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A COMPARATIVE ^{1}H-N.M.R. STUDY OF MurNAc–L-Ala–D-iGln (MDP) AND ITS ANALOGUE MURABUTIDE: EVIDENCE FOR A STRUCTURE INVOLVING TWO SUCCESSIVE β-TURNS IN MDP

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ABSTRACT

The active principle, MurNAc–L-Ala–D-iGln (MDP), of complete Freund's adjuvant and its analogue, MurNAc–L-Ala–D-Gln-O*n*Bu (murabutide), which express immunomodulatory as well as other biological properties, have been studied by 2D-^{1}H-n.m.r. spectroscopy at 500 MHz. The results suggest the presence in MDP of two successive turns involving the MurNAc–L-Ala and L-Ala–D-iGln moieties, respectively, whereas only the former turn persists in murabutide. This turn mimics the type II β-turn found in L-D depsipeptides, whereas the other is a typical type II β-turn for L-D peptides.

INTRODUCTION

The active principle of Freund's complete adjuvant is a portion of the mycobacterial peptidoglycan sub-unit, namely, *N*-acetylmuramyl–L-alanyl–D-isoglutamine (MurNAc–L-Ala–D-iGln or MDP)[1]. This simple glycopeptide can elicit immunomodulatory and other biological effects[2,3], and there have been numerous studies of structure–activity relationships[3,4] which help to predict the biological status of new molecules. Thus, some analogues are adjuvant without being active against infectious challenge, and *vice versa*, whereas others are more effective against tumours. Moreover, in certain analogues, including *N*-acetylmuramyl–L-alanyl–D-glutamine-*n*-butyl ester (MurNAc–L-Ala—D-Gln-O*n*Bu or murabutide), the immunomodulating activity is dissociated from the unwanted pyrogenic activity[5].

The few ^{13}C- and ^{1}H-n.m.r. studies of MDP indicate the possible existence of non-random structures in solution, although no precise information has

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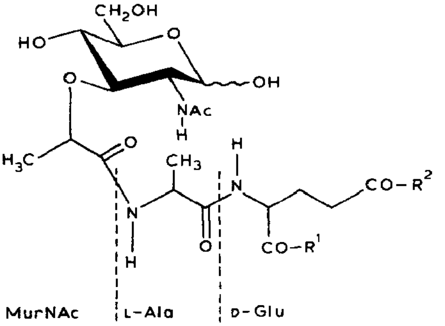

1	$R^1 = NH_2$, $R^2 = OH$	(MDP)
2	$R^1 = O(CH_2)_4H$, $R^2 = NH_2$	(Murabutide)
3	$R^1 = OH$, $R^2 = OH$	(MDPA)
4	$R^1 = OH$, $R^2 = NH_2$	(MDPG)
5	$R^1 = NH{-}Me$, $R^2 = OH$	(MDPA methylamide)

emerged[6–9]. If a structural basis for the biological functions of MDP could be inferred from spectroscopic studies, it would be of value for the design of analogues.

We now report a 500-MHz ^{1}H-n.m.r. study of MDP (**1**) and murabutide (**2**).

RESULTS

The $\alpha\beta$-ratio for fresh solutions of MDP and murabutide in $(CD_3)_2SO$, derived from the integrated intensities of various proton resonances, was similar to that found[6,9] for solutions of MDP in water and methyl sulfoxide. However, the $\alpha\beta$-ratio, which remains constant for aqueous solutions, changes with time for solutions in methyl sulfoxide, although the rates of $\beta \rightarrow \alpha$ conversion were not the same in MDP and in murabutide: at 20°, equilibration required 3 days for the former and 1 month for the latter. This effect is being investigated further.

The chemical shift data are given in Table I. Some of the values can be compared to those previously reported[8,9]. The resonances of the L-Ala, but not of the D-iGln residue were affected by the α- and β-sugar moieties. The resonances of the pyran ring of the α-anomer were generally at lower field than those of the β-anomer. In both the α and the β forms, the HO-4,6 resonances were broad for MDP, but narrow for murabutide. The resonances for the amide protons were found at higher field for the β-anomers, possibly reflecting differences in hydrogen bonding.

The coupling constants are reported in Table II. For the pyranose ring, the values of $J_{2,3}$ were 0.4 Hz larger in the α-anomers. For the peptide backbone, the $^3J_{H,NH}$ values also reflected the influence of the α,β-anomerism on the N-acetyl group, whereas the L-Ala residue was affected only weakly and the second amino

TABLE I

CHEMICAL SHIFT DATA FOR THE GLYCOPEPTIDES [$(CD_3)_2SO$, INTERNAL Me_4Si, 20°]

Moiety	*Proton*	*Murabutide*		*MDP*	
		α-anomer	*β-anomer*	*α-anomer*	*β-anomer*
GNAC	H-1	4.92	4.40	4.95	4.41
	H-2	3.64	3.47	3.66	3.5
	H-3	3.44	3.26	3.44	3.28
	H-4	3.24	3.18	3.24	3.18
	H-5	3.58 } 2nd	3.09	3.6 } 2nd order	3.11
	H-6	3.61 } order	3.67		3.67
	H-6′	3.50	3.45	3.49	3.45
	HO-1	6.57	6.68	~6.6b	
	HO-4	5.21	5.23	~4.24b	
	HO-6	4.48	4.60	vb	
	Ac NH	8.06	7.93	8.05	7.93
	Ac CH_3	1.78	1.77	1.78	1.77
D-Lactoyl	Hα	4.29	4.18	4.26	4.13
	CH_3	1.25	~1.25	1.24	1.23
L-Alanyl	NH	7.60	7.49	7.63	7.51
	Hα	4.35	4.31	4.26	4.22
	CH_3	1.22	1.22	1.21	1.24
D-Glx	NH	8.40	8.37	8.17	8.1
	Hα	4.18		4.13	4.14
	Hβ	1.94		1.94	1.94
	Hβ′	1.74		1.69	1.69
	Hγ,γ′	2.11		2.21	2.20
	$CONH_2$ { H_E	γ { 7.28		α { 7.33	
	$CONH_2$ { H_Z	γ { 6.80		α { 7.06	7.11
n-Butyl	H-1,1	4.03			
	H-2,2	1.54			
	H-3,3	1.31			
	H-4,4,4	0.88			

acid residue was not affected. Conversely, the effects of the chemical modification of the D-Glx residue did not extend beyond the L-Ala residue. One $^3J_{H,OH}$ value conformed to a non-averaged structure around the sugar-OH bond, namely, that (4.29 Hz) for $J_{1,HO\text{-}1}$ (*cf.* 6–6.5 Hz for the other hydroxyl groups) in the α-anomer. In the D-Glx unit, the *J* values (Table IId) showed that the side chain was involved in the conformational difference between MDP and murabutide.

The temperature-dependence coefficients of the resonances of the amide protons are given in Table III. The values are in the range -5×10^{-3} to -0.7×10^{-3} p.p.m./K. Murabutide showed similar behaviour with the differences depending on the α and the β configurations except for the D-Glx residue. Usually,

TABLE II

$^3J_{H,H}$ VALUES (Hz) FOR THE GLYCOPEPTIDES [$(CD_3)_2SO$, 20°]

(a) Amide protons

Compound	N-*Acetyl* J_{NH,CH_2}		L-*Alanyl* $J_{NH,H\alpha}$		D-*Glx* $J_{NH,H\alpha}$	
	α	β	α	β	α	β
Murabutide	7.9	8.62	7.93	7.77	7.65	7.67
MDP	7.95	8.7	7.03	6.93	8.2	8.3

(b) Sugar ring[a]

Compound	$J_{1,2}$		$J_{2,3}$		$J_{3,4}$		$J_{4,5}$		$J_{5,6}$		$J_{5,6'}$	
	α	β	α	β	α	β	α	β	α	β	α	β
Murabutide	3.38	8.2	10.7	10.3	8.7	8.65	9.6	9.5	5.0	2.2	5.0	5.7
MDP	3.34	8.28	10.8	10.4	8.6	8.6	10 ±0.2	9.8 ±0.2	5.0	2.2	5.0	5.9

(c) Hydroxyl protons

Compound	$J_{1,HO\text{-}1}$		$J_{4,HO\text{-}4}$		$J_{6,HO\text{-}6}$		$J_{6',HO\text{-}6'}$	
	α	β	α	β	α	β	α	β
Murabutide	4.29	6.08	6.75	6.45	5.5 or 6.3	5.8	5.5 or 6.3	5.8
MDP	Not observed (broad signals)							

(d) Side chains

Compound	D-*Lactoyl* $J_{H\alpha,Me}$		L-*Alanyl* $J_{H\alpha,Me}$		D-*Glx* $J_{\alpha,\beta}$		$J_{\alpha,\beta'}$		$J_{\gamma,\beta} = J_{\gamma,\beta'}$	
	α	β	α	β	α	β	α	β	α	β
Murabutide	6.7	6.85	7	7	5.0	N.o.[b]	9.4	N.o.	7	7
MDP	6.7	6.7	7.1	7.1	5.1	5.1	9.3	9.3	8.1	8.1

[a] Unless stated otherwise, the precision is ±0.1 Hz. [b] N.o., not observed.

for the amide protons, the smaller chemical shifts corresponded to the weaker temperature-dependence coefficients and this is reflected by the resonances of the amide protons of the *N*-acetyl group and L-Ala residue, when the α and the β forms are compared.

TABLE III

TEMPERATURE-DEPENDENCE COEFFICIENTS OF THE AMIDE PROTONS (× 10^{-3} p.p.m./K) IN THE GLYCOPEPTIDE

Compound	N-*Acetyl*		L-*Alanyl*		D-*Glx*		*CONH*$_2$[a]	
	α	β	α	β	α	β	E[b] α	Z β
Murabutide	−5.3	−2	−2.6	−0.7	−5	−4.9	−3.7	−4.7
MDP	−5.3	−2.25	−2.6	−0.7	−5.3	−5.3	−4.1	−4.6 −4

[a]α-Oxamide in MDP and γ-oxamide in murabutide. [b]*E* amide proton *anti* to oxygen of carbonyl.

DISCUSSION

The most useful parameters for depicting the conformations of MDP and murabutide are the $^3J_{\mathrm{H,NH}}$ values, which reflect the dihedral angles in the peptide moiety, and the $^3J_{\mathrm{H,H}}$ values associated with the pyranose ring. The latter moiety determined the conformation of the MDP molecule through its influence on the spatial orientation of the *N*-acetyl and lactoyl groups at positions 2 and 3, respectively.

The conformation of the pyranose ring in MDP was derived using the relationship between coupling constants and dihedral angles established by Deber *et al.*[11] (Table IV). The principal conformations for solutions in methyl sulfoxide were similar to those reported[9] for aqueous solutions of MDP and several analogues. The alternation of positive and negative θ values shows that the ring preferentially adopted the $^4C_1$ conformation and that the ring in the β-anomer was more flattened than that in the α-anomer and also more than that found in *N*-acetyl-α-D-muramic acid in the solid state[12] (Table IV). Although the *N*-acetyl and the lactoyl groups were both equatorial, their spatial locations differed in the α- and the β-anomers, because of the differing influence of HO-1 on the vicinal *N*-acetyl group.

TABLE IV

DIHEDRAL ANGLES (DEGREES) IN THE SUGAR RING OF MDP COMPARED TO THOSE OF *N*-ACETYLMURAMIC ACID (NAM) IN THE SOLID STATE

Pyranose		θ^1 *O-5–C-1–C-2–C-3*	θ^2 *C-1–C-2–C-3–C-4*	θ^3 *C-2–C-3–C-4–C-5*	θ^4 *C-3–C-4–C-5–O-5*
NAM		62	−59	53.7	−49.5
MDP	α-anomer	60	−41	25	−35
	β-anomer	24	−36	25	−34

TABLE V

POSSIBLE DIHEDRAL-ANGLE VALUES (DEGREES) DERIVED FROM THE $^3J_{NH,H}$ VALUES IN MDP AND MURABUTIDE

Compound	N-*Acetyl*[a]		L-*Alanyl*		D-*Glx*	
	α	β	α	β	α	β
Murabutide	−86, −154	−92, −148	−86, −154	−85, −155	+84, +156	+84, +156
MDP	−86, −154	−92, −148	−81, −159	−80, −160	+88, +152	+88, +152

[a] In the *N*-acetyl moiety, ϕ corresponds to C′N–C-2,3.

The dihedral angle ϕ for C-3–C-2–NCO was derived from published relationships[13,14], and the possible values shown in Table V confirm that the *N*-acetyl group did not have the same spatial orientation in the α- and the β-anomers.

The geometry of the D-lactoyl group was not easily amenable to ^{1}H-n.m.r. study since it has no vicinal protons. However, X-ray analysis showed[15,16] that this group is accommodated in the second part of a β-turn with dihedral angles of ~90° for ϕ and −10° for Ψ.

The dihedral angles which could be adopted by the L-Ala and the D-Glx residues are given in Table V. There were only subtle differences between the various α- and the β-anomers, yet the two compounds displayed different molecular conformations as shown by the possible ϕ values for L-Ala in MDP (−81°, −159°) and murabutide (−86°, −154°). For the D-Glx residue, the site of the chemical modification, the possible values of ϕ were 88° and 152° in MDP, which became 84° and 156° in murabutide.

The dihedral angles and the temperature coefficients for MDP and murabutide can be interpreted in terms of folded structures stabilised by intramolecular hydrogen-bonds. The L-Ala residue and the *N*-acetyl group donate the hydrogen bonds. However, their role could be distinguished in the α- and the β-anomers. For instance, the hydrogen bond involving the *N*-acetyl group was formed according to temperature coefficients (-2×10^{-3} p.p.m./K) only in the β-anomer. There are two plausible acceptors in the backbone, namely, the lactoyl carbonyl group and HO-1. The former results in a hydrogen bond involving an 8-membered ring (Fig. 1a) similar to that found in crystalline *N*-acetyl-α-D-muramic acid[12] and also in the type IV β-turn present in cyclo[(D-isoleucyl–lactoyl–isoleucyl–D-hydroxyisovaleryl)$_2$][17]. The second possible acceptor is HO-1. After a rotation of ~180° of the *N*-acetyl group, HO-1 replaces the 8- with a 5-membered ring (Fig. 1b).

The hydrogen bond involving the amide proton in the L-Ala is stronger in the β- (temp. coeff., -0.7×10^{-3} p.p.m./K) than in the α-anomer (temp. coeff., -2.6×10^{-3} p.p.m./K). However, the *N*-acetyl carbonyl group is the best acceptor of the hydrogen bond forming a 10-membered ring (Fig. 1b). This folded structure, which

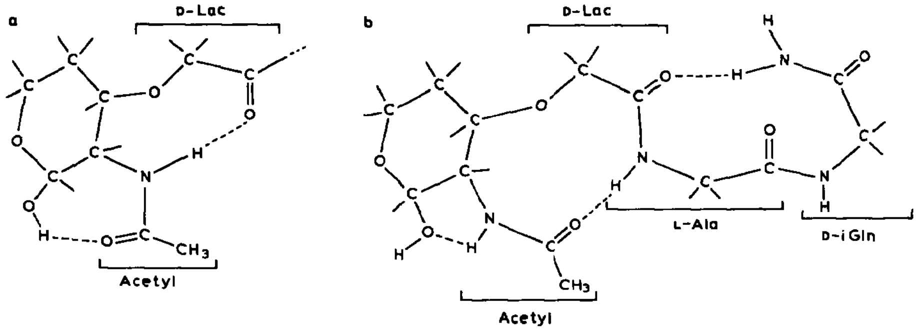

Fig. 1. Possible structures of MDP: (*a*) 7- and 8-membered hydrogen-bonded structures in the β-anomer, (*b*) "S" shape structure with two successive β-turns stabilised with a 10-membered hydrogen-bonded structure for both the α- and β-anomers and a 5-membered ring involving HO-1 and the *N*-acetyl amide proton.

is similar to that found by theoretical studies in disaccharide–peptides[18,19], has the same stability in MDP and murabutide, despite the large rotation that the butyl ester produces at the C-terminus on the N–Cα bond (φ angle) of the L-Ala residue.

The 10-membered ring has similarities with the type II β-turns found in peptides and depsipeptides. Only the L-amino acid occupying the first corner position (i + 1 residue) is replaced by the -N–C-2,3 moiety arising partly from the *N*-acetyl group and partly from the pyranose ring. The N and C-3 atoms are similar to Nα (amino) and C′ (carbonyl), and C-2 mimics Cα of the L-amino acids. At the second corner of the turn, the D-lactoyl residue is similar to that in L-Pro–D-Lac depsipeptides[15,16]. The possible angles for this structure in the α-anomer of MDP are given in Table VI. The lower stability of the above structure compared to that found in the β-anomer can be explained by the geometrical differences observed for the sugar and the *N*-acetyl group. They are more or less favorable for the formation of the 10-membered ring and therefore for the stability of the turn.

While there is no evidence from the temperature-variation experiments for a hydrogen-bonded structure in the C-terminal part of MDP (the amide protons in

TABLE VI

THE VALUE (DEGREES) OF THE φ, Ψ DIHEDRAL ANGLES IN THE "S"-SHAPE STRUCTURE OF MDP

First turn				*Second turn*			
NAG		D-*Lactoyl*		L-*Alanyl*		d-*iGln*	
ϕ_1[a]	Ψ_1[b]	ϕ_2	Ψ_2	ϕ_1[a]	Ψ_1	ϕ_2[a]	Ψ_2
−86	101	90	−10	−81	120	88	0

[a] The φ experimental values have been selected from Table V. [b] In NAG, Ψ_1 corresponds to N,C-2–C-3,O. Its value has been derived from θ_2 C-1,2–C-3,4 41° (Table IV).

the α-$CONH_2$ group and the Glx residue display temperature coefficients of -5×10^{-3} p.p.m./K), the conformational changes produced on the L-Ala residue by the modification of MDP to give murabutide strongly suggests the implication of the L-Ala–D-iGln dipeptide in a geometrically defined conformation stabilised by long-range interactions. According to the $^3J_{H,NH}$ values, this conformation corresponds to a type II β-turn. It was characterised by the experimental (ϕ) and model-derived (Ψ) values of −80°, 120° in L-Ala and 90°, −15° in D-iGln (Table VI). Within this geometry, the *E* proton (*anti* to the carbonyl oxygen) in the α-$CONH_2$ group may interact with the D-lactoyl carbonyl group, again giving a 10-membered hydrogen-bond ring.

The occurrence of a second 10-membered hydrogen-bond ring in MDP is not supported by the temperature coefficients. In the analogues MDPA (**3**), MDPG (**4**), and MDPA(αCONHMe) (**5**), where D-iGln has been replaced with D-Glu, D-Gln, and D-iGln-α-CONHMe, respectively, there was a change in the ϕ angle of L-Ala in **3** and **4** which is similar to that found in murabutide, whereas there was no change in **5** (Table IIa). The proton in the α-CONHMe group of **5** formed a hydrogen bond as shown by its temperature coefficient of -3×10^{-3} p.p.m./K, which did not occur in MDP, probably, in part, due to the differences in solvation or in the dihedral angles involved in the formation of the hydrogen bond.

All the compounds in which the α-amide group was replaced had the same backbone conformations, which were different from those of MDP but identical to those of murabutide. No particular structural role was assigned to the D-Glx side-chain nor did the bulky *n*-butyl ester group appear to be involved in murabutide.

Thus, several results were consistent with a succession of two β-turns in MDP, giving an "S" shape to the molecule. Whether the second turn was stabilised by a 10-membered hydrogen-bond ring is still unclear. L-Ala is located at the end of the first turn and at the first corner of the second turn, and plays a crucial structural role although it is the peptide bond linking lactoyl to alanine in the center of the molecule. Similar successions of turns have been described for synthetic short peptides and poly(α-amino acids) built up with the alternation of D- and L-residues[20] and they have been proposed for the linear L,D peptide Gramicidin to explain some of its pore-forming properties[18,20]. This kind of structure could explain the involvement of MDP in ion transport through the lipid membranes of the target cells, as in the macrophages[21].

The deletion of the second turn and its consequences on the geometry of L-Ala in murabutide could explain some of its properties, as compared to those of MDP[5]. This hypothesis is being assessed with the help of a large series of analogues which display dissociated activities.

EXPERIMENTAL

Materials. — The syntheses of MDP, MDPA, MDPA-α-methylamide, MDPG[10], and murabutide[5] have been reported.

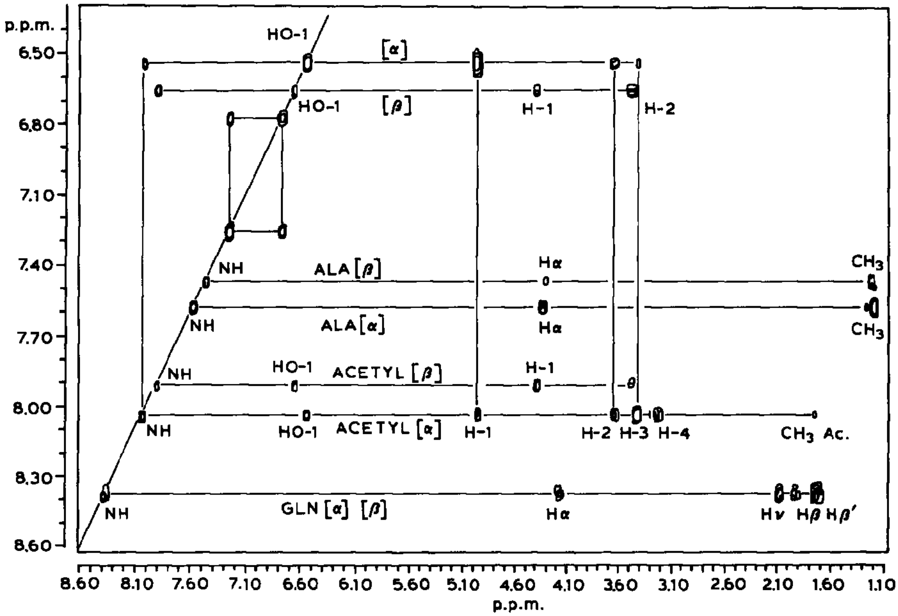

Fig. 2. Double-relay homonuclear correlation (amide region) of murabutide in $(CD_3)_2SO$. Assignments for the α- and the β-anomers.

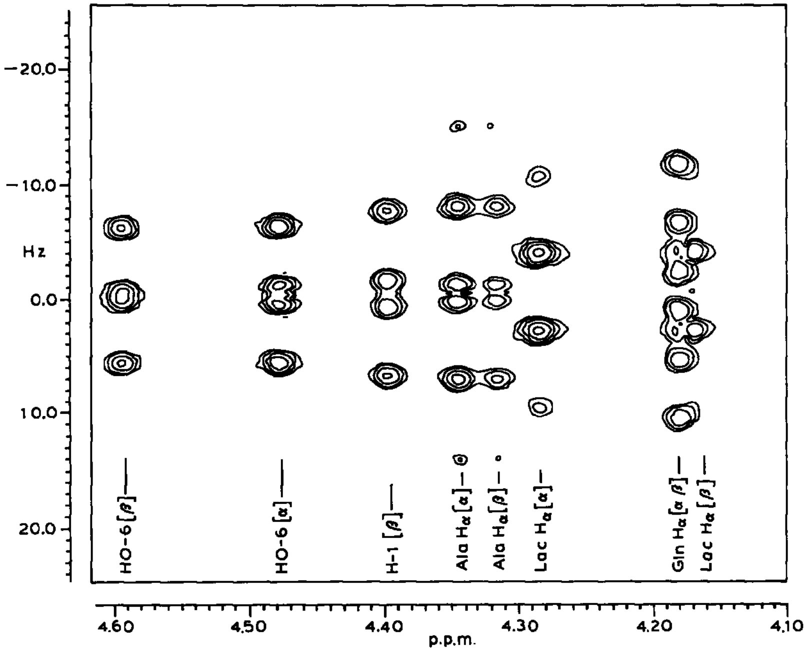

Fig. 3. Homonuclear J-resolved partial-contour plot of D_2O-exchanged murabutide in Me_2SO for both the α- and β-anomers.

Methodology. — 0.01M Solutions of MDP (as the free acid), murabutide, and other analogues in $(CD_3)_2SO$ containing a trace of Me_4Si were used. Exchange of labile protons, when necessary, was effected by three successive lyophilisations of solutions in D_2O.

^{1}H-N.m.r. spectra (500.13 MHz) were recorded with a Bruker WM-500 spectrometer at temperatures in the range 290–330 K (±0.5 K). Spectra collected using 32k data points were processed with an optimised gaussian window. The *J* values are accurate to ±0.1 Hz.

Complete spectral assignment was performed using 2D techniques. For homonuclear correlation, 1024 × 1024 data-point matrices were used. Contour plots were shown in absolute value modes after processing with square sine bell windows. COSY (45° mixing pulse) and single- and double-relay correlation experiments[22] allowed a complete and unequivocal assignment of all protons. Fig. 2 shows a typical partial-contour plot of a double-relay experiment.

Coupling constants were obtained from selected high-resolution experiments under homonuclear decoupling. Complete analysis of the overcrowded sugar-proton region could be achieved only by using homonuclear *J*-resolved experiments (Fig. 3).

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