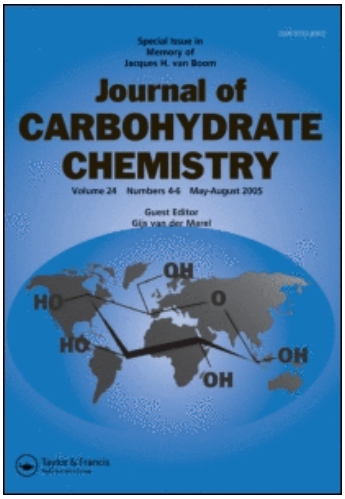

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COMPLETE ASSIGNMENT OF THE 360 MHz ^{1}H NMR SPECTRA OF SOME OLIGOMANNOSIDES

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ABSTRACT

The 360 MHz ^{1}H NMR spectra of four closely related synthetic oligomannosides have been completely assigned. This was achieved by a combination of spin-tickling difference spectroscopy and spectral simulation. The compounds are: methyl 3-O-(α-D-mannopyranosyl)-α-D-mannopyranoside (II3), methyl 6-O-(α-D-mannopyranosyl)-α-D-mannopyranoside (II6), methyl 3,6-di-O-(α-D-mannopyranosyl)-α-D mannopyranoside (III) and methyl 3-O-(α-D-mannopyranosyl)-6-O-(3-O-α-D-manno-pyranosyl-α-D-mannopyranosyl)-α-D-mannopyranoside (IV). These oligomannosides are analogues of the high mannose structures occurring naturally in the N-linked glycopeptides of glycoproteins. A number of long range chemical shift perturbations were observed which are interpreted as being

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caused by specific interactions arising from the three-dimensional structure of the oligosaccharides.

INTRODUCTION

The determination of the primary structure of glycopeptides by NMR is based on the observation that chemical shift perturbations can be attributed to well defined structural variations.[1,2] These variations may arise by direct substitution on the hexose in question or by substitution at more remote sites in the molecule. The direct substitution effects will depend on the nature of the aglycon and the glycon, and on the nature of the linkage. The longer range effects spanning more than two residues could be due to a number of causes. The possible mechanisms are: (i) proximity of two residues in space which are remote in the primary structure, (ii) a change in linkage conformation (change in the torsion angles ϕ, ψ or ω),[3] or (iii) an altered solvation cage[4] brought about by a distant substitution. The consistent observation of these long range effects strongly suggests that glycopeptides occur in well defined three dimensional forms since otherwise motional averaging would reduce these effects to a negligible level.[1,5]

Since glycopeptides are postulated to be receptors in cellular membrane interactions and in many other recognition phenomena,[6] the knowledge of their three dimensional structures is essential. However, due to the complexity of their primary structure which is reflected in the ^{1}H NMR spectra, model compounds which are analogues of glycopeptides are more suitable for a detailed analysis of their structural properties by NMR than the glycopeptides themselves. Hence, as an essential background to the nuclear Overhauser experiments about which we shall report later, we present in this paper a complete assignment of the 360 MHz ^{1}H NMR spectra

of a methyl mannotrioside, methyl 3,6-di-O-(α-D-mannopyranosyl)-α-D-mannopyranoside (III); the related disaccharides, methyl 6-O-(α-D-mannopyranosyl)-α-D-mannopyranoside (II6) and methyl 3-O-(α-D-mannopyranosyl)-α-D-mannopyranoside (II3); and a tetrasaccharide, methyl 3-O-(α-D-mannopyranosyl)-6-O-(3-O-α-D-mannopyranosyl-α-D-mannopyranosyl)-α-D-mannopyranoside (IV) (see Table 1 for structures and nomenclature). The validity of using shift increments due to different substitutions to predict the chemical shifts in glycopeptides is also discussed, and applied to the detection of long range effects in a methyl mannotrioside and a methyl mannotetraoside.

RESULTS AND DISCUSSION

Assignments of the ^{1}H NMR spectra of the mannosides in Table 1 were primarily made using spin-tickling difference spectroscopy, which consisted of a 90° pulse, with selective excitation by the decoupler of a given transition during acquisition, followed by a 90° pulse with the decoupler turned off. The two resulting free induction decays are subtracted to show the effect of the irradiating pulse on the spectrum. Any transitions which are connected to the irradiated transition by spin-spin coupling are split into doublets centred on their original frequency.[7] An example is shown in FIG. 1. Irradiation of the low-field transition of the H-6 doublet of doublets of methyl β-D-mannoside (I), results in the connected H-5 and H-6 transitions being split into doublets(FIG. 1b and 1c).

Hence, from the difference spectra obtained by irradiating each peak in the spectrum, estimates of chemical shifts and coupling constants for the various protons in the molecule can be obtained. In the methyl mannotrioside and the α1-6 linked methyl mannobioside (II6), overlapping lines and strong coupling sometimes prevented unambiguous assignments. However,

TABLE 1A

^{1}H Chemical Shifts[1] of Oligomannosides

Compound	H-1	H-2	H-3	H-4	H-5	H-6	H-6'	OMe
Mβ1-OMe βI	4.575	3.982	3.632	3.560	3.373	3.934	3.733	3.541
Mα1-OMe I	4.762	3.930	3.756	3.636	3.611	3.899	3.754	3.407
Mα1-6	4.913	3.989	3.847	3.661	3.710	3.894	3.736	
Mα1-OMe II6	4.754	3.941	3.744	3.744	3.744	3.772	3.977	3.402
Mα1-OMe II3	4.742	4.078	3.867	3.752	3.657	3.900	3.766	3.412
Mα1-3'	5.110	4.064	3.878	3.662	3.754	3.883	3.760	
Mα1-6	4.908	3.994	3.838	3.661	3.689	3.892	3.763	
Mα1-OMe III	4.729	4.089	3.856	3.898	3.804	3.737	4.017	3.407
Mα1-3'	5.097	4.062	3.879	3.665	3.760	3.883	3.761	
Mα1-3	5.154	4.069	3.885	3.661	3.763	3.880	3.760	
Mα1-6	4.894	4.141	3.944	3.783	3.735	3.894	3.774	
Mα1-OMe IV	4.730	4.088	3.860	3.905	3.804	3.749	4.013	3.408
Mα1-3'	5.099	4.069	3.885	3.661	3.763	3.880	3.760	

[1] in ppm, at 23°, relative to internal acetone set at 2.225 ppm. The average error is considered to be 0.003 ppm. Throughout the Tables, M represents a -D-mannopyranose residue

TABLE 1B

Coupling Constants[1] of Oligomannosides

Compound		$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$	$J_{5,6'}$	$J_{6,6'}$
Mβ1-OMe	βI	1.0	3.2	9.8	10.2	2.3	6.7	-12.2
Mα1-OMe	I	1.7	3.5	9.6	9.8	2.2	6.0	-12.3
II6,II3,III,IV except for the Mα1-OMe residue of II6,III,IV		1.7	3.4	9.5	9.8	2.2	6.0	-12.3
		1.7	3.4	9.5	9.8	1.8	4.5	-11.5

[1] in Hz, at 23°.

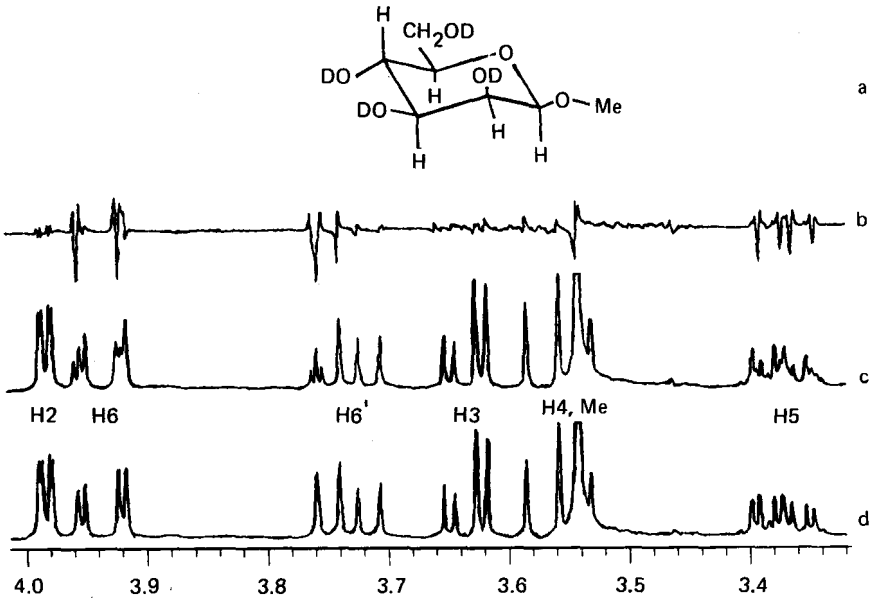
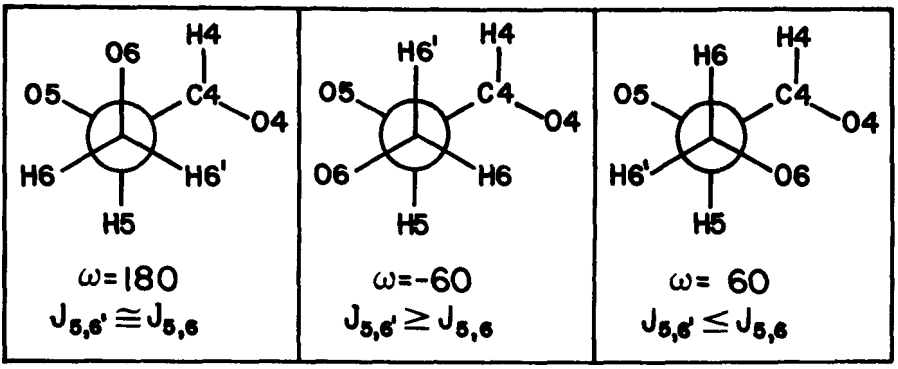


FIG. 1. SPIN-TICKLING EXPERIMENTS FOR MANβ1-OMe: (a) STRUCTURE, D REFERS TO DEUTERIUM, (b) THE DIFFERENCE BETWEEN THE SPIN TICKLED (AT 3.76 PPM) SPECTRUM, (c), AND THE NORMAL SPECTRUM, (d).

the approximate chemical shifts and coupling constants could be inferred from a comparison with the spectra of related sugars.

The chemical shifts and coupling constants thus obtained for the various compounds were then refined by a seven spin simulation using the Nicolet NTCSIM programme, until a good agreement between the observed and calculated spectra was obtained. The refined parameters obtained from the simulation of the various compounds studied are given in Table 1. Spin simulation is essential to obtain accurate chemical shifts because, in the presence of strong coupling, first order assignments can change by as much as 0.01 ppm.[8] The stereochemical assignment of the H-6 and H-6' signals in

Table 1 was based on recent studies of the rotamer population about the C5-C6 bond in several aldohexopyranoses[8,9] and from the conformational analysis of α-D(1-6) linked glucans.[3] From these studies, it can be concluded that the coupling constant $J_{5,6}$ should be smaller than the coupling constant $J_{5,6'}$. An epimerization of C-2 (i.e. in mannose) should not affect these conclusions. As a result, in Table 1, the proton signal of the hydroxymethyl group which has the smaller vicinal coupling constant is assigned to H-6 and the other one to H-6'.



Thus for an unsubstituted CH_2OH group, the H-6 signal resonates at lower field than the H-6' signal. However for a substitution at C-6 (compounds II6, III, and IV), the H-6 signal of the substituted residue occurs at higher field than the H-6' signal. This reversal can readily be explained by considering the orientation of the Manα1-6 residue with respect to the substituted hydroxymethyl group. From nuclear Overhauser experiments on the 1-6 linked compounds II6 and III and from carbon-13 enrichment of the C-1 of the Man 1-6 residue in the methyl mannotrioside, the linkage conformation about the C1-O1 and O1-C6 bonds has been determined.[10] The resulting orientation places the H-1 of the 1-6 linked pyranose ring in close proximity (2.4 A) to the H-6 of the substituted residue. Also the ring oxygen of

the αl-6 linked mannopyranoside is near (2.4 A) the H-6' of the substituted mannopyranoside. Referring to Table 1A, this orientation provides a plausible explanation for the shielding of H-6 by -0.17 ppm and the deshielding of H-6' by 0.26 ppm (see the shift increments for a 3,6 di-substitution in Table 2). The reciprocal shielding of the H-1 signal of Manαl-6 residue in compound III of -0.27 ppm (5.180 ppm in α-D-mannose vs 4.908 ppm in III) can also be readily explained by this orientation and is similar to the one deduced from the spectra of the α-D(1-6)-linked glucans.[11]

Some coupling constants also underwent changes upon a substitution at C-6 (Table 1B). The vicinal coupling constants $J_{5,6}$ and $J_{5,6'}$ changed from 2.2 and 6.0 Hz to 1.8 and 4.5 Hz respectively, while the geminal coupling constant, $J_{6,6'}$ changed from -12.3 to -11.5 Hz. These changes are generally attributed to a redistribution in the rotamer population about the C5-C6 bond upon a C-6 substitution.

The spin simulations for the three residues in the trisaccharide (III) shown in FIG.2b. As can be seen, they reproduce quite satisfactorily the observed spectrum in FIG. 2a. The disaccharides were invaluable in assigning the methyl mannotrioside spectrum, since as can be observed in Table 1, the chemical shifts to the terminal mannose residues in the trisaccharide were quite similar to those of their respective disaccharides.

A practice often employed in the analysis of the spectra of complex glycopeptides is the summation of shift increments, derived from similar compounds, to predict the chemical shifts of more complex structures.[12] This approach assumes that the effect of each substitution is independent of all others. However, as shown in Table 2, the shift increments for the Man 1-OMe residue of the methyl mannotrioside are not the

TABLE 2

Shift Increments on Substitution of the Man 1-OMe Residue

	Shift increments in ppm						
	H-1	H-2	H-3	H-4	H-5	H-6	H-6'
(II6 - I)[1] = 6	-.008	.011	-.012	.108	.133	-.127	.223
(II3 - I)[1] = 3	-.020	.148	.111	.116	.046	.001	.012
(III - I)[1] = 3,6	-.033	.159	.100	.262	.193	-.172	.263
Δ3 + Δ6	-.028	.159	.099	.224	.179	-.126	.235
Δ3,6 - (Δ3 + Δ6)	-.005	.000	.001	.038	.014	.046	.030

[1] chemical shifts of the Manα1-OMe residue in Table 1A

TABLE 3

Predicted and Observed Chemical Shifts for the Manα1-6 Residue in the Methyl Mannotetraoside

	Chemical shifts in ppm						
	H-1	H-2	H-3	H-4	H-5	H-6	H-6'
Predicted: III[1] + Δ3	4.888	4.142	3.949	3.777	3.735	3.893	3.775
Observed: IV[1]	4.894	4.141	3.944	3.783	-[2]	-[2]	-[2]

[1] chemical shifts of the Manα1-6 residue in Table 1A
[2] strong coupling and overlapping signals prevented accurate assignments

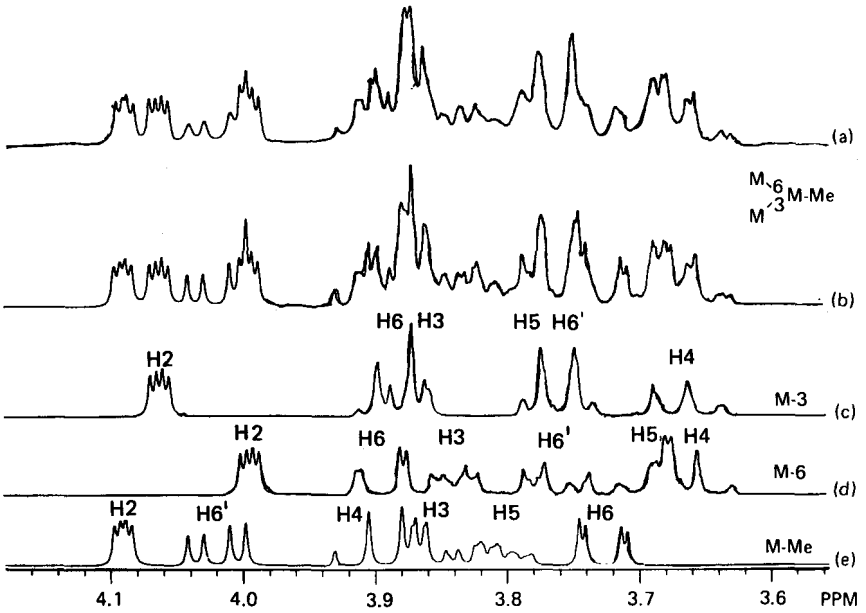


FIG. 2. METHYL MANNOTRIOSIDE: (a) OBSERVED SPECTRUM, AND, (b) SIMULATED SPECTRUM. THE CONTRIBUTIONS TO THE SIMULATED SPECTRUM ARE SHOWN IN (c), FOR THE MANα1-3 RESIDUE, IN (d) FOR THE MANα1-6 RESIDUE, AND IN (e), FOR THE MANα1-OMe RESIDUE.

sums of its constituent disaccharide shift increments. For the H-1, H-2 and H-3 signals they agreed, but for the rest they differed by as much as 0.04 ppm. The breakdown of this method arises most probably from long range interactions.

The H-5 chemical shift of the Man 1-6 residue also differed by 0.02 ppm in the disaccharide (II6) and the trisaccharide (III). Since this proton and H-4, H-5, H-6 and H-6' of the Manα1-OMe residue are in the locality of the α1-6 linkage, the poor agreement between the observed and the predicted chemical shifts can be attributed to an altered conformation about this glycosidic bond or to a

change in the population of conformers about this bond. Although changes in rotamer population about the C5-C6 bond should be reflected in changes of $J_{5,6}$ and $J_{5,6'}$, the H-6 and H-6' resonances of II6 were too severely broadened by virtual coupling effects[8] and their coupling constants could not be accurately measured.

Since most of the chemical shifts of the Manα1-3 and Manα1-6 residues in III are the same as those found for their respective disaccharides, these two residues are unlikely to be in close proximity in the methyl mannotrioside. However, chemical shift perturbations are still observed in the locality of the α1-6 linkage of III. One possible explanation for this long range effect could be a different orientation of the hydroxyl group at C-4 of the Man 1-OMe residue in the trisaccharide and the disaccharide II6. This mechanism termed 'conformational transmission' was initially proposed[13] to explain some long range chemical shift perturbations observed in the ^{1}H NMR spectra of glycosphingolipids. The different conformation of the hydroxyl group at C-4 might also lead to an altered solvation cage, since hydroxyl groups in sugars appear to be extensively hydrogen bonded with surrounding water molecules.[4]

In FIG. 2c and 2d strong coupling and virtual coupling effects clearly lead to line broadening and the appearance of new transitions. However, the H-6 and H-6' signals of the Manα1-OMe residue show an anomalous line broadening (FIG. 2a vs FIG. 2b). This might be due to long range coupling, although the other signals of the Manα1-OMe residue do not appear to be broadened. Conformational averaging at an intermediate rate about the α1-6 bond might also lead to such an effect.

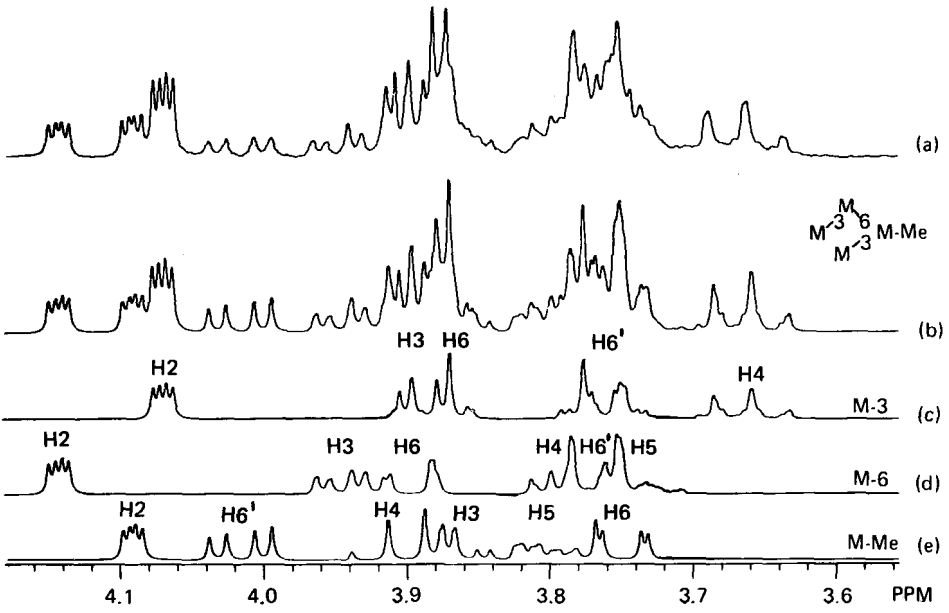


FIG. 3. TETRAMANNOSIDE SPECTRA: (a) OBSERVED SPECTRUM AND (b) SIMULATED SPECTRUM. THE CONTRIBUTIONS TO THE SIMULATED SPECTRUM ARE SHOWN IN (c) FOR THE TWO MANα1-3 RESIDUES, IN (d), FOR THE MANα1-6 RESIDUE, AND IN (e) FOR THE MANα1-OMe RESIDUE.

The observed and the simulated spectra for the methyl mannotetraoside are shown in FIG. 3. Due to strong coupling between the transitions of H-5 and H-6' in the Manα1-6 and the two Manα1-3 residues, these signals could not be assigned accurately. Their chemical shifts were inferred from those of the methyl mannotrioside and the shift increments for an α1-3 substitution (Table 3). Because of these limitations there is less agreement between the observed and the simulated spectra near 3.76 ppm (FIG. 3a vs. FIG. 3b).

The linkage conformation for the two Manα1-3 residues was deemed to be similar because, except for the H-1 resonances, the chemical shifts for the H-2, H-3 and H-4 signals were

found to be identical by spin tickling experiments. Also, as can be noted in Table 3, for those protons of the 3-O-substituted Manα1-6 residue which could be located accurately by spin tickling, the observed chemical shifts are in good agreement with those predicted from shift increments. The difference of 0.055 ppm between the two H-1 resonances of the Manα1-3 residues is attributed to a long range shielding effect of the methoxy group. This interpretation is supported by the observation of substantial differences in chemical shifts between Manα1-OMe (I) and Manα1-6 of II6 (Table 1). In particular, for the H-3 resonances the difference is 0.091 ppm. Since, for an α1-3 linkage, the H-1 of the glycon is proximal to the H-3 of the aglycon,[10] the H-1 signal should also be affected but to a lesser extent, as observed.

Large perturbations on the resonances of four protons in the locality of the α1-6 linkage in the methyl mannotrioside were detected from a comparison of the chemical shifts of the methyl mannotrioside with those of the α1-6 linked disaccharide (Table 2). These perturbations were attributed to a change in the linkage conformation about the α1-6 bond as a result of the substitution at C-3 on the aglycon. For the methyl mannotetraoside (IV) compared to the methyl mannotrioside III, perturbations occur only for the H-4 and H-6' signals of the Manα1-OMe residue (0.027 ppm and 0.022 ppm respectively). These differences are much less pronounced than the ones observed between the disaccharide II6, and the trisaccharide (Table 2). Hence, the biggest alteration in the conformational equilibrium about the α1-6 linkage was not brought about by the substitution on the glycon (i.e., C-3 substitution on the Manα1-6 residue), but by the substitution on the aglycon (i.e., C-3 substitution on Manα1-OMe).

In conclusion, the use of this series of closely related synthetic mannosyl oligosaccharides has permitted the complete assignment of their 360 MHz ^{1}H NMR spectra. These assignments, in turn, have revealed the existence of several chemical shift perturbations which are related to alterations in the Man 1-6 linkage conformation.

EXPERIMENTAL

Methyl α-D-mannoside was obtained from Sigma (St. Louis, Mo). Methyl β-D-mannoside and the other oligomannosides shown in Table I were synthesized in our laboratory.[14,15]

For ^{1}H NMR, solutions of the sugars (previously exchanged twice with D_2O) were prepared in 99.96% D_2O (Merck, Sharpe and Dohme, Montreal). ^{1}H NMR spectra were recorded at 23 ± 1°C on a 360 MHz Nicolet spectrometer at the Toronto Biomedical NMR Centre, using a spectral width ± 500 Hz and 8K data points resulting in an average error of 0.2 Hz. For a 10mM solution of sugar, 128 transients were accumulated for normal and spin-tickling difference spectroscopy. A pulse angle of 90° was used in all cases and the free induction decays were exponentiated with a line broadening factor of 0.1. Chemical shifts were measured relative to internal acetone at 2.225 ppm from internal DSS. The spin simulations were performed on a Nicolet 1180 computer using the Nicolet programme NTCSIM. A linewidth of 1.2 Hz was used in all cases.

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