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STRUCTURE ELUCIDATION OF THE CORE OCTASACCHARIDE FROM *Citrobacter* PCM 1487 WITH THE AID OF 500-MHz, TWO-DIMENSIONAL PHASE-SENSITIVE CORRELATED, RELAYED-COHERENCE TRANSFER, DOUBLE-QUANTUM, TRIPLE-QUANTUM FILTERED, AND N.O.E. ^{1}H-N.M.R. SPECTRA

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ABSTRACT

The main features of the primary structure of the octasaccharide, α-D-Glc*p*-(1→2)-α-D-Glc*p*-(1→2)-[α-D-Gal*p*NAc-(1→3)]-α-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→3)-[α-LD-Hep*p*-(1→7)]-α-LD-Hep*p*-(1→3)-α-LD-Hep, have been determined in the *ab initio* manner by ^{1}H-n.m.r. spectroscopy without resorting to biochemical methods of analysis. Several nontypical interresidue n.O.e. values point to a preferred solution conformation of the molecule.

INTRODUCTION

Recently, we reported the determination of the primary structure of the title octasaccharide **1** by methylation analysis–mass spectrometry, chemical degradations, and simple methods of n.m.r. spectroscopy[1]. The purpose of the present paper is to demonstrate that the main features of the structure can be elucidated more easily by use of the newer n.m.r. techniques alone, without resorting to other methods. Whereas the previous sample was inhomogeneous to some extent owing to a partial splitting off of the diphosphorylethanolamine residue, the oligosaccharide reported herein was completely dephosphorylated.

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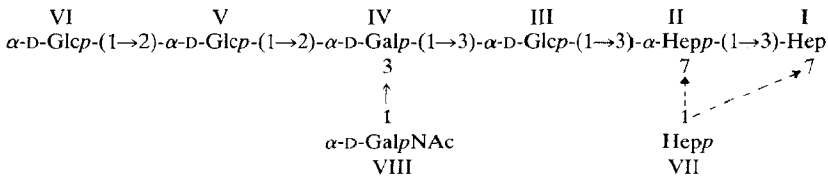

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The first step in the structural elucidation of an oligosaccharide consists in the recognition of n.m.r. signals belonging to closed-spin systems, *i.e.*, to individual sugar residues, and this can be best achieved with the aid of correlated spectroscopy. Since all nonanomeric proton signals of native oligosaccharides are located in a narrow spectrum region, COSY spectra[2] are usually inadequate in this respect. In such cases, relayed coherence transfer[3] (RCT) and double-quantum[4,5] (DQ) spectroscopy can help continue the tracing of the connectivity paths lost in the crowded areas of a COSY spectrum.

For the identification of the sugar components and their anomeric configuration, the coupling constants between neighboring protons around the sugar ring must be determined. A useful tool to achieve this goal is the new technique of pure absorption, phase-sensitive, shift-correlated spectroscopy[6] (PS-COSY).

Once individual resonances have been assigned to specific sugar residues, then the sites of glycosidic linkage and the sequence can be determined by searching for n.O.es, between each of the anomeric protons, and the relevant protons of the adjacent glycosidically linked sugar residue in a one-[7] or two-dimensional[8] n.O.e. (NOESY) spectrum. Moreover, these and other interresidue n.O.es. provide a basis for the elucidation of a three-dimensional structure of the oligosaccharide[9].

RESULTS AND DISCUSSION

The one-dimensional ^{1}H-n.m.r. spectrum (Fig. 1) showed the resonances of the anomeric protons between δ 4.9 and 5.9, the signal of an acetyl group of an amino sugar at δ 2.04, as well as the remaining resonances strongly overlapping between δ 3.4 and 4.4. The location of the anomeric proton signals in low field, as well as the small $^3J_{1,2}$ coupling constants suggested that all sugar components are α linked.

Further protons could be assigned with the aid of COSY[2,10] which was obtained in the phase-sensitive mode[6,10,12] and will be discussed in more detail farther below, and relayed-COSY spectra[3,10–12]. Starting from a given proton resonance, cross-peaks leading not only to the next one, but also to the next but one proton can be seen, *e.g.*, from H^{IV}-1 to H^{IV}-2 *and* H^{IV}-3. Therefore, after H-2 has been determined in a COSY spectrum, H-3 can be assigned in a relayed-COSY spectrum in the anomeric region (Fig. 2). Sometimes, an otherwise invisible long-range coupling may extend the correlation beyond the expected two-step level. This may

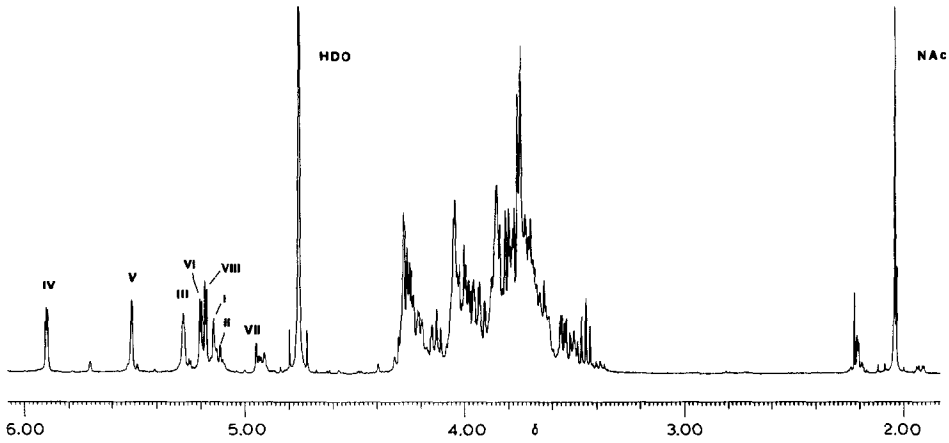

Fig. 1. 1D 500-MHz ^{1}H-n.m.r. spectrum of the dephosphorylated core octasaccharide (**1**) from *Citrobacter* PCM 1487; see scheme for numbering of ^{1}H of residues. The acetone peak was set at δ 2.225 as internal standard.

cause confusion, as in the case of Glc^V, for which a cross-peak originating from long-range magnetization transfer, V^1_4, was at first mistaken for the expected V^1_3 one (for labelling, see figure legends). The H^V-3 resonance, which partly overlaps with the H^V-2 one, was then correctly assigned in the phase-sensitive COSY spectrum *(vide infra)*. By measuring double- and triple-relayed COSY spectra, the signals for H-4 and H-5 could also be assigned. Fig. 3 shows the anomeric region of

TABLE I

CHEMICAL SHIFTS (δ) AND COUPLING CONSTANTS (Hz) OF OCTASACCHARIDE **1**

Chemical shift	*Hep(I)*	*Hep(II)*	*Glc(III)*	*Gal(IV)*	*Glc(V)*	*Glc(VI)*	*Hep(VII)*	*GalNAc(VIII)*
H-1	5.15	5.12	5.29	5.91	5.52	5.21	4.96	5.19
H-2	4.06	4.25	3.73	4.21	3.76	3.56	3.97	4.27
H-3	3.97	4.03	4.14	4.28	3.77	3.78	3.86	4.00
H-4			3.81		3.51	3.46	3.62	4.06
H-5					3.85	3.95		
H-6					3.78	3.78		
H-6′					3.93	3.86		
Coupling constant								
$J_{1,2}$	2.0	2.0	4.1	3.8	3.0	3.8	1.8	3.7
$J_{2,3}$	3.2	2.9	10.2	10.4	9.8	9.9	2.8	11.0
$J_{3,4}$			8.4	3.8	8.3	8.8		3.1
$J_{4,5}$			10.6		10.5	10.4		
$J_{5,6a}$					5.4	5.1		
$J_{5,6b}$					2.2	2.6		
$J_{6a,6b}$					12.0	12.3		

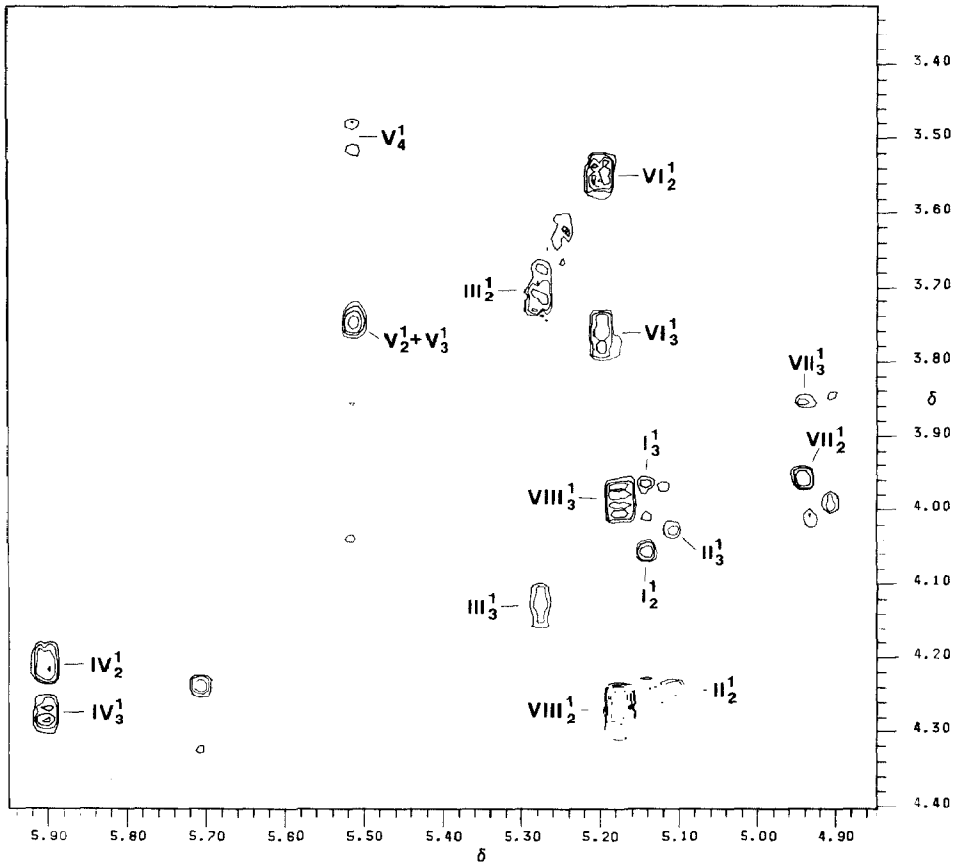

Fig. 2. Anomeric region of a 500-MHz, relayed-COSY spectrum of octasaccharide **1**. Off-diagonal cross-peaks at δ_A/δ_B indicate a scalar coupling between two nuclei resonating at δ_A (horizontal scale) and δ_B (vertical scale), respectively; *e.g.*, IV_2^1 indicates the coupling of H-1 (5.91) with H-2 of IV (δ 4.21). Along with one-step connectivities known from the PS-COSY spectrum (*e.g.*, IV_2^1), two-step connectivities (*e.g.*, IV_3^1) are visible for each of the sugar residues I–VIII.

the triple relayed-COSY spectrum of **1**. Owing to the simultaneous appearance of COSY and relayed-COSY cross-peaks, the region of the remaining protons naturally becomes even more overcrowded than in the COSY spectrum.

Apart from the relayed-COSY experiments, two-dimensional, double-quantum (DQ) spectra[4,5,10,12,13] are also suitable for unequivocal assignments of proton resonances (see Fig. 4). In the two-dimensional DQ spectrum, direct connectivities between two coupled protons A and M can be seen at $\delta_A/\delta_A + \delta_M$ and $\delta_M/\delta_A + \delta_M$. These peaks are equidistant from the (dotted) skew diagonal, $F_1 = 2F_2$. Furthermore, DQ transitions can occur in AMX systems at $\delta_M/\delta_A + \delta_X$ for remote connectivities, *e.g.*, DQ $VIII^2_{1+3}$ (as in Fig. 2, superscripts and subscripts refer to chemical shifts in the F_2 and F_1 dimensions, respectively). They can be easily identified by the lack of pair signals equidistant from the diagonal.

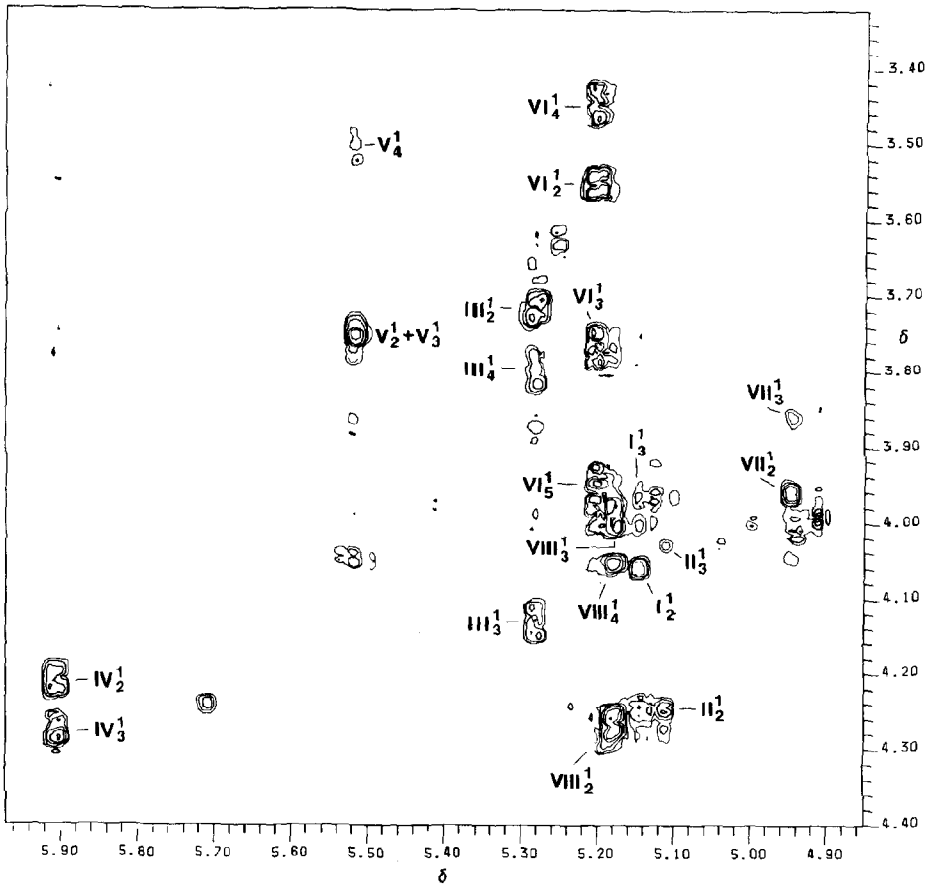

Fig. 3. Anomeric region of a 500-MHz, triple-relayed COSY spectrum of octasaccharide **1**. Labelling by analogy with Fig. 2. Owing to relaxation and an incomplete coherence transfer (*cf.*, Experimental section), the assignments are restricted to less than the theoretically possible five protons for some of the connectivity paths.

In contrast to the various COSY spectra, DQ spectra have no diagonal peaks. Therefore, signals of coupled nuclei which, on account of a small difference in the chemical shift, are lying in the COSY spectrum too closely to the diagonal to be resolved, can be recognized separately in the DQ spectrum, *e.g.*, H^{VIII}-3 and H^{VIII}-4.

The splitting patterns of the cross-peaks gave indications on the nature of the observed sugar component. For example, DQ VI^3_{1+2}, the DQ transition from H-1 to H-2 *via* the $^4J_{1,3}$ long-range coupling, showed a triplet fine structure due to the two axial–axial couplings from H-3 to H-2 and H-4, which are similar in magnitude, whereas DQ $VIII^3_{1+2}$ of the GalNAc group showed a doublet fine structure, because of a large axial–axial coupling between H-3 and H-2, and a small, barely resolved axial–equatorial coupling between H-3 and H-4.

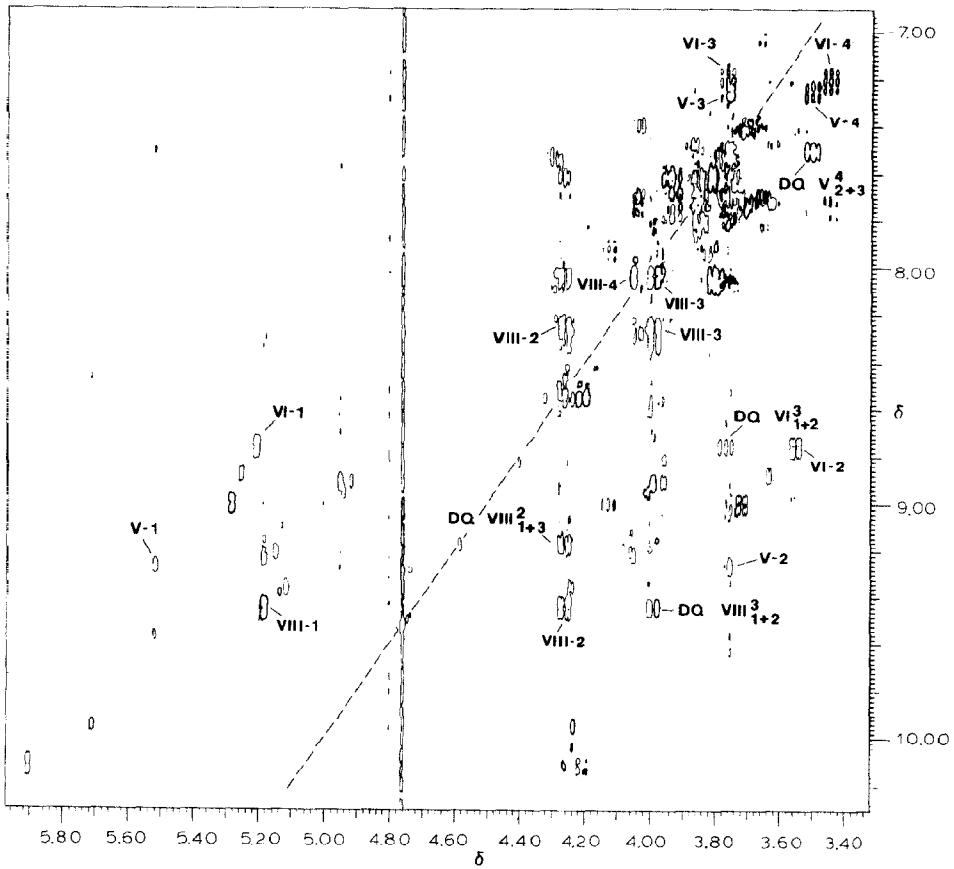

Fig. 4. Two-dimensional 500-MHz double-quantum spectrum of octasaccharide **1**. In order to keep this figure readable, only some of the cross-peaks have been labelled. A detailed interpretation is given in the text.

Numerical values of coupling constants can be obtained with the aid of a phase-sensitive COSY experiment[6,10,12]. Owing to the better digital resolution, the appearance of positive and negative signals within cross-peaks, and pure absorption lineshapes, it is much easier to analyze the splitting patterns of cross-peaks in PS–COSY than in usual (magnitude mode) COSY spectra. A crosspeak at δ_A/δ_B in the F_2/F_1 coordinate system contained all of the couplings of the nuclei A and B. Cross-sections through this peak parallel to F_2 showed the multiplet pattern of the A resonance, and cross-sections parallel to the F_1 axis gave the multiplet of the B resonance. The coupling $J_{A,B}$ (active coupling) appeared as antiphase splitting along both frequency axes, and the couplings of A or B to other protons (passive couplings) gave additional in-phase splitting along the F_2 and F_1 axes, respectively (See Table I for the coupling constants).

The structures of the Glc_I^2 and Gal_I^2 cross-peaks were similar (Fig. 5a) because

both these sugar units exhibited an equatorial–axial *(ea)* coupling of about 4 Hz between H-1 and H-2 (α-D configuration) and a large *aa* coupling, $J_{2,3} \sim 10$ Hz. On the other hand, the Hep_I^2 cross-peak exhibited two small (*ee* and *ea*) couplings of ~2–3 Hz because of its mannose configuration. Thus, sugar residues having the *gluco* and *galacto* configuration could be distinguished from heptopyranose residues (Fig. 5c). To distinguish a Glc from a Gal residue, their differing $^3J_{3,4}$ values were read along the F_1 axis from the H-2–H-3 cross-peaks, where these couplings are passive (see Fig. 5b).

The merits of phase-sensitive correlated spectra were clearly demonstrated by the assignment of the Glc^V spin system, which could not be deciphered by relayed-COSY spectra, as pointed out earlier. The cross-peak at $\delta(F_2)$ 5.52/$\delta(F_1)$ 3.51 in the relayed spectra (Figs. 3 and 4) specified the chemical shift of one of the *farther* protons belonging to the same spin system as the anomeric proton resonating at δ5.52, as H-2 was directly assigned from the $\delta(F_2)$ 5.52/$\delta(F_1)$ 3.76 cross-peak of the PS-COSY spectrum. In the latter spectrum, there were only two cross-peaks at $\delta(F_2)$ 3.51 (Fig. 5d); hence this chemical shift cannot correspond to H-5, which would have shown three connectivities. The $\delta(F_2)$ 3.51/$\delta(F_1)$ 3.85 cross-peak was given the V_5^4 assignment, as its twin V_4^5 cross-peak, which is better resolved along the F_2 axis, exhited *two different* passive couplings, *i.e.*, $J_{5,6a}$ and $J_{5,6b}$. Consequently, the second cross-peak peak at $\delta(F_2)$ 3.51/$\delta(F_1)$ 3.77 corresponds to the V_3^4 connectivity. Owing to the small chemical shift difference δ(H-3) − δ(H-2), it has a second-order character along the F_1 axis, where the passive H-2–H-3 coupling was displayed, but splitting along the F_2 axis may be explicitly analyzed, as active couplings in this cross-peak were the same as passive couplings in the V_5^4 cross-peak, and *vice versa*. The δ(H-5) coordinate determined from the V_5^4 cross-peak facilitated the localization of δ(H-6a) and δ(H-6b), which lied in a crowded region of the spectrum, close to the diagonal. These assignments were obtained with the aid of a triple-quantum filtered spectrum[10,12,14,15] (not shown here), in which only the systems of three mutually coupled spins (*i.e.*, H-5, -6a, and -6b) passed the filter. As follows from the aforementioned, phase-sensitive COSY spectra enabled the identification of the components of an oligosaccharide by analyzing their sets of coupling constants. Neither reasoning by analogy, nor any reference data was necessary.

The sequence and glycosylation sites were found with the aid of a two-dimensional n.O.e. experiment (NOESY) (Fig. 6 shows the anomeric region of the NOESY spectrum). Along with the intra-residue n.O.e. between H-1 and H-2 of each of the sugar components, the inter-residue n.O.e. between anomeric protons and the relevant protons of the adjacent glycosidically linked residue were clearly seen. For example, the IV-1–III-3 cross-peak showed the linkage of Gal^{IV} to O-3 of Glc^{III}. The VIII-1–IV-3 linkage may seem equivocal, because, for a glycosyl link to O-3 of the galactosyl residue, the n.O.e. is usually stronger for H-4 than for H-3. This is due to a preferred conformation that locates the anomeric proton of the $GalNAc^{VIII}$ residue H-3 and its equatorial neighbor H-4 of the Gal^{IV} residue closer to the latter. Although this problem has been discussed several times[7,9,16–20], no

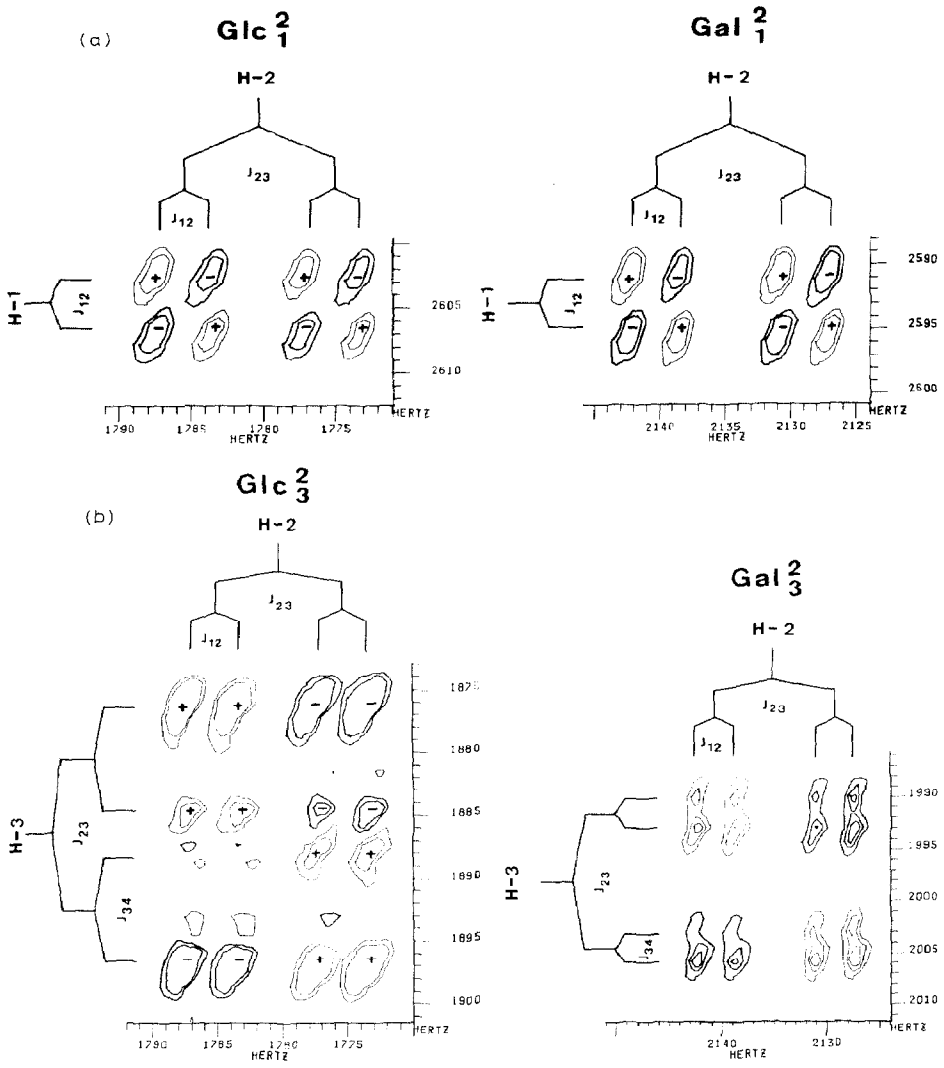

Fig. 5. Diagnostic patterns of cross-peaks in the phase-sensitive COSY spectrum of octasaccharide **1** (positive and negative multiplet components are drawn in thin and fat lines, respectively). A detailed interpretation is given in the text.

clear conclusion of the use of H-3 and H-4 n.O.es. for linkage analysis of the Gal residuehas been reached. Actually, the n.O.e. observed for H-3 was always diagnostic of the (1→3) linkage, because it could not arise from the (1→4) linkage, H-3 being in axial position and lying on the opposite side of the ring with respect to H-1 of the (1→4)-linked substituent. For a (1→3) linkage, therefore, it is meaningless whether the H-4 n.O.e. was simultaneously observed or not, as in the pre-

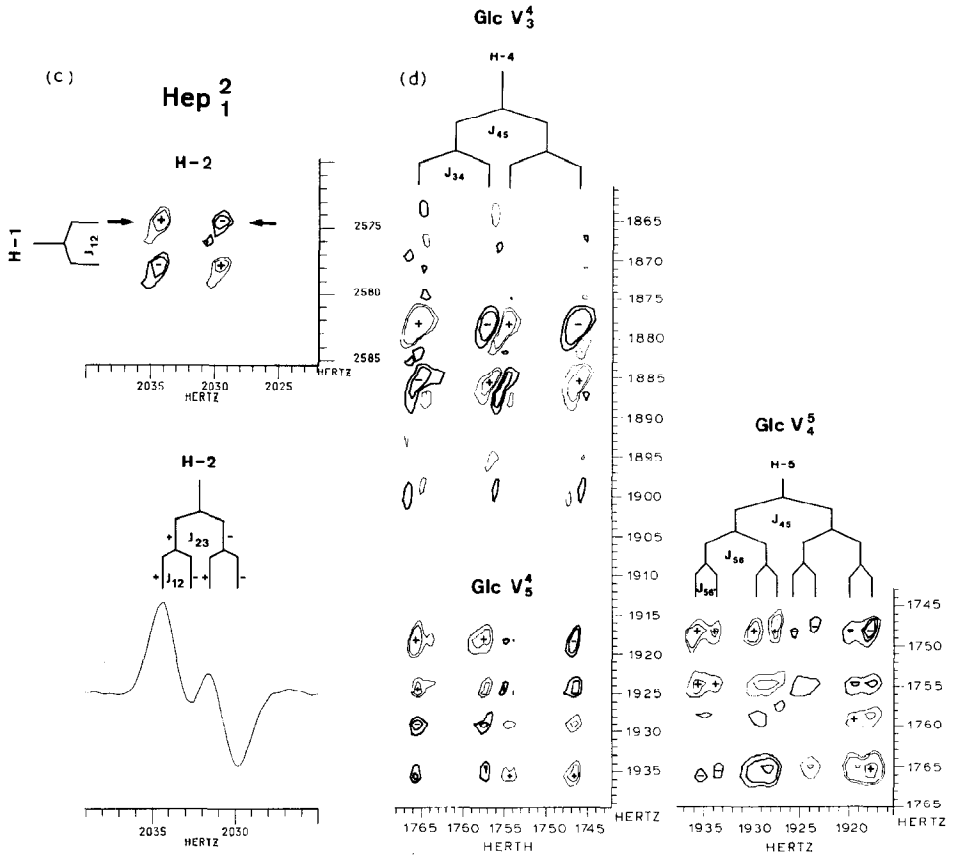

Fig. 5 (continued)

sent case*. Ambiguity could only arise in a reverse situation, with the n.O.e. for H-4 being observed and that for H-3 missing, because this could have resulted from two diametrically different reasons: a (1→4) linkage is indeed present, or a (1→3) linkage is present, but the H-4 n.O.e. is so much stronger than that of H-3 that the latter could not be observed under the experimental conditions applied.

Interestingly, there were also intense cross-peaks between the anomeric proton resonances VI-1 and V-1, and V-1 and IV-1. Here again, H-1 of the glycosyl residue is located between the proton at the bridge (H-2) and the equatorial neighboring proton (H-1) of the glycosyl-linked sugar residue. Such less common n.O.es. were observed several times for a similar steric situation in α-D-mannose residues linked by a glycosyl group at O-2[21–25]. In the present spectrum, the strong VI-1–III-4 cross-peak revealed the proximity in space of two sugar rings that are distant from each other in the

*The failure to detect this signal is probably due to a short transverse-relaxation time, T_2, characteristic of sugar residues located at points of branching[10].

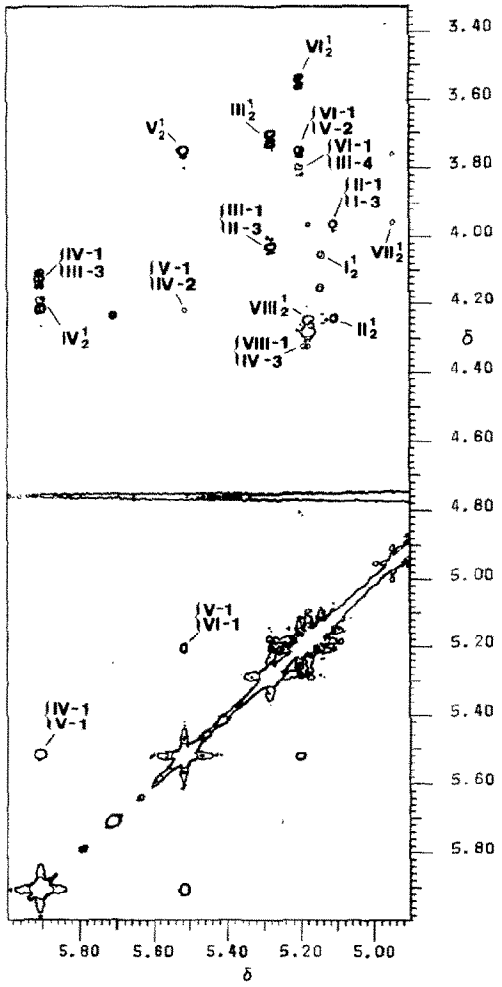

Fig. 6. Anomeric region of the 500-MHz magnitude-mode NOESY spectrum of octasaccharide **1**. Labelling of cross-peaks referring to *intra*-residue n.O.es. is the same as for COSY (Fig. 5) and relayed COSY (Figs. 2 and 3), *e.g.*, IV$_2^1$. Cross-peaks originating from *inter*-residue n.O.es. are marked by braced proton labels. A detailed interpretation is given in the text.

primary structure. All these findings reduce the number of possible conformations of the oligosaccharide under study, and will certainly be useful in calculations aiming at the determination of the three-dimensional structure of octasaccharide **1**.

EXPERIMENTAL

Material. — The core oligosaccharide[1] was dephosphorylated by a treatment with 40% HF at 4° during 4–5 days and purified, after lyophilization, on a Sephadex G-25 column (3 × 34 cm).

^{1}H-N.m.r. spectroscopy. — The samples were repeatedly treated with ^2H_2O, with intermediate lyophilization, and then dissolved in ^2H_2O (0.3 mL) containing a trace of acetone, which was used as internal reference (δ 2.225). All spectra were measured at 303 K with a Bruker AM-500 spectrometer, equipped with an Aspect 3000 computer and an array processor using Bruker standard software.

For the relayed coherence-transfer spectrum the accordion variant, *i.e.*, the incrementing of the coherence-transfer delay (τ) in concert with t_1 was applied in order to cover a wider range of coupling constants[3,11]. The pulse sequence was $90 - t_1 - 90 - (\tau_1 + n\tau_2) - 180 - (\tau_1 + n\tau_2) - 90 - t_2$ (n 1, 2, . . . 256). The sum $(\tau_1 + n\tau_2)$ was incremented stepwise from 0.031 to 0.125 s to optimize transfer *via J* ~2–8 Hz. Relaxation delay between the pulse sequences was 2 s. A spectral width of 1650 Hz in both dimensions was used with quadrature detection to collect a 256 × 1024-data matrix with 64 transients for each t_1 delay. The matrix was zero-filled in the t_1 dimensions and transformed in the magnitude mode by use of the sine-bell window function in both dimensions. Digital resolution in the resulting 512 × 512 matrix was 3.2 Hz per point. A double-relayed experiment was performed analogically, with one more $-(\tau_1 + n\tau_2) - 180 - (\tau_1 + n\tau_2) - 90$ sequence inserted before t_2.

The triple relayed COSY measurement was performed both in the accordion mode and with fixed delays of 0.02 s for each step. Against expectations, the latter variant gave slightly better results, although fixed delays obviously cannot optimally match all the coupling constants occurring in Glc, Gal, GalNAc, and Hep*p* residues.

The double-quantum spectrum was measured by the method of Mareci and Freeman[4], by applying a 90°-reconversion pulse and quadrature detection in both dimensions. Spectral width was 2660 Hz in the F_1 dimension and 1330 Hz in the F_2 dimension. A 256 × 2048-data matrix with 64 transients for each t_1 delay was acquired. The time domain data were multiplied by a sine-bell window function in both dimensions and zero-filled in the F_1 dimension. The magnitude-mode spectrum was calculated after Fourier transformation. The F_2 dimension was calibrated relative to the acetone peak, and twice as large a chemical shift was set in the F_1 dimension. With this calibration, each of the contours appeared at an F_1 coordinate equal to the sum of the chemical shifts of the protons involved in double-quantum transitions.

The phase-sensitive COSY spectrum with pure absorption line shapes and quadrature detection in both dimensions was obtained by the use of time-proportional phase increments[6,26]. The spectral width was 1330 Hz in both dimensions, and the spectral size in the time domain was 1 K × 4 K; 96 transients for each t_1 were accumulated. Prior to Fourier transformation, the time-domain data were multiplied by phase-shifted sine-bell window functions (phase shifts of $\pi/8$ and $\pi/16$ were used in t_1 and t_2 dimensions, respectively), and zero-filled in both dimensions. Digital resolutions in the resulting 2 K × 8 K-data matrix were 0.32 and 1.3 Hz per point in the F_2 and F_1 dimensions, respectively.

NOESY spectra were obtained both in the phase-sensitive[27] and the magnitude mode[28] with 96 and 32 transients for each t_1, respectively. The mixing delay t_m 0.5 s

was randomly varied by ±5% of its value to suppress remaining *J*-coupling effects. For other details, see relayed COSY.

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REFERENCES

1 E. ROMANOWSKA, A. GAMIAN, AND J. DABROWSKI, *Eur. J. Biochem.*, 161 (1986) 557–564.
2 W. P. AUE, E. BARTHOLDI, AND R. R. ERNST, *J. Chem. Phys.*, 64 (1976) 2229–2246.
3 G. EICH, G. BODENHAUSEN, AND R. R. ERNST, *J. Am. Chem. Soc.*, 104 (1982) 3731–3732.
4 T. H. MARECI AND R. FREEMAN, *J. Magn. Reson.*, 51 (1983) 531–535.
5 L. BRAUNSCHWEILER, G. BODENHAUSEN, AND R. R. ERNST, *Mol. Phys.*, 48 (1983) 535–560;.
6 D. NEUHAUS, G. WAGNER, M. VAŠÁK, J. H. R. KÄGI, AND K. WÜTHRICH, *Eur. J. Biochem.*, 151 (1985) 257–273.
7 P. HANFLAND, H. EGGE, U. DABROWSKI, S. KUHN, D. ROELCKE, AND J. DABROWSKI, *Biochemistry*, 20 (1981) 5310–5319.
8 J. H. PRESTEGARD, T. A. W. KOERNER, JR., P. C. DEMOU, AND R. K. YU, *J. Am. Chem. Soc.*, 104 (1982) 4993–4495.
9 R. U. LEMIEUX, K. BOCK, L. T. J. DELBAERE, S. KOTO, AND V. S. RAO, *Can. J. Chem.*, 58 (1980) 631–653.
10 J. DABROWSKI, in W. R. CROASMUN AND R. M. CARLSON (Eds.), *Methods in Stereochemical Analysis; Two-Dimensional NMR Spectroscopy for Chemists and Biochemists*, VCH Publishers, 1987, pp. 349–386.
11 S. W. HOMANS, R. A. DWEK, D. L. FERNANDES, AND T. W. RADEMACHER, *Proc. Natl. Acad. Sci. U.S.A.*, 81 (1984) 6286–6289.
12 J. DABROWSKI, A. EICHART, M. KORDOWICZ, AND P. HANFLAND, *Magn. Reson. Chem.*, 25 (1987) 338–346; and references cited therein.
13 S. W. HOMANS, R. A. DWEK, D. L. FERNANDES, AND T. W. RADEMACHER, *Biochim. Biophys. Acta*, 798 (1984) 78–83.
14 U. PIANTINI, O. W. SØRENSEN, AND R. R. ERNST, *J. Am. Chem. Soc.*, 104 (1982) 6800–6801.
15 K. HIKICHI, *JEOL News*, [20A] (1984) No. 2, 19–23.
16 J. DABROWSKI, P. HANFLAND, H. EGGE, AND U. DABROWSKI, *Arch. Biochem. Biophys.*, 210 (1981) 405–411.
17 J. DABROWSKI, P. HANFLAND, AND H. EGGE, *Methods Enzymol.*, 83 (1982) 69–86.
18 H. VAN HALBEEK, J. F. G. VLIEGENTHART, H. WINTERWERP, W. M. BLANKEN, AND D. H. VAN DEN EIJNDEN, *Biochem. Biophys. Res. Commun.*, 110 (1983) 124–131.
19 H. EGGE, J. DABROWSKI, AND P. HANFLAND, *Pure Appl. Chem.*, 56 (1984) 807–819.
20 V. K. DUA, B. N. N. RAO, SHING-SHING WU, V. E. DUBE, AND C. A. BUSH, *J. Biol. Chem.*, 261 (1986) 1599–1608.
21 A. A. GREY, S. NARASHIMHAN, J.-R. BRISSON, H. SCHACHTER, AND J. P. CARVER, *Can. J. Biochem.*, 60 (1982) 1123–1131.
22 J.-R. BRISSON AND J. P. CARVER, *J. Biol. Chem.*, 258 (1983) 1431–1434.
23 S. W. HOMANS, R. A. DWEK, D. L. FERNANDES, AND T. W. RADEMACHER, *FEBS Lett.*, 164 (1983) 231–235.
24 S. N. BHATTACHARYYA, W. S. LYNN, J. DABROWSKI, K. TRAUNER, AND W. E. HULL, *Arch. Biochem. Biophys.*, 231 (1984) 72–85.
25 H. PAULSEN, T. PETERS, V. SINWELL, R. LEBUHN, AND B. MEYER, *Justus Liebigs Ann. Chem.*, 1985, 489–509.
26 D. MARION AND K. WÜTHRICH, *Biochem. Biophys. Res. Commun.*, 113 (1983) 967–974.
27 G. BODENHAUSEN, H. KOGLER, AND R. R. ERNST, *J. Magn. Reson.*, 58 (1984) 370–388.
28 R. BAUMANN, G. WIDER, R. R. ERNST, AND K. WÜTHRICH, *J. Magn. Reson.*, 44 (1981) 402–406.