR spectra of some of the spin adducts described here have been reported elsewhere (Rees *et al.*, 2003); those from glucosamine-*N*-sulfate and glucosamine have not been characterised previously. This previous report did not discuss the structural basis of the observed hyperfine splitting patterns. These are analysed in detail here with particular emphasis on adduct isomerism and chiral effects on the observed spectra; these processes result in unexpectedly complex EPR spectra.

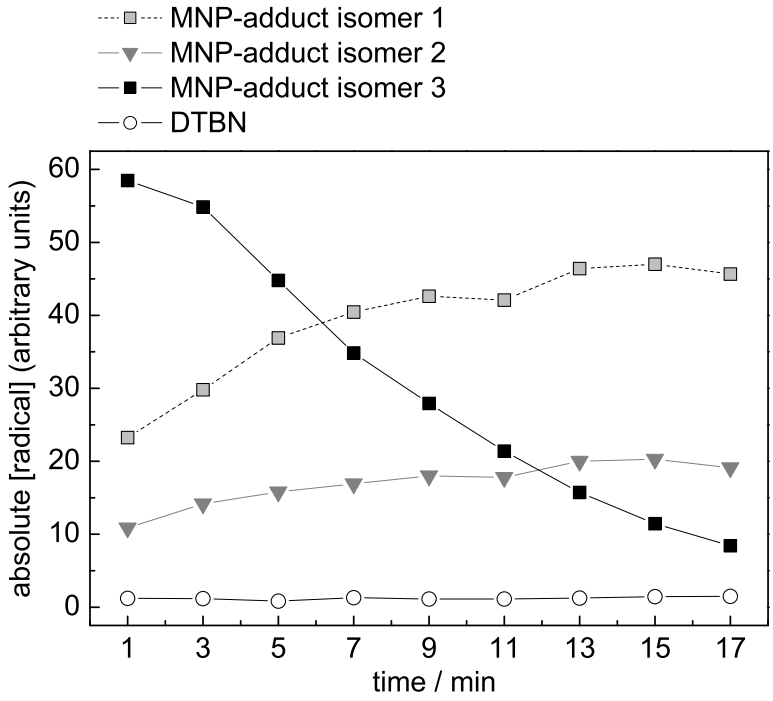

Fig. 3. Time-dependent changes in abundance of MNP adducts of the *N*-acetyl-[2-^{13}C]-glucosamine C-2 carbon-centred radical (reprinted in part with permission from Rees et al., 2003; Copyright 2003, American Chemical Society). Abundances of radical adducts in sequential spectra (t = 1 – 17 min after initiation of radical formation by addition of Cu^{+}: see text): fractional abundances were determined by computer simulation with three isomeric MNP-adducts of the *N*-glucosamine C-2 carbon-centred radical (for the hyperfine splitting parameters of these signals, see Table 1) and the spin trap degradation product DTBN; total radical concentrations were determined by double integration of spectra. At each time point, values for the (absolute) radical concentrations were obtained by multiplying the fractional radical abundances by the ratio of the total radical concentration to that at t = 1 min.

Monosaccharides and oligosaccharides

Decomposition of the *N*-acetylglucosamine chloramide in the presence of MNP gave rise to EPR signals which have been assigned to three carbon-centred radical adducts, each of which possessed a second triplet splitting (in addition to that from the nitroxide nitrogen) due to coupling to a substrate-derived nitrogen [*adduct 1*: *a*(N) 1.56, *a*(H) 0.10, *a*(N) 0.26 mT; *adduct 2*: *a*(N) 1.56, *a*(N) 0.26 mT; *adduct 3*: *a*(N) 1.54, *a*(H) 0.09, *a*(N) 0.30 mT]. When the corresponding compound with a ^{13}C-label at C-2 was examined, additional doublet splittings were observed in each case due to coupling to the ^{13}C nucleus [*adduct 1*: *a*(^{13}C) 0.48 mT; *adduct 2*: *a*(^{13}C) 0.48 mT; *adduct 3*: *a*(^{13}C) 0.30 mT]. With DMPO as the spin trap, two carbon-centred radical adducts with similar hyperfine splitting constants were detected [*adduct 1*: *a*(N) 1.56, *a*(H) 2.29 mT; *adduct 2*: *a*(N) 1.55, *a*(H) 2.12 mT]; the partial superposition of these signals gave rise to an asymmetric triplet of doublets pattern. With the ^{13}C-2 labelled compound each of these signals possessed an additional doublet splitting from the ^{13}C nucleus [*adduct 1*: *a*(^{13}C) 0.74 mT; *adduct 2*: *a*(^{13}C) 0.82 mT]. These signals have been assigned to *isomeric* adducts of a single parent radical - the *N*-acetylglucosamine C-2 carbon-centred radical - for reasons outlined below. This species is believed to be formed via an intramolecular 1,2-hydrogen atom shift to an initial (undetected) amidyl radical (Fig. 2) (Rees *et al.*, 2003, 2004).

The substrate-derived nitrogen splittings in the MNP adduct signals [*adduct 1*: *a*(N) 0.26 mT; *adduct 2*: *a*(N) 0.26 mT; *adduct 3*: *a*(N) 0.30 mT] are attributed to coupling with the amide nitrogen.

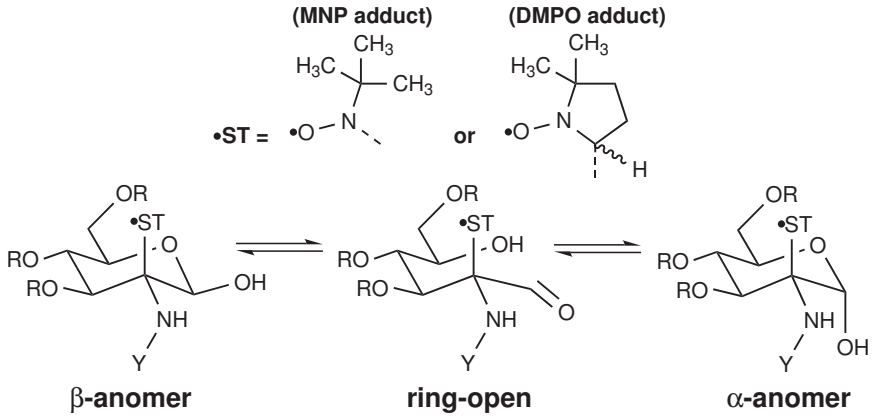

Fig. 4. Proposed structures of spin trap adducts formed via trapping of C-2 carbon-centred radicals on reducing glycosamine residues by MNP and DMPO (cf. parent radical structures: Fig. 2).

The substrate-derived hydrogen splitting present in two of the three signals [*adduct 1*: *a*(H) 0.10 mT; *adduct 3*: *a*(H) 0.09 mT] are postulated to arise via a long-range γ-coupling to a ring-derived C-H hydrogen located at C-1 or C-3 (Rees et al., 2003). An alternative assignment of this coupling to the amide N-H hydrogen has been discounted as a result of experiments carried out in a mixed D_2O/H_2O solvent system (4:1, ca. 22°C, 1 h incubation), where no exchange effects were detected. The size of this postulated γ-hydrogen splitting is large when compared to previously reported γ-

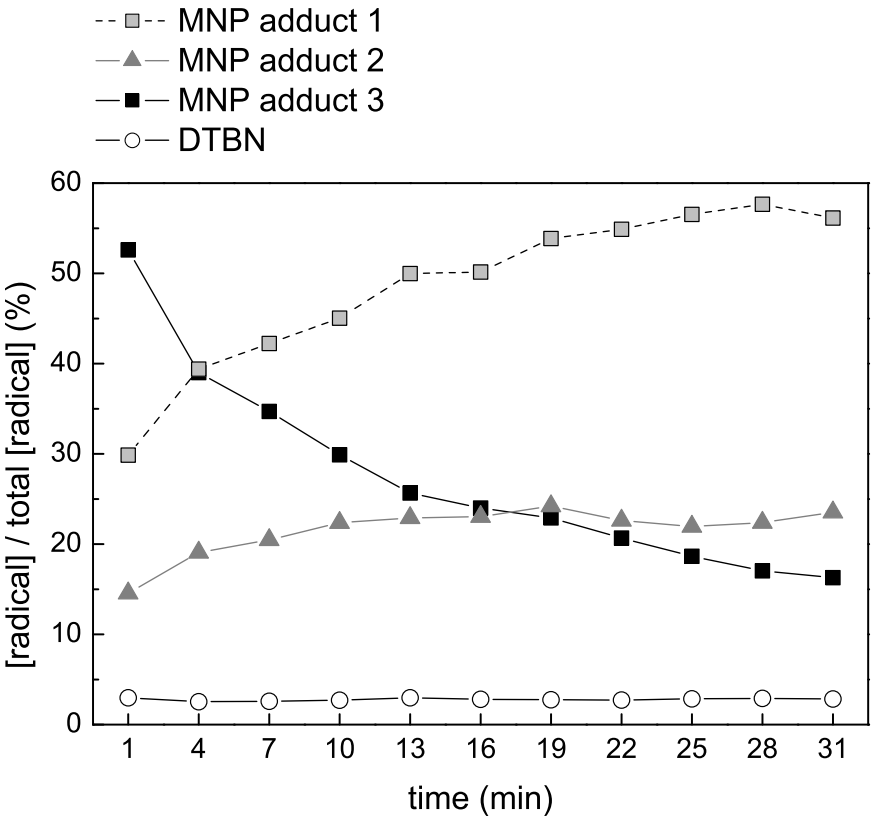

Fig. 5. Time-dependent changes in abundance of MNP adducts of the 2-acetamido-2-deoxy-3-*O*-methyl-D-glucopyranose C-2 carbon-centred radical. Fractional abundances of radical adducts in sequential spectra (t = 1 – 17 min after initiation of radical formation by addition of Cu^+: see text) as determined by computer simulation with three isomeric MNP-adducts of the 2-acetamido-2-deoxy-3-*O*-methyl-D-glucopyranose C-2 carbon-centred radical (for the hyperfine splitting parameters of these signals, see Table 1) and the spin trap degradation product DTBN.

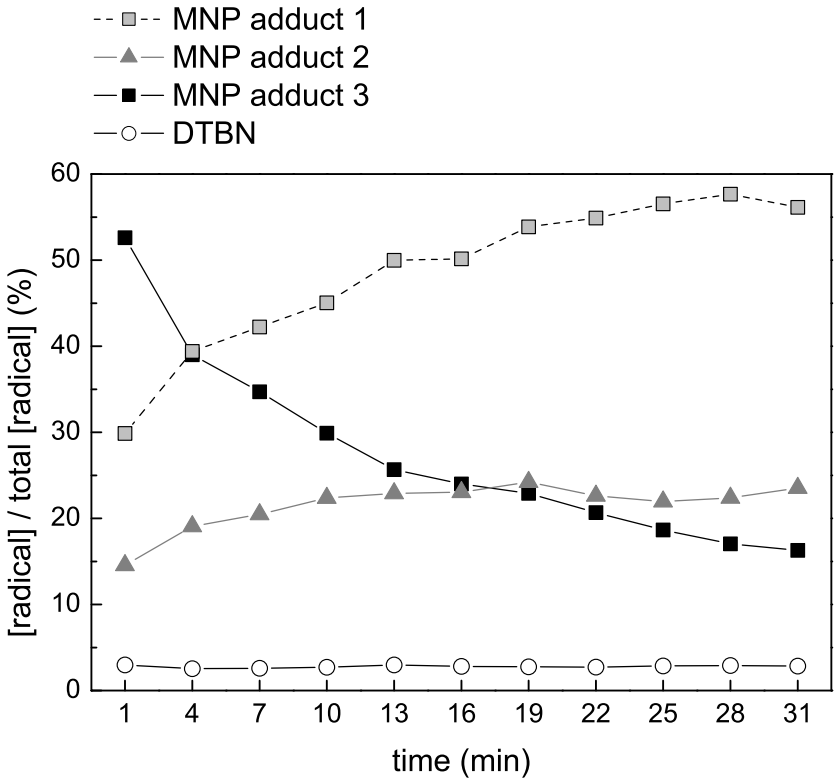

Fig. 6. Time-dependent changes in abundance of MNP adducts of the *N*-acetylallolactosamine C-2 carbon-centred radical. Fractional abundances of radical adducts in sequential spectra (t = 1 – 31 min after initiation of radical formation by addition of Cu^{+}: see text) as determined by computer simulation with three isomeric MNP-adducts of the *N*-acetylallolactosamine C-2 carbon-centred radical (for the hyperfine splitting parameters of these signals, see Table 1) and the spin trap degradation product DTBN.

hydrogen splittings for nitroxides; very often, these splittings are smaller than the signal linewidth and are not observed. However, MNP adducts with γ-hydrogen splittings of this magnitude, or greater, are known, particularly in the radical addends with rigid ring or hydrogen-bonded structures. Thus, γ-hydrogen couplings have been observed for MNP adducts of α-hydroxyalkyl radicals derived from cyclohexanol and 2-methylcyclohexanol [a(γ-H) 0.075 – 0.165 mT] and an α-amidoalkyl radical adduct from cyclo-(Gly-Gly) [a(γ-H) 0.06 mT] (Madden and Taniguchi, 1993; C. Hawkins, unpublished data). The former pair of γ-hydrogen splittings (from cyclohexanol- and 2-methylcyclohexanol) have been assigned to couplings to both ring-derived C-H hydrogens [a(γ-H) 0.075 – 0.165 mT] and to the O-H hydrogen [a(γ-H) 0.108 – 0.165 mT] (Madden and Taniguchi, 1993). The γ-hydrogen splitting observed from the cyclo-(Gly-Gly)-derived adduct has been assigned to an amide N-H hydrogen [a(γ-H) 0.06 mT] (C. Hawkins, unpublished data); ε-coupling to one of the ring-hydrogens is unlikely to be responsible for this splitting as no previous examples exist of ε-hydrogen splittings in the EPR spectra of nitroxides.

The relative concentrations of the three isomeric MNP adducts of the *N*-acetylglucosamine C-2 carbon-centred radical change dramatically over time, though the total adduct concentration remained constant (as assessed by double integration of the EPR spectra) (Fig. 3). Two of the adduct isomers accumulated at the expense of the third, with the ratio of these species approaching a constant value by 20 min. Similar behaviour was observed with the DMPO adducts. These phenomena are consistent with interconversion of these isomeric adducts, indicating that their stereochemical configuration must be identical at the *N*-acetylglucosamine C-2 carbon (i.e. trapping by MNP and DMPO occurs selectively on one face of the radical). Interconversion of the DMPO adducts is also consistent with a common configuration at the pyrrolidine β-carbon (i.e. trapping occurs selectively on one face of DMPO). Differences in the

thermodynamic stability of the adduct isomers would provide the driving force for the observed time-dependent interconversion via mutarotation at the C-1 hemiacetal function.

The facial selectivity of the addition of the spin traps to the *N*-acetylglucosamine C-2 carbon-centred radical is likely to be controlled by steric factors. The parent radical is predicted to exist principally in its two ring-closed (α and β) anomeric forms retaining the chair conformation of the parent sugar with the *N*-acetyl function adopting an equatorial position to minimise diaxial interactions with the ring hydrogens (Praly, 2000). The configuration of the MNP and DMPO adducts at the *N*-acetylglucosamine C-2 carbon is likely to be that which arises from the (less hindered) axial approach of the spin traps to the radical centre (cf. facial selectivity of addition of pyranosyl carbon-centred radicals to alkenes; Praly, 2000). The three MNP adduct isomers are therefore assigned to two (ring-closed) anomers plus a species which is either a second conformer of one of the anomers or the ring-open form (Fig. 4). Nitroxides which have a single configuration have previously been shown to form conformers in aqueous solution that give distinct EPR signals; examples include MNP adducts of some amide-derived carbon-centred radicals (restricted rotation about C-N bonds) (Rustgi & Riesz, 1978) or DMPO-OOH (pyrollidine ring conformers) (Rosen, Beselman, Tsai, Pou, Mailer, Ichikawa, Robinson, Nielsen, Halpern & MacKerell, 2004). Formation of the ring-open form might be favoured due to relief of steric crowding in the ring-closed forms. However, it is generally observed that the ring-open forms of reducing pyranoses are present in very low abundance in aqueous solution (Angyal, 1984). By similar reasoning the two DMPO adduct isomers are proposed to be the (ring-closed) α- and β-anomers.

Analogous behaviour was observed on decomposition of the chloramide derivatives of 3-O-methyl *N*-acetylglucosamine, *N*-acetylallolactosamine, di-*N*-acetylchitobiose, tri-*N*-acetylchitotriose and hexa-*N*-acetyl chitohexaose, with three MNP adducts detected in each case. These have been assigned, in each case, to isomeric adducts of C-2 carbon-centred radicals formed on the reducing *N*-acetylglucosamine residue via an intramolecular 1,2-hydrogen shifts to an (undetected) amidyl radical (Fig. 2). The parameters of these adducts are almost identical to those detected from *N*-acetylglucosamine (Table 1). The relative abundance of each set of adducts changed over time in a manner consistent with their interconversion (Figs. 5 and 6), and are likewise attributed to two anomers plus a species which is either a second conformer of one of the anomers or the ring-open form.

Signals assigned to adducts of C-2 carbon-centred radicals derived from the (reducing) monosaccharides glucosamine-*N*-sulfate and glucosamine have also been detected upon decomposition of the glucosamine-*N*-sulfate *N*-chlorosulfonamide, and glucosamine chloramine, in the presence of MNP or DMPO (Rees and Davies, unpublished data). Formation of these C-2 carbon-centred radicals is attributed to the occurrence of intramolecular 1,2-hydrogen shifts to the glucosamine-*N*-sulfate sulfonamidyl radical ($R\text{-}N^{\bullet}\text{-}SO_3^-$) and the glucosamine aminyl radical ($R\text{-}NH^{\bullet}$) (Rees and Davies, unpublished data).

The parameters of the glucosamine-*N*-sulfate-derived C-2 MNP adducts [*adduct 1*: *a*(N) 1.52, *a*(H) 0.05, *a*(N) 0.18 mT; *adduct 2*: *a*(N) 1.53, *a*(N) 0.18 mT; *adduct 3*: *a*(N) 1.50, *a*(H) 0.08, *a*(N) 0.22 mT] are again similar to those of the *N*-acetylglucosamine C-2 carbon-centred radical adducts and showed similar time-dependent changes in abundance (data not shown). It is proposed that these adducts are, as above, two anomers plus a species which is either a second conformer of one of the anomers or the ring-open form. Of note is that the sulfonamide nitrogen splittings in these signals [*a*(N) 0.18 – 0.22 mT] are smaller than the amide nitrogen splittings in the signals of the analogous *N*-acetylglucosamine-derived C-2 MNP adducts [*a*(N) 0.26 – 0.30 mT]. This presumably reflects either the electron-withdrawing properties of the sulfonamide and amide functions, or differences in the dihedral angle between the N-O and C-N bonds.

In equivalent experiments with DMPO as spin trap, three carbon-centred radical adducts were detected [*adduct 1:* *a*(N) 1.61, *a*(*β*-H) 2.33 mT; *adduct 2:* *a*(N) 1.61, *a*(*β*-H) 2.14 mT; *adduct 3:* *a*(N) 1.54, *a*(*β*-H) 2.31 mT] which have been assigned to isomeric adducts of the glucosamine-*N*-sulfate C-2 carbon-centred radical. The glucosamine C-2 carbon-centred radical also formed isomeric adducts with DMPO (but was not trapped by MNP); assignment of these DMPO adducts was based on the detection of large ^{13}C-splittings with the ^{13}C-labelled C-2 compound [*adduct 1*: *a*(N) 1.56, *a*(*β*-H) 2.52, *a*(^{13}C) 0.66 mT; *adduct 2*: *a*(N) 1.56, *a*(*β*-H) 2.34, *a*(^{13}C) 0.69 mT; *adduct 3*: *a*(N) 1.54, *a*(*β*-H) 2.25, *a*(^{13}C) 0.80 mT]. With the above-mentioned DMPO adducts, the relative abundance of two of the three adduct isomers changed whilst that of the third remained fixed (data not shown). The former two isomers are likely to be anomers of each other whilst the third may be a diastereoisomer of the anomers (e.g.

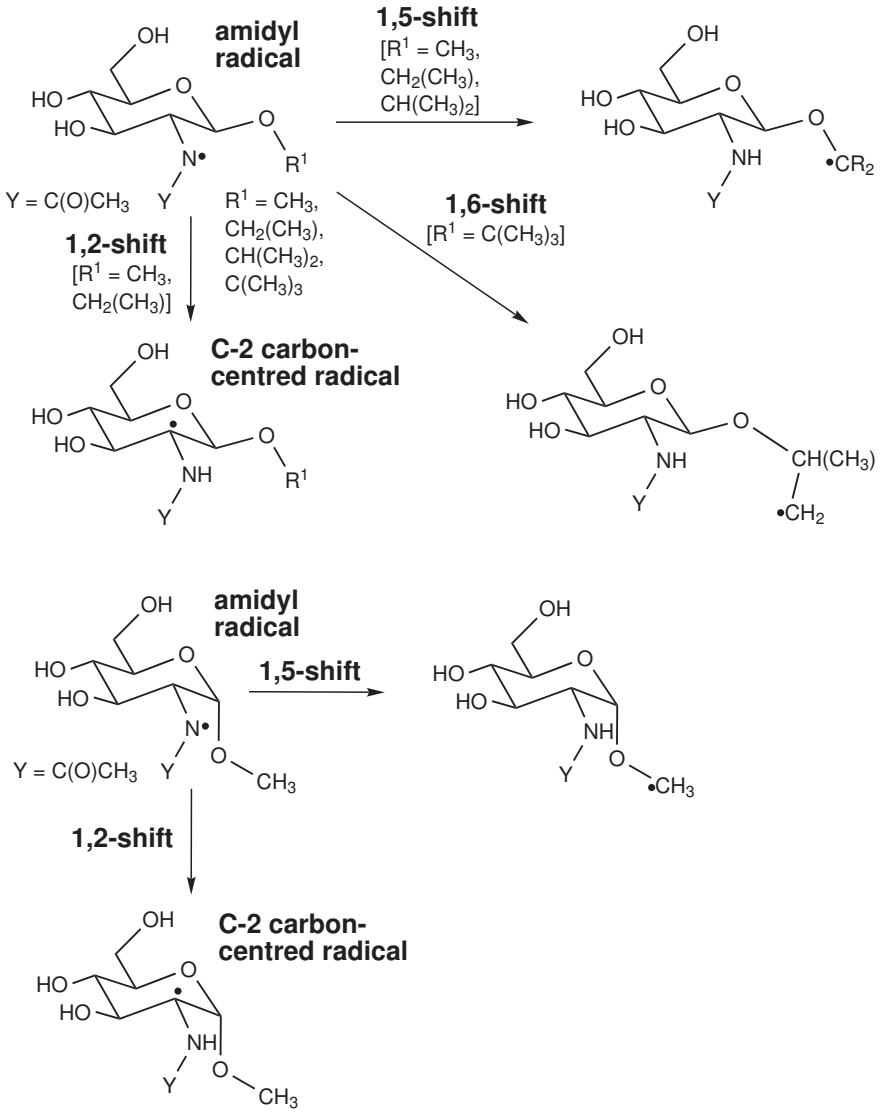

Fig. 7. Formation of glycosamine C-2 carbon-centred radicals and glycosidic alkyl radicals on *N*-acetylglucosamine C-1 alkyl glycosides (*N*-acetylglucosamine β-methyl glycoside, *N*-acetylglucosamine β-ethyl glycoside, *N*-acetylglucosamine β-*iso*-propyl glycoside, *N*-acetylglucosamine β-*tert*-butyl glycoside, *N*-acetylglucosamine α-methyl glycoside) via intramolecular 1,2-, 1-5- and 1,6-hydrogen shifts to initial amidyl radicals (Rees *et al*., 2003).

formed via trapping on the opposite face of the DMPO molecule and or parent radical).

C-1 alkyl glycosides

Amidyl radicals formed from chloramides located on *N*-acetylglucosamine residues that bear C-1 alkyl substituents have been shown to undergo 1,2-hydrogen shift reactions to give ring-derived C-2 carbon-centred radicals, as well as 1,5- and 1,6-hydrogen shift reactions that yield carbon-centred radicals on the C-1 alkyl substituent (Fig. 7) (Rees *et al.*, 2003). DMPO and MNP adducts of carbon-centred radicals formed via these processes have been detected with a series of *N*-acetylglucosamine C-1 alkyl glycosides (Table 1 and Table 2).

The MNP adduct of the *N*-acetylglucosamine β-methyl glycoside C-2 carbon-centred radical and the corresponding adducts derived from the *N*-acetylglucosamine α-methyl glycoside and the *N*-acetylglucosamine β-ethyl glycoside each gave a single symmetrical EPR signal [*β-methyl glycoside*: *a*(N) 1.54, *a*(H) 0.13, *a*(N) 0.25 mT; *α-methyl glycoside*: *a*(N) 1.54, *a*(H) 0.12, *a*(N) 0.26 mT; *β-ethyl glycoside*: *a*(N) 1.54, *a*(H) 0.12, *a*(N) 0.25 mT). It is proposed that each of the glycoside-

derived C-2 MNP adducts are formed via stereoselective reaction of MNP with one face of the parent radical (if the reaction was unselective, two EPR signals arising from two possible diasteromers should be detected). The stereochemical configuration of the C-2 carbon in the glycoside-derived adducts is expected to be identical to that proposed for the equivalent *N*-acetylglucosamine-derived adducts (i.e. that which arises from axial approach of the spin trap to chair-like C-2 carbon-centred radicals).

The hyperfine splitting parameters of the signals of the glycoside-derived C-2 MNP adducts are almost identical to those of two of the three *N*-acetylglucosamine-derived C-2 MNP adducts of the (*N*-acetylglucosamine *adduct 1* and *N*-acetylglucosamine *adduct 3* - see above and Table 1; *N*-acetylglucosamine *adduct 2* gives a signal which lacks a γ-hydrogen splitting but possesses similar nitrogen couplings to those assigned to its isomers). In the case of the β-methyl glycoside, an additional ^{13}C-splitting in the signal of the C-2 MNP adduct was observed when ^{13}C-labelling of the C-2 carbon was employed [a(^{13}C) 0.47 mT]. This splitting is almost identical to the ^{13}C-splitting detected in the signals of the (^{13}C-labelled) *N*-acetylglucosamine *adduct 1* [a(^{13}C) 0.48 mT] and the (^{13}C-labelled) *N*-acetylglucosamine *adduct 2* [a(^{13}C) 0.48 mT] but is quite different to the ^{13}C-splitting in the signal of the (^{13}C-labelled) *N*-acetylglucosamine *adduct 3* [a(^{13}C) 0.30 mT]. The hydrogen responsible for the small hydrogen splitting in the signal of the β-methyl glycoside C-2 MNP adduct was not exchanged in D_2O (D_2O/H_2O = 4:1, ca. 22°C, 1 h incubation).

These data offer further insight into the possible structures of the *N*-acetylglucosamine-derived adduct isomers. The identity between the hyperfine splitting parameters of the β-methyl glycoside C-2 adduct signal and the *N*-acetylglucosamine *adduct 1* signal (particularly the ^{13}C-splittings) suggests that *N*-acetylglucosamine *adduct 1* is the (ring-closed) β-anomer. Based on similar reasoning, the *N*-acetylglucosamine *adduct 3* is likely to be the α-anomer (if this reasoning holds, a ^{13}C-splitting of ca. 0.30 mT would be expected in the signal of the α-methyl glycoside-derived C-2 adduct if ^{13}C-labelling of the C-2 carbon was employed); *N*-acetylglucosamine *adduct 2* is likely to be the ring-open form or a conformer of one of the anomers. Interestingly, whilst the α-anomer of the parent monosaccharide *N*-acetylglucosamine is the most stable anomer in aqueous solution (Angyal, 1984), the C-2 MNP adduct β-anomer (*N*-acetylglucosamine *adduct 1*) appears to be the most stable species as evidenced by its accumulation over time (Fig. 3).

With the *β-tert*-butyl glycoside C-2 carbon-centred radical, two MNP adduct isomers were detected [*adduct 1*: a(N) 1.57, a(H) 0.13, a(N) 0.23 mT; *adduct 1*: a(N) 1.56, a(H) 0.19 , a(N) 0.23 mT]. These isomers may be conformers or diastereomers formed by reaction of MNP with both faces of the *β-tert*-butyl glycoside C-2 carbon-centred radical; the former interpretation is favoured in the light of data which suggests trapping of related C-2 radicals is stereospecific (see above).

With some, but not all, of the carbon-centred radicals formed on the alkyl substituents of the glycosides, trapping by MNP resulted in the detection of asymmetric EPR adduct signals attributed to the presence of overlapping signals of two adduct isomers.

With the $^{\bullet}CH_2$-O-sugar radicals derived from the *β*-methyl- and *α*-methyl glycosides, only single MNP adduct signals were detected in each case [*β-methyl glycoside*: a(N) 1.45, a(*β*-H_a) 0.53, a(*β*-H_b) 0.39 mT; *α-methyl glycoside*: a(N) 1.46, a(*β*-H_a) 0.60, a(*β*-H_b) 0.40 mT]. This is consistent with the fact that trapping of these anomeric radicals by MNP can produce adducts with only one configuration. The β-methylene hydrogens in these adducts are rendered magnetically non-equivalent by the chiral sugar moiety (*t*-Bu-NO$^{\bullet}$-CH_2-O-*sugar; *denotes the presence of a chiral centre) (Franchi Lucarini, Pedulli & Bandini, 2002).

With the *β*-ethyl-glycoside $^{\bullet}CH(CH_3)$-O-sugar radical, two MNP adducts were detected [*adduct 1*: a(N) 1.48, a(*β*-H) 0.14 mT; *adduct 2*: a(N) 1.51, a(*β*-H) 0.13 mT] whose superimposed signals gave rise to an asymmetric triplet of doublets splitting pattern. These signals are believed to arise from a pair of adducts generated by trapping of opposite faces of the pro-chiral radical centre (*t*Bu-NO$^{\bullet}$-*$CH(CH_3)$-O-*sugar). Of note is that the observation of non-equivalent EPR spectra with these diastereomeric nitroxides would involve recognition of chirality in the sugar moiety: enantiomeric nitroxides (i.e. which differ in structure at only one chiral centre) give identical EPR spectra in achiral media (Schuler, Schaber, Stegmann & Janzen, 1999).

With the *β-iso*-propyl glycoside $^{\bullet}C(CH_3)_2$-O-sugar radical, two MNP adducts were detected [*adduct 1*: a(N) 1.60 mT; *adduct 2*: a(N) 1.62 mT] whose superimposed signals gave rise to an asymmetric triplet splitting pattern. As trapping of this radical can produce adducts with only one configu-

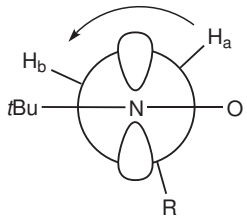

Fig. 8. Sterochemical projection of a nitroxide with the general structure *t*Bu-NO$^{\bullet}$-CH_2-R.

ration (*t*Bu-NO$^{\bullet}$-$C(CH_3)_2$-O-sugar), the two MNP adducts are believed to be conformers.

With the *β-iso*-propyl glycoside $^{\bullet}CH_2CH(CH_3)$-O-sugar radical, a single MNP adduct was detected which gave rise to a symmetrical EPR signal with equivalent β-hydrogen splittings [*a*(N) 1.72, *a*(2*β*-H) 0.71 mT]. Of note is that although the β-hydrogens in this adduct are diastereotopic as a result the presence of an adjacent chiral centre and the chiral sugar moiety (*t*Bu-NO$^{\bullet}$-CH_2*$CH(CH_3)$-O-*sugar), this is not recognised in the EPR signal. In addition, though it is likely that two diastereomeric methylene radicals are trapped to form diastereomeric adducts that possess opposite configurations at the β-carbon, only one symmetrical EPR signal is observed.

Each of the glycoside derivatives gave DMPO adduct spectra with an assymmetric triplet of doublets splitting pattern, consistent with the presence of two (or more) carbon-centred radical adducts (Rees *et al.*, 2003); although these spectra could be satisfactorily simulated as a composite of signals from two species, the presence of other minor components cannot be excluded. The two major DMPO adducts detected with each glycoside are assigned to isomeric adducts of carbon-centred radicals formed on the glycosidic groups via 1,5-hydrogen shifts (i.e. $^{\bullet}CR^1R^2$-O-sugar) (Rees *et al.*, 2003). Trapping of the C-2 ring radicals appears to be inefficient, possibly as a result of steric hindrance. This conclusion is supported by the observation that no DMPO adduct signals containing a ^{13}C-splitting were observed with the 2-^{13}C-labelled β-methyl glycoside. The origins of isomerism in the DMPO adducts of the $^{\bullet}CR^1R^2$-O-sugar radicals are uncertain: isomerism may arise in the same way as the corresponding MNP adducts as well as via reaction of the radicals with opposite faces of the DMPO molecule (i.e. the DMPO adducts may consist of up to four diastereomers).

Recognition of Adduct Isomerism and Chirality

A number of examples have been presented in the present work where trapping of a "single" radical by MNP or DMPO has been shown to yield multiple adduct isomers that give distinct EPR signals (i.e. "recognition of adduct isomerism"). In the EPR signals of some, but not all, adducts bearing diastereotopic β-methylene hydrogens, non-equivalent β-hydrogen splittings were observed (i.e. "recognition of adduct chirality"). The recognition of adduct diastereomerism, anomerism and chiralty in these carbohydrate-derived nitroxides stems from a common structural basis: the influence of one or more chiral centres present on the sugar moiety on the magnetic environment of the unpaired electron which is largely localised to the nitroxide N-O bond. The recognition of the presence of adduct conformers arises from the slow exchange between inter-convertible structures in which the magnetic environment of the nitroxide moiety is different and is not necessarily dependent on chirality. In the following section, the structural basis of the recognition of adduct isomerism and chirality is discussed in further detail; the latter phenomena is addressed first as it will illuminate the former.

The magnetic non-equivalence of methylene hydrogens in chiral radicals arises from two contributions: an intrinsic term and a term depending on the conformer populations. Recently, it has been demonstrated that the latter term is predominant in determining the magnitude of this non-equivalence (Franchi *et al.*, 2002). In nitroxides with the partial structure *t*Bu-NO$^{\bullet}$-CH_2-R (such as the *t*Bu-NO$^{\bullet}$-CH_2-O-sugar species derived from the α-methyl and β-methyl glycosides described in the present study), the magnitude of the β-methylene hydrogen hyperfine couplings are principally determined by the dihedral angles between the nitroxide N-O bond and the β-methylene C-H bonds (Fig. 8). On the EPR timescale, rotation about the α-C-N bond in nitroxides is usually fast in solution, and the magnitude of such couplings represent time-averaged values determined by the residence time of the radical in various conformations. The residence time in particular conformations is a function of their energy, with the longest residence times being associated with the lowest energy conformations.

Where the R substituent in radicals with the structure *t*Bu-NO$^{\bullet}$-CH_2-R bears a chiral centre, the time-averaged dihedral angles between the nitroxide and each of the methylene C-H bonds will differ (except if these angles are equal by "accident") due to steric interactions, stereo-electronic effects or hydrogen bonding. In this way, the in-

duction of restricted rotation about the nitroxide α-C-N bond can lead to a divergence in the time-averaged values of each of the β-methylene hyperfine splittings. If chiral substituents do not appreciably affect the energy of rotation about the nitroxide α-C-N bond, the intrinsic magnetic non-equivalence term will dominate. As this term is small, the difference in the β-methylene hyperfine couplings may be less than the line width of the EPR spectrum (i.e. chiral recognition may not occur).

Just as the recognition of chirality depends mainly on interactions between one or more chiral centres and the nitroxide moiety that affect conformer populations rather than intrinsic magnetic non-equivalence (Franchi *et al.*, 2002), the observation of non-equivalent EPR spectra with diastereomeric nitroxides appears to be determined principally by interactions between two or more chiral centres and the nitroxide moiety that affect conformer populations (e.g. at the nitroxide α-C-N bond) rather than intrinsic magnetic non-equivalence (Schuler *et al.*, 1999). Similar intramolecular interactions are likely to participate in both recognition processes (i.e. steric hindrance, stereo-electronic effects and hydrogen bonding).

Hydrogen-bonding can play a key role in determining the conformation of nitroxides in non-aqueous and aqueous solution (Davies & Timmins, 2000; DeGray & Mason, 1994; Haire *et al.*, 1987; Janzen, 1990; Janzen & I-Ping Liu, 1973; Janzen & Lopp, 1972; Kotake *et al.*, 1979; Madden & Taniguchi, 1993; Taniguchi & Madden, 1998, 1999, 2000; Timmins & Davies, 1998). Though nitroxides may be expected to form predominantly inter-molecular hydrogen-bonds to water molecules in aqueous solution, intra-molecular hydrogen-bonds have been shown to persist in some cases (e.g. α- and β-hydroxyalkyl carbon-centred radical adducts to MNP and DMPO; Madden & Taniguchi, 1993; Taniguchi & Madden, 1998, 2000). It is also possible that distant chiral centre(s) can influence the conformation of nitroxide moieties via interactions that are mediated by the solvent (e.g. inter-molecular hydrogen-bonding). Solvent interactions are also important in determining the conformation of polar substituents on carbohydrates (e.g. pyranose C-6 -CH_2-OH groups Kirschner & Woods, 2001).

The intramolecular interactions which underlie the recognition of chirality in the *t*Bu-$NO^{\bullet}$-CH_2-O-*sugar species derived from the α- and β-methyl glycosides are likely to be similar to those which underlie the recognition of diastereomerism in the two *t*Bu-$NO^{\bullet}$-*$CH(CH_3)$-O-*sugar species derived from the β-ethyl glycoside. It is difficult to account for the influence of the remote, sugar-derived chiral centre(s) on the conformation of the nitroxide moiety in these species in terms of steric interactions: in each case, the nearest of these chiral centres is at the γ-position (the ring-derived C-1 carbon). Inspection of molecular models suggests that direct or solvent-mediated hydrogen-bonding of the nitroxide oxygen to the amide N-H hydrogen may be favourable. Direct hydrogen bonding would involve via a seven-membered ring with a rigid segment of three bonds belonging to the sugar ring. This suggestion is supported by the detection of analogous intramolecular hydrogen-bonds in structurally-related DMPO adducts of β-hydroxalkyl carbon-centred radicals (Taniguchi & Madden, 1999).

Although rare, other examples exist of chiral recognition in radicals with γ- and even δ-chiral centres (e.g. *t*Bu-$NO^{\bullet}$-CH_2-CH_2-*CH(Et)-OH (Madden & Taniguchi, 1993) and $NO_2^{-\bullet}$-CH_2-CO-NH-*$CH(CH_3)$-COO^-; Taniguchi, 1984). As with the *t*Bu-$NO^{\bullet}$-CH_2-O-*sugar adduct species described above, it is unlikely that steric interactions are responsible for chiral recognition with these adducts; inter- or intra-molecular hydrogen-bonding is a potential mechanism via which the remote chiral centre exerts its effects. In contrast to the above, numerous examples exist of chiral recognition in nitroxides with β-chiral centres (i.e. *t*Bu-$NO^{\bullet}$-CH_2-*CXYZ; see (Franchi *et al.*, 2002) and references cited therein). With these nitroxides, it is plausible that chiral recognition occurs mainly as a result of steric interactions between the nitroxide function and substituents on the β-chiral centre. However, where bulky substituents on the β-chiral centre are absent, other interactions such as hydrogen-bonding or stereo-electronic effects may also be important (e.g. *t*Bu-$NO^{\bullet}$-CH_2-*$CH(CH_3)$OH or *t*Bu-$NO^{\bullet}$-CH_2-*$CH(CH_3)$Cl; Gilbert & Trenwith, 1973).

Given that chiral recognition occurs with the *t*Bu-$NO^{\bullet}$-CH_2-O-*sugar adduct species from the α- and β-methyl glycosides where the remote chiral centre is in the γ- or greater position, it is interesting that chiral recognition does not appear to occur with the *t*Bu-$NO^{\bullet}$-CH_2*$CH(CH_3)$-O-*sugar species derived from the *β-iso*-propyl glycoside, despite the β-chiral centre on the C-1 alkyl substituent. The strength of the interaction between the nitroxide and the (chiral) sugar moiety (e.g. via inter- or intra-molecular hydrogen-bonding) may be critical in inducing chiral recognition in these adducts; such an interaction may be weaker for the *t*Bu-$NO^{\bullet}$-CH_2*$CH(CH_3)$-O-sugar species compared to the *t*Bu-$NO^{\bullet}$-CH_2-O-sugar species due to the gre-

greater distance between the nitroxide function and the sugar moiety.

With the isomeric adducts of C-2 carbon-centred radicals formed on *N*-acetylglucosamine with MNP and DMPO, and on glucosamine with DMPO (anomers plus conformer or ring-open form), resolution of distinct EPR spectra was assisted by introduction of additional non-equivalent ^{13}C-splittings upon ^{13}C-labelling of the C-2 carbons. Recognition of isomerism in these adducts is likely to involve the influence of substituents on the C-1 carbon on the conformation of the nitroxide moiety and, possibly independently, the spin density at the C-2 carbon as evidenced by differences in the ^{13}C-splittings.

CONCLUSIONS

EPR spectroscopy in combination with spin trapping with MNP and DMPO is a valuable technique in the investigation of the chemistry of carbohydrate-derived radicals. However, as is evident from the present work, the interpretation and assignment of the EPR spectra of carbohydrate-derived spin adducts can be challenging due to the complexity of the spectra obtained. The use of specifically ^{13}C-labelled carbohydrates can assist in both the identification of carbon-centred radical addends, and the analysis of complex spectra by introducing additional ^{13}C-splittings that resolve overlapping signals.

It has been shown that trapping of a "single" carbohydrate-derived radical can result in the formation of multiple isomeric adducts that give closely-related, overlapping EPR signals. The potential sources of isomerism in the spin trap adducts of carbohydrate radicals include: diastereomerism arising from addition of the spin trap to opposite faces of pro-chiral radical centres; conformational isomerism arising from restricted rotation about the nitroxide α-C-N bond; and, in the case of adducts formed on reducing carbohydrates, anomeric isomerism arising from mutarotation of the hemiacetal function. Evidence has been presented for the occurrence of each of these classes of isomerism. The recognition of adduct isomerism is attributed primarily to intramolecular interactions between the nitroxide group and the radical addend that affect adduct conformations. Similar intramolecular interactions are proposed to give rise to the non-equivalent methylene hydrogen couplings in the spectra of some carbohydrate-derived MNP adducts i.e. recognition of adduct chirality. The nature of the interactions which give rise to recognition of isomerism and chirality in these adducts is amenable to further investigation by the examination of solvent- and temperature-effects on their EPR spectra.

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