STRUCTURE DETERMINATION OF A CERAMIDE PENTADECASACCHARIDE BY TWO-DIMENSIONAL *J*-RESOLVED AND *J*-CORRELATED NMR SPECTROSCOPY

Janusz DABROWSKI and Peter HANFLAND*

Max-Planck-Institut für Medizinische Forschung, 6900 Heidelberg and **Institut für Experimentelle Hämatologie und Bluttransfusionswesen der Universität Bonn, 5300 Bonn, FRG*

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1. Introduction

The variables of oligosaccharide structure: (a) the sugar type variations, (b) linkage anomerity, (c) linkage position and (d) sequence, lead to a practically infinite number of possible structural combinations already for molecules of medium size. Hence, it is essential for an unequivocal determination of the structure of a complex oligosaccharide derivative by NMR data that possibly full spectral information be extracted from the spectra, i.e., that spectral parameters on as many protons as possible be obtained and analyzed. Since only the anomeric proton signals, and a few others in special cases, are recognizable by inspection [1,2], the remaining resonances have to be found and identified in the bulk of unresolved spectral lines. We have solved this problem by the combined use of spin decoupling and nuclear Overhauser difference spectroscopy. In this way, the structures of a ceramide decasaccharide from rabbit erythrocyte membranes [3] and of a number of plasmatic Lewis blood-group active glycosphingolipids [4] were elucidated. For selectivity reasons, it is necessary for those techniques to be successfully applied that the resonances of the irradiated protons be well separated from others or, at least, not heavily overlapped. As structure and spectra of higher oligomers get more complex, this requirement becomes hard to be fulfilled. A suitable solution to this problem offers the two-dimensional *J*-correlated NMR spectroscopy [5] since no irradiation by a second *rf* field is needed to obtain information on connectivities between coupled nuclei.

The combined application of 2D *J*-resolved [6] and 2D *J*-correlated spectroscopy enabled us to assign almost all resonances in the spectrum of the title compound. It follows that structural analysis of complex oligosaccharides can greatly be facilitated by the use of the 2D NMR methods.

Abbreviations: NMR, nuclear magnetic resonance; 2D, two-dimensional; *J*, interproton coupling constant; δ, chemical shifts in ppm from internal reference Me_4Si; SECSY, spin-echo correlated spectroscopy; NOE, nuclear Overhauser effect, or enhancement

2. Materials and methods

The ceramide pentadecasaccharide (*para* BI_{rab}-II, **1**; see formula in table 1) was isolated from rabbit erythrocyte membranes as described for the ceramide decasaccharide [3]. Details will be reported elsewhere.

For NMR spectroscopy the deuterium-exchanged sample was dissolved in Me_2SO-d_6 containing 2% D_2O.

The 360 MHz ^1H NMR spectra were obtained at 333 K on a Bruker HX-360 spectrometer by using quadrature detection. For the conventional one-dimensional spectrum (fig.1) 16-k memory capacity of the Aspect 2000 computer was used. Digital resolution was 0.35 Hz. For resolution enhancement the free induction decay was multiplied by the Lorentzian–Gaussian transformation function [7] included into the Bruker software package. Although the accuracy of the measurements was 0.001 ppm, the chemical shifts were rounded to 0.01 ppm to conform with the presentation in [3,4].

The software used for 2D spectra was the Bruker November 1980 version based on the programs developed in [5]. The data size of the time domain 2D *J*-resolved spectrum was a 128 × 8192 matrix. To improve the spectral resolution these data were multiplied in both the t_1 and t_2 directions with the Gaussian

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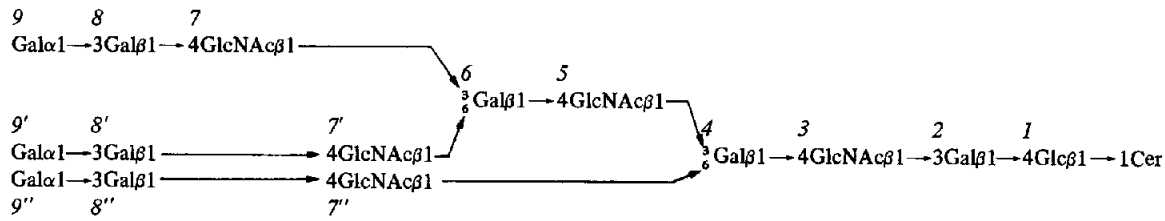
Table 1

Chemical shifts (ppm from Me_4Si) of *para*-BI_{rab}-II (I blood-group active), as obtained by two-dimensional ^1H NMR spectroscopy



H-1	4.84	4.30	4.68	4.42	4.30	4.68	4.30	4.68	4.28	4.17
H-2	3.59	3.44	3.48	3.44	3.44	3.48	3.44	3.43	3.44	3.04
H-3	3.64	3.50	3.54	3.50	3.46	3.54	3.46	3.54	3.50	3.33
H-4	3.75	3.85			3.81		3.81		3.85	3.33
H-5	4.02	3.33	3.33	3.33	3.59	3.33	3.59	3.33	3.36	3.33
H-6	3.45	3.44	3.61	3.61	3.59	3.61	3.59	3.61	3.44	3.61
H-6'	3.53	3.52	3.78	3.78	3.81	3.78	3.81	3.78	3.51	3.78

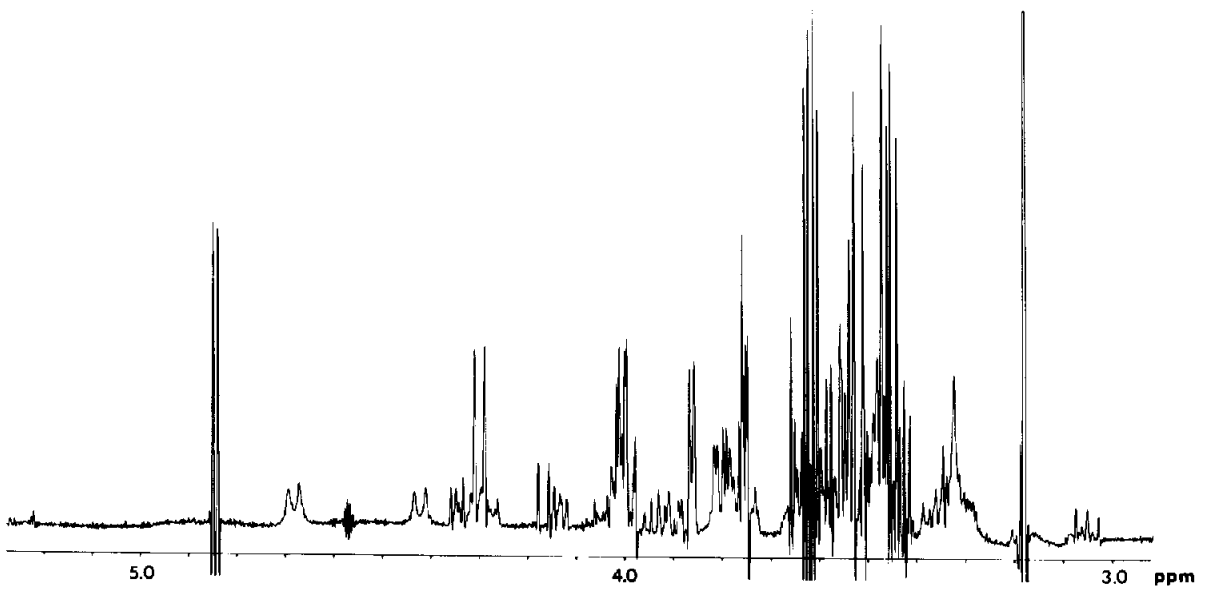


Fig.1. The resolution-enhanced 360 MHz ^1H NMR spectrum of *para*-BI_{rab}-II (1) measured in Me_2SO-d_6 at 333 K.

window function. Fourier transformation was performed with zero filling in both directions. The 512 X 2048 data matrix of the time domain 2D *J*-correlated (SECSY, [5]) spectrum was processed in a similar way. The latter spectrum was analysed in form of the contour plot (fig.2) and cross-sections through individual resonances parallel to the ω_1-axis [8]. Cross sections through individual multiplets of the 2D *J*-resolved spectrum were also obtained but some of them were unanalyzable because of their second-order character and overlap with other signals. Since the 'three-dimensional' stacked plots have a chiefly ornamental value, we refrain from reproducing them.

3. Results and discussion

In the sugar protons part of the one-dimensional spectrum of **1** (fig.1; cf. table 1) the signals of the 15

anomeric protons and of glucose H-2 lie outside of the bulk but the resonances of the remaining 90 sugar protons and of 4 sphingosine protons [9] are severely crowded within a narrow interval between 3.2–4.9 ppm. Although most of these resonances can be resolved in the second frequency dimension in form of individual multiplets (e.g., α-galactose residues in fig.3), this is of little use for spectral assignments unless the connectivities between those multiplets have been established. The connectivity information was obtained with the aid of the SECSY variant [5] of the 2D *J*-correlated spectroscopy. The contour plot (fig.2) shows the SECSY spectrum of 1 viewed from the top of the signals. The one-dimensional spectrum is displayed along the ω_2-axis in the centre of the plot. The off-line contours equally spaced (with opposite signs) from this axis indicate coupled nuclei, e.g., α-gal H-1 at δ4.84 coupled to H-2 at δ3.59. A number of connectivities between resonances located at large $\Delta\nu$ distances were readily recognizable in this way and could be visualized by linking the contours by lines of 135° slope (like H-3/H-4, H-4/H-5, H-5/H-6, and H-5/H-6* of α-gal) but this was not possible for closely spaced resonances (e.g., H-2/H-3 of α-gal) because of contour overlap. In those cases SECSY cross-sections were analyzed [8]. In a cross-section

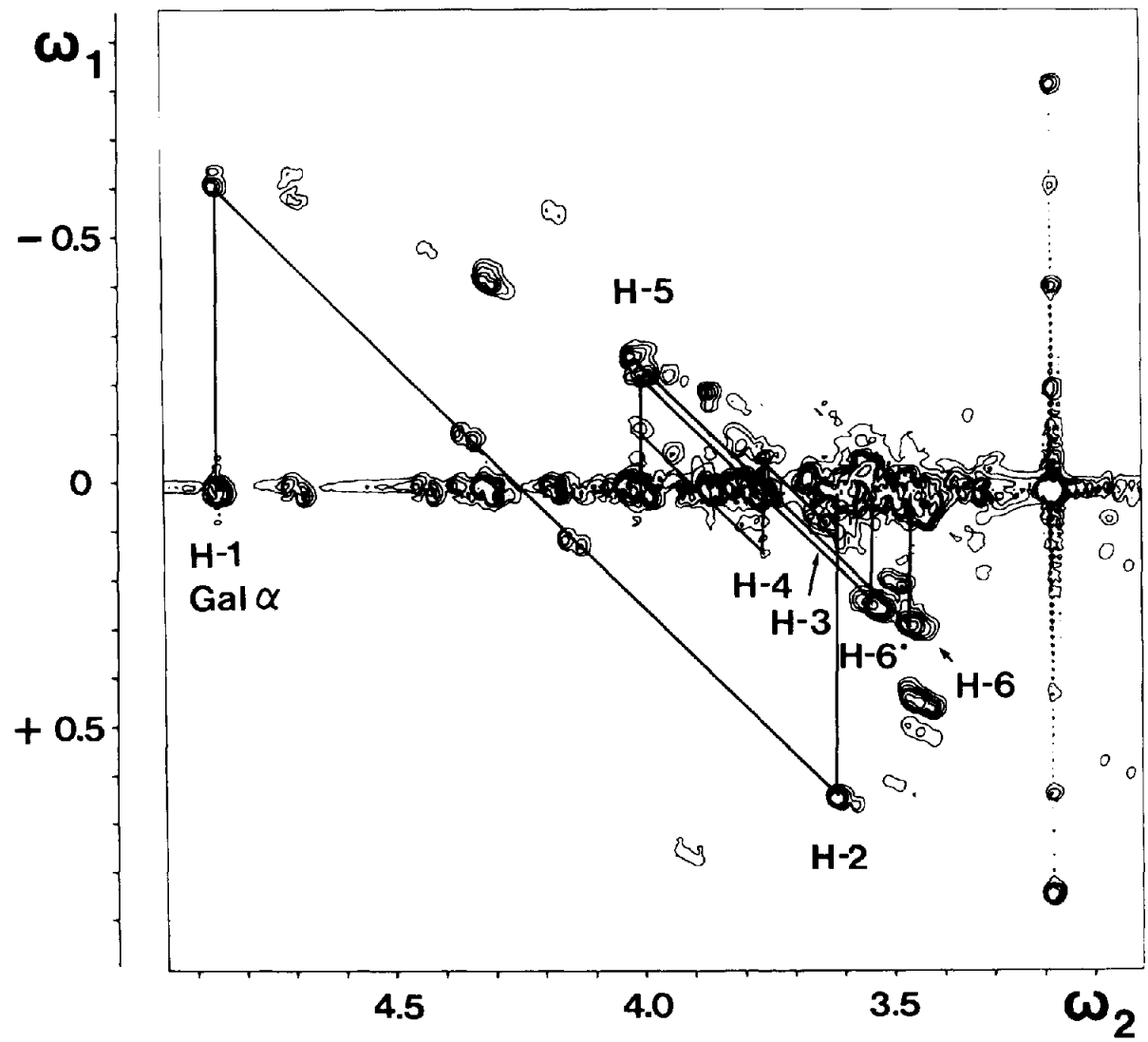


Fig.2. Contour plot of the ^{1}H 2D SECSY spectrum of *para*-BI_{rab}-II (1). Scale in ppm.

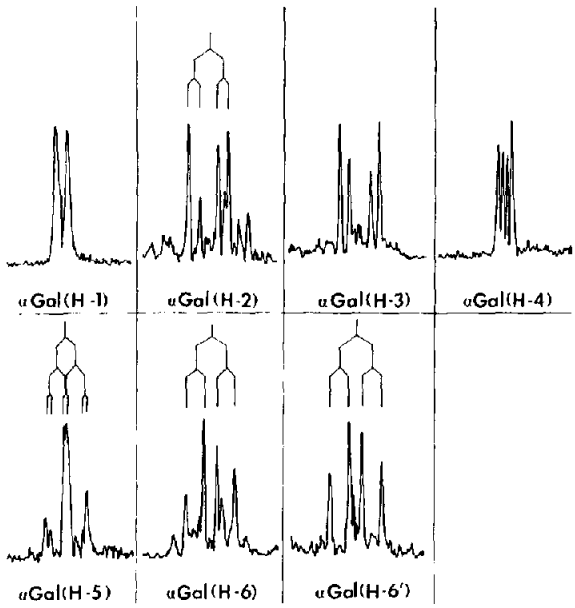


Fig.3. Cross-sections through the resonances of α-Gal-*9*, -*9'* and -*9''* in the 2D–*J*-resolved spectrum of *para*-BI_{rab}-II (1).

through a resonance the signal of the latter is located in the centre and its $\Delta\omega_1$ distance from the cross-peak (corresponding to the off-line contour of the contour plot) can directly be measured in Hz or ppm units. The resonance of the pair-coupled nucleus has to be looked for at the double distance on the ω_2-axis where another cross-section shows the cross-peak at a $-\omega_1$ distance (i.e., $\Delta\omega_2 = 2|\Delta\omega_1|$). This cross-section may contain further cross-peaks if the given nucleus is additionally coupled to still other nuclei. The procedure of establishing connectivities can then be continued until the entire coupled spin system is traced. When analysing a given SECSY cross-section it is helpful to consult the 2D–*J* cross-section of the same resonance for confirmation of the assignment. Fortuitous overlap by resonances belonging to other spin systems may still take place despite the enormously increased resolution but such problems can in most cases be successfully handled by utilizing the knowledge on spectra–structure relationships found for series of related substances [3,10]. For example, δ3.33 is common to H-5 of all GlcNAc and of β-Gal-*8*, -*8'* and -*8''* units hence the SECSY spectrum alone does not provide the information whether the two H-6,6' resonance pairs obtained from the cross-section at δ3.33 should be ascribed to the GlcNAc and Gal residues in the order shown in table 1 or rather in the reverse order. However, since δ3.74 and 3.62–3.64 was found [11] or H-6,6' of *N*-acetylglucosamine residues whereas 3.53–3.55 and 3.43–3.50 was characteristic for H-6,6' of galactose residues, there can be little doubt as to the validity of the choice made for table 1. That δ3.78 and 3.61 constitute a pair, was shown by tracing back in the cross-section at δ3.78. It should be added that since the H-4 resonance of *N*-acetylglucosamine units could not be found, tracing of its H-5, H-6 and H-6' resonances had to be started by first finding H-5 with the aid of one-dimensional NOE (H-1 was preirradiated). Another example of possible difficulties is the set of β-Gal-*4* and -*6* chemical shifts of H-4–H-6 constituting a 'vicious circle' from the point of view of tracing the signals. The assignment offered can only be substantiated by a negative evidence, viz., by the lack of further peaks in the cross-section at δ3.59. A confirmation independent of the SECSY method is provided by the one-dimensional NOE difference signals at δ3.59 and 3.81 obtained upon preirradiation of H-1 of the subsequent, 1 → 6 linked *N*-acetylglucosamine residues *7'* and *7''*.

It is seen that two-dimensional NMR spectroscopy is by no means a panacea for all imaginable signal overlap problems and should be reasonably combined with other methods, where necessary.

The assignments derived in the above way are gathered in table 1. Both table 1 and fig.1 suggest that **1** is closely related to the bi-antennary ceramide decasaccharide, BI_{rab}-I, whose structure was established in our recent work [3]. The integration of the signals of the anomeric protons of **1** shows the presence of 15 sugar residues in groups of 1 + 1 + 5 + 2 + 3 + 3 (going downfield). The one-proton H-1 resonances at 4.17 and 4.28 ppm can be ascribed beyond any doubt to β-glucose-*1* and β-galactose-*2*, respectively, of the lactose head group which occurred in all glycosphingolipids investigated in [3,4,10]. The 3-proton H-1 resonance at 4.84 ppm and the whole set of chemical shifts of this spin system can be attributed with the same degree of certainty to 3 terminal α-galactose-*9*, -*9'* and -*9''* units [3] thus pointing to a tri-antennary structure. Another evidence of this structure is the 3:2 intensity ratio between the H-1 signals of GlcNAcl → 3 and GlcNAcl → 6 [3]. The previously inaccessible H-6 resonances are very useful in that their large downfield shift in the case of two β-galactose residues directly confirm the 1 → 6 linkage.

Other linkage and sequence problems were solved by applying NOE and using chemical shift data, simi-

larly as in [3]. However, one question remains open, viz., whether the two branches starting with GlcNAc-5 and -7'' are linked to sites 3 and 6 of Gal-4 in the order indicated in table 1 or rather in the reverse order. The study of this problem is being continued.

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