ASSIGNMENT OF ^1H AND ^{13}C NMR SPECTRA BY A COMBINATION OF HOMO- AND HETERONUCLEAR SHIFT CORRELATED TWO-DIMENSIONAL NMR. APPLICATION TO OLIGOSACCHARIDES RELATED TO ARABINOXYLANE

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It is shown that a combination of homo- and heteronuclear shift correlated 2 D NMR yields correct and complete assignment of ^{1}H and ^{13}C NMR spectra. The amount of the work necessary can be considerably reduced if some of the lines can be unambiguously assigned on the basis of other information or if suitable model compounds are available. The pattern of homonuclear cross-peaks is characteristic for molecular fragments and can be used with advantage to trace out the lines in the spectral regions with heavy overlap. The exactly assigned ^{13}C chemical shifts of disaccharide and trisaccharides related to arabinoxylan agree satisfactorily with the shifts predicted according to the shifts found in the spectra of model mono- and disaccharides measured under the same conditions.

Undoubtedly, ^{13}C NMR spectroscopy owes its wide acceptance to the relative simplicity of ^{13}C NMR spectra. While the ^{1}H NMR spectrum of an organic molecule of a moderate size is complex and difficult to analyse in terms of chemical shifts and coupling constants, the ^{13}C NMR spectrum measured with proton broadband decoupling consists only of so many lines as there are nonequivalent carbon atoms present in the molecule and the position of each of the lines is equal to its chemical shift. The simplicity, however, comes at the expence of information content (coupling information is absent) which makes assignment of the chemical shifts to individul carbon atoms in the molecule more difficult. The chemist can then either resort to empirical assignment rules (*e.g.* chemical shift additivity) or employ some additional NMR experimental technique. The second alternative is receiving most attention lately because of large progress in NMR instrumentation and methodology. Especially interesting in this respect are various forms of two-dimensional NMR spectroscopy (2 D NMR)[1,2] which are general and flexible at the same time. It appears that with the present arsenal of 2 D NMR methods virtually all problems of spectral analysis and assignement can be solved providing that sufficient amount of the compound is available, that spectrometer time is not limited, and that the operating frequency is adequately high. When these conditions are not met the experimentalist must carefully select the method which serves the purpose and yet complies with the experiment's economy. Very powerful are various combinations of different 2 D NMR methods, especially those which include ^1H NMR spectroscopy as they (usually) require less spectrometer time. In the present paper we will describe the use of one such combination, namely combination of chemical shift correlated homo- and heteronuclear 2 D NMR, to solve the problem of assigning ^{13}C chemical shifts in two tri- and one disaccharide related to arabinoxylane.

Several 2 D NMR methods have been applied to various carbohydrates: homonuclear *J*-resolved 2 D NMR spectroscopy was used by Hall and coworkers[3-6] and others[7] to enhance the resolution in proton spectra[3-5,7] and to distinguish between homonuclear and heteronuclear scalar spin couplings[6], *J*-resolved ^{13}C 2 D NMR spectroscopy was used for determination of proton multiplet structure[8] and $^{13}C-{}^1H$ coupling constants[9], the ^1H NMR spectra were assigned and analysed with the help of homonuclear correlated 2 D NMR spectra (both of COSY and SECSY variants)[10-13]. Morris and Hall demonstrated[14] that chemical shifts of ^{13}C and ^1H can be exactly assigned (and measured) from two-dimensional heteronuclear correlation maps if the exact assignment of the complementary (*i.e.* ^1H and ^{13}C, respectively) spectrum is available. In another study Hall and coworkers[5] proposed a synergistic combination of heteronuclear correlation 2 D NMR with homonuclear *J*-resolved 2 D spectroscopy by which both ^{13}C and ^1H chemical shifts can be measured and assigned. The combination yields exact assignment of all the lines in the spectra if there are no two coupling constants which differ by less than the achieved resolution (in *J*-domain) and if strong coupling involves only pairs of protons the spectra of which do not overlap. Otherwise, some additional information is necessary for complete assignment. In such cases when several coupling constants are similar it would be advantageous to combine the heteronuclear correlated 2 D NMR spectra with homonuclear (proton) chemical shift correlated spectroscopy rather than with *J*-resolved 2 D NMR. The results described here demonstrate the power of this combination as applied on derivatives of methyl-D-xylopyranoside in which several vicinal coupling constants are usually very close[15,16].

EXPERIMENTAL

Preparation and properties of the studied saccharides are described elsewhere[17-20], monosaccharide (*5*) was a commercial sample (Lachema, Brno).

The 2 D NMR spectra were measured in approximately 0·3—0·4M solutions in D_2O (99·99%

USSR product) with no reference. The values of chemical shifts were obtained from routine 1 D NMR spectra which were measured in the same solutions but to which sodium 3,3-dimethyl--3-silapropylsulfonate was added to serve as an internal reference.

All the spectra were measured on a Varian XL-200 spectrometer operating at 200·05 MHz for proton and at 50·30 MHz for carbon spectra. In all cases the standard software provided by the manufacturer (H—1Z version) was employed. All the 2 D NMR spectra shown here were plotted in the absolute value mode using 1 024 × 1 024 data matrices.

Homonuclear chemical shift correlated spectra were measured using HOMCOR pulse sequence (with 90° flip angles) which incorporates quadrature detection in both domains. Water

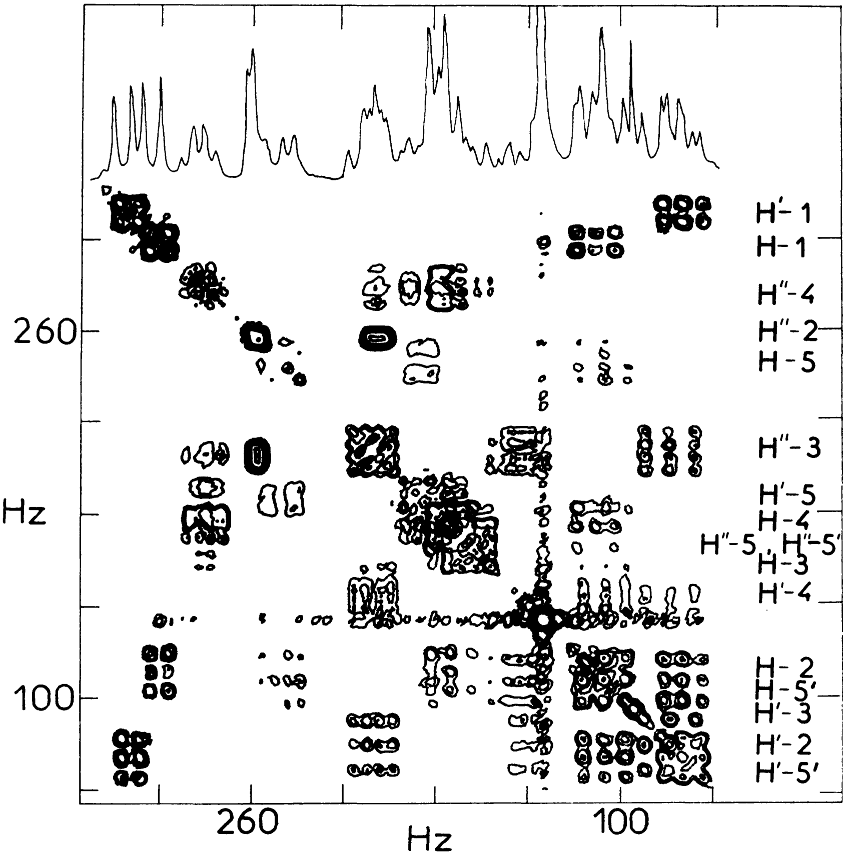


FIG. 1

Partial ^1H NMR spectra of methyl 3-O-α-L-arabinofuranosyl-4-O-β-D-xylopyranosyl-β-D-xylopyranoside (*1*). (The part corresponding to H″—1 proton is omitted here). Upper spectrum — ordinary 1 D ^1H NMR spectrum, lower spectrum — contour plot of 2 D homonuclear shift correlated 2 D ^1H NMR Scales are in Hz units with an arbitrary reference. The assignment given on the right side reffers to the corresponding dia-peak. For the details and proton labeling see the text signal was eliminated by a presaturation through equilibration period lasting 2 s. Spectral width of 600 Hz and pseudo-echo transformation was used in both dimensions. In total, 512 FIDs were acquired each for 0·854 s of acquisition time. The resulting spectra were symmetrized around the main diagonal.

Heteronuclear chemical shift correlated spectra were measured by HETCOR pulse sequence which also incorporates quadrature detection in both domains. FIDs were sampled with the rate corresponding to spectral width of 3500 Hz (^{13}C-axis), t_1 delay time was incremented according to the spectral width of 600 Hz (^{1}H-axis), 512 FIDs were recorded with the fixed delays corresponding to coupling $J(^{1}H—^{13}C) = 150$ Hz. Exponential weighting corresponding to line-broadening of 5 Hz was employed in the first transformation, various lower line-broadenings were tried in the second transformation.

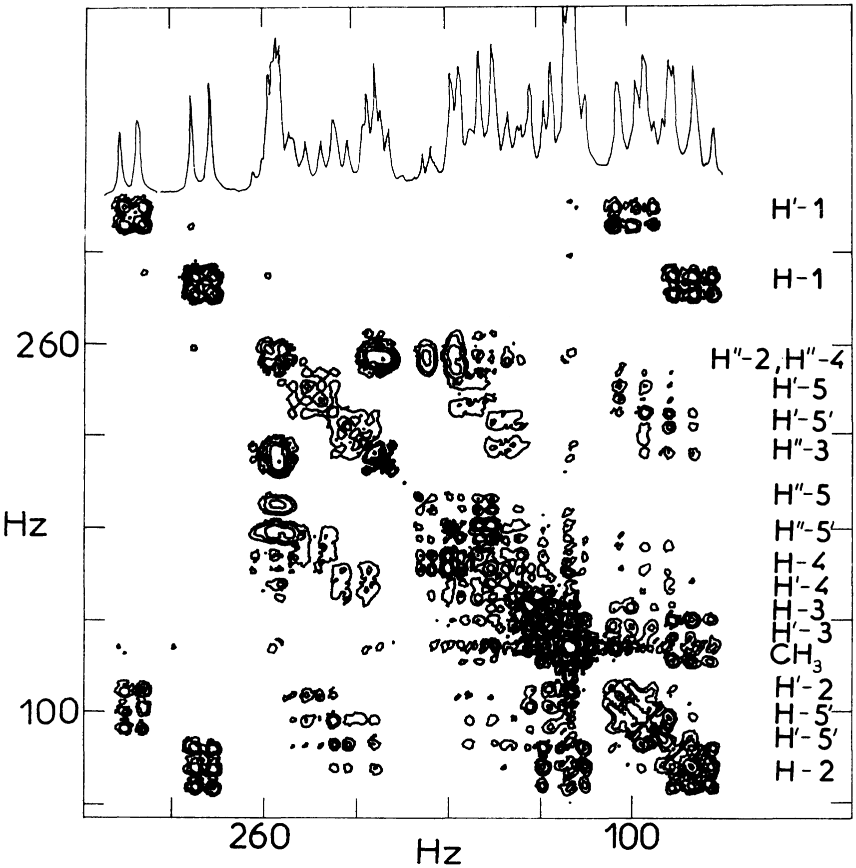


Fig. 2

Partial ^{1}H NMR spectra of methyl 4-O-[3-O-(α-L-arabinofuranosyl)-β-D-xylopyranosyl]-β-D-xylopyranoside (*2*). For explanations see Fig. 1

RESULTS AND DISCUSSION

^{1}H NMR (1 D *and chemical shift correlated* 2 D) *spectroscopy.* The relevant parts of proton 1 D and homonuclear correlated 2 D NMR spectra of methyl-3-O-α-L-arabinofuranosyl-4-O-β-D-xylopyranosyl-β-D-xylopyranoside (*1*), methyl 4-O-[3-O-(α-L-arabinofuranosyl)-β-D-xylopyranosyl]-β-D-xylopyranoside (*2*), and methyl 2-O-α-L-arabinofuranosyl-β-D-xylopyranoside (*3*) are shown in Figs 1–3. The cross-peaks in the 2 D spectra were used to establish connectivity between the dia-peaks and thus to assign the 1 D spectra which, in turn, were later used to assign ^{13}C NMR spectra through heteronuclear correlated 2 D NMR. Since the practical problems encountered here in deciphering the 2 D spectra are general for such spectra with heavy overlap, we will describe our approach in some detail.

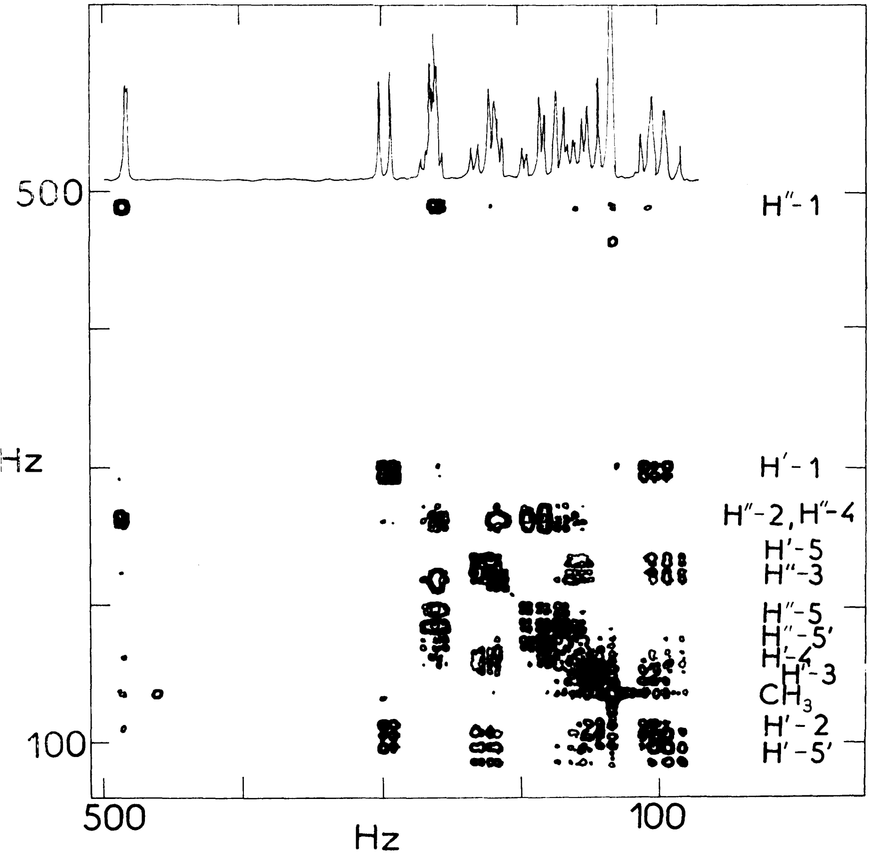


FIG. 3

Complete ^{1}H NMR spectra of methyl 3-O-α-L-arabinofuranosyl-β-D-xylopyranoside (*3*). (HDO signal suppressed by presaturation, compare Fig. 6). For explanations see Fig. 1

In principle, the correct assignment can be reached starting from any dia-peak and proceeding through cross-peaks to other dia-peaks. In the process the assignment which is not self-consistent has to be rejected and a new one tried. The number of such trials can be greatly reduced if some other information can be put to a use in assigning the starting dia-peak.

In carbohydrate chemistry quite reliable starting points are the peaks which correspond to anomeric protons, they occur at the lowest field (highest frequency)[21] of all skeletal CH protons, have simple splitting pattern, and the splitting is characteristic for the conformation of the monosaccharide unit[21].

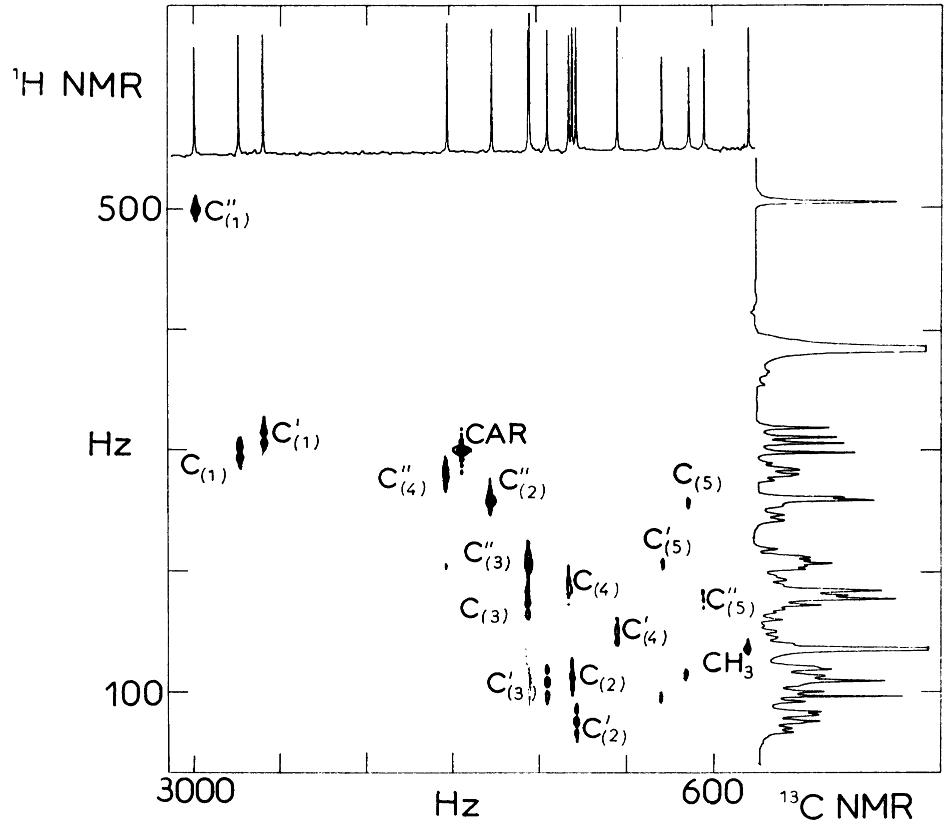


Fig. 4

^{13}C and ^{1}H 1 D NMR spectra and their correlation map obtained as a contour plot of heteronuclear shift correlated 2 D NMR spectrum of methyl 3-O-α-L-arabinofuranosyl-4-β-D-xylopyranosyl-β-D-xylopyranoside (*1*). (Carbon atoms labelled as indicated in the structural formula, for the assignement see the text. CAR denotes carrier frequency)

The closely spaced doublet of H″ − 1 proton of the arabinofuranoside unit (all the atoms of this unit are denoted by double prime here) is found at the highest frequency (Fig. 3), some 200 Hz higher then the anomeric protons of xylopyranoside units. Since the H″ − 2 proton lines (around 260 Hz) are easily identified through distinct (H″ − 1, H″ − 2) cross-peak (Fig. 3), only partial spectra of *1* and *2* are shown in Figs 1 and 2. There, the highest frequency doublets are characteristic for anomeric protons H − 1 and H′ − 1 of xylopyranoside units. (For the assignment of single primed and unprimed atoms to the two xylopyranoside units see below). Protons H − 2 and H′ − 2 are easily found through the well developped (H − 1, H − 2) and (H′ − 1, H′ − 2) cross-peaks, respectively, so as are H − 3 and H′ − 3 protons through (H − 2, H − 3)

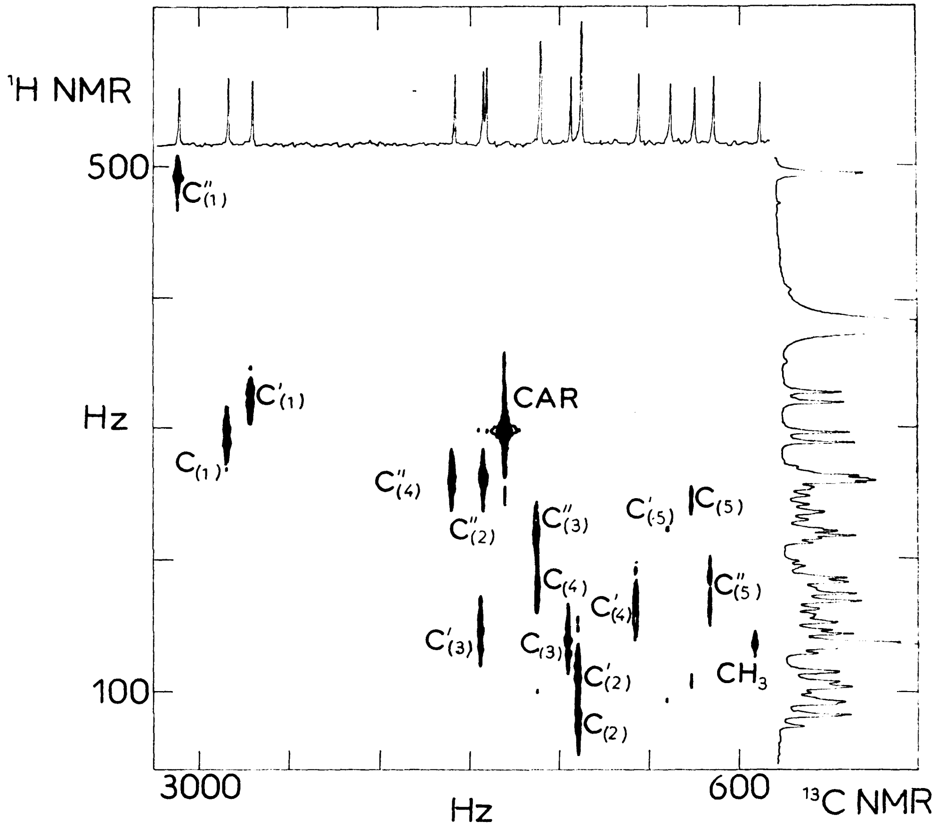


FIG. 5

^{13}C and ^{1}H 1 D NMR spectra and their correlation map obtained as a contour plot of heteronuclear shift correlated 2 D NMR spectrum of methyl 4-O-[3-O-(α-L-arabinofuranosyl)-β-D-xylopyranosyl]-β-D-xylopyranoside (*2*). (For explanations see Fig. 4) and (H′−2, H′−3) cross-peaks. Localizing the remaining protons in the heavily overlapped spectra of *2* and *3* is more complicated.

Since the appearance of the cross-peaks depends essentially only on the ratio of the appropriate coupling constant to the chemical shift difference and on the flip angle[1], the shape of the cross-peaks between protons sufficiently remote from the site of substitution is characteristic for the given pair of protons in the structural subunit. Therefore, the characteristic cross-peak pattern can be utilized for fast localization of the protons of the pair if the pattern is known from some model compound measured under the same experimental conditions. For example, the 2 D NMR spectrum of compound *1* shows only limited overlap of arabinofuranosyl

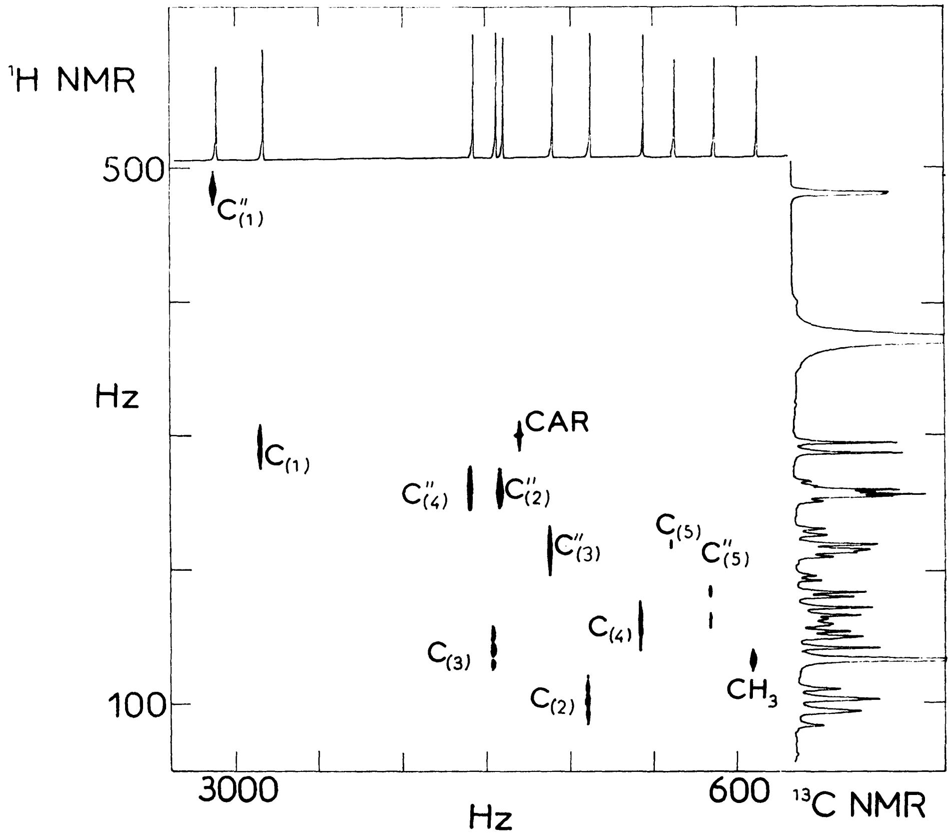


FIG. 6

^{13}C and ^{1}H 1 D NMR spectra and their correlation map obtained as a contour plot of heteronuclear shift correlated 2 D NMR spectrum of methyl 2-O-α-L-arabinofuranosyl-β-D-xylopyranoside (*3*). (For explanations see Fig. 4) proton multiplets and all dia- and cross-peaks are readily identified as described above. The cross-peaks (H″−2, H″−3), (H″−3, H″−4), (H″−4, H″−5), and (H″−4, H″−5) in the region of 250 290 Hz have characteristic appearance. In the spectra of compounds *2* and *3* the cross-peaks around 260 Hz can be visualized as a superposition of the mentioned cross-peaks caused by the overlap of H″−2 and H″−4 proton multiplets. Similarly, the assignment in the overcrowded region of 130−190 Hz in the spectrum of *2* is greatly facilitated by a comparison with the cross-peak patterns in the spectra of *1* and *3* (or more simple models can be utilized, if necessary).

Correctness of the assignment of the proton spectra is verified not only by its consistency but also by the ensuing assignment of ^{13}C 1 D and 2 D NMR spectra. Spreading ^1H NMR spectra along the ^{13}C chemical shift axis in the 2 D spectra permits multiplet separation and so the assignments in the overlapping regions can be verified (see *e.g.* the triplet, structure of $C'_{(3)}$, $C_{(2)}$, and $C'_{(2)}$ overlapping ridges in the spectra in Fig. 4). The assignment of anomeric carbon lines is obvious[22] so as is the assignment of the lines belonging to carbon atoms in positions 5. In fact, the chemical shifts of anomeric $C_{(1)}$ and $C'_{(1)}$ carbon atoms which are known from previous studies[23] permit the assignment of all unprimed chemical shifts to atoms

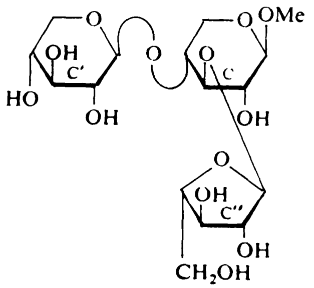
TABLE I

^{13}C NMR chemical shifts in tri- (*1*, *2*), di- (*3*, *4*), and monosaccharides (*5*, *6*)[a]

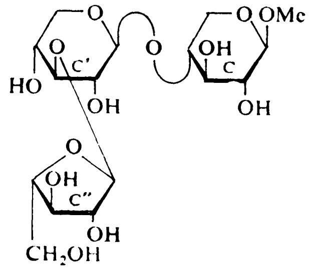
Saccharide	Ring[b]	$C_{(1)}$	$C_{(2)}$	$C_{(3)}$	$C_{(4)}$	$C_{(5)}$	CH_3
1	C	106·24	75·89	79·93	76·20	65·25	59·80
	C′	104·03	75·54	78·19	71·78	67·70	
	C″	110·20	83·24	79·82	87·34	63·90	
2	C	106·51	75·53	76·46	79·16	65·59	59·89
	C′	104·43	75·53	84·21	70·48	67·71	
	C″	110·84	83·90	79·16	86·69	63·92	
3	C	106·41	75·47	84·53	70·50	67·53	59·82
	C″	110·80	83·78	79·11	86·61	63·88	
4[c]	C	106·43	75·49	76·39	79·03	65·51	59·84
	C′	104·49	75·39	78·23	71·81	67·84	
5[d]	C	106·53	75·53	78·30	71·77	67·58	59·75
6[e]	C″	110·99	83·37	79·02	86·57	63·87	57·59

[a] Chemical shifts in δ scale, approximate error ±0·02 ppm. For the assignment of *1*—*3* see the text. [b] For the labeling of xylopyranose rings see the structural formula, C″ arabinofuranose unit. [c] Assignment taken from ref.[23]. [d] Assignment taken from ref.[16]. [e] Assignment taken from ref.[24].

in the reducing unit and of single primed shifts to the non reducing xylopyranoside unit (in accord with the labels shown in the structural formulas).



(1)



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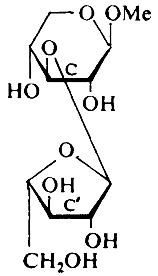

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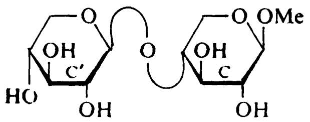

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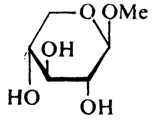

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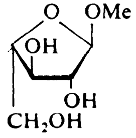

^{13}C NMR (1 D *and heteronuclear correlated* 2 D) *spectroscopy*. The spectra of compounds *1*–*3* are shown in Figs 4–6, the chemical shifts are summarized in Table I where are also given the chemical shifts determined for simpler model compounds: methyl 4-O-β-D-xylopyranosyl-β-D-xylopyranoside (*4*), methyl β-D-xylopyranoside (*5*), and methyl α-L-arabinofuranoside (*6*). (The 1 D NMR spectra of model compounds 4–6 were remeasured here under the same conditions as the spectra of *1*–*3*, but the published[16,23,24] assignments were accepted). Assignment of the ^{13}C chemical shifts in the spectra of compounds *1*–*3* from the 2 D spectra is straightforward with the only exception of assignment of $C''_{(2)}$ and $C''_{(4)}$ in the spectra of *2* and *3*. Since the attached protons ($H''-2$ and $H''-4$) have almost exactly the same chemical shifts, the assignment had to be made on the basis of the shape of the cross-peaks along the ^1H axis (a broad multiplet for $H''-4$ and a narrow doublet for $H''-2$). The assignment is consistent with the above mentioned points of verification and agrees with the accepted additivity rules (see below).

The values of ^{13}C chemical shifts given in Table I differ from literature values in a systematic manner, all our values are somewhat larger, *e.g.* our values for compound *6* are 1·6 ± 0·2 ppm larger than the literature[24] values, similarly for *5* the values are larger by 2·7 ± 0·3 ppm than in ref.[25] and for *4* by 1·4 ± 0·1 ppm than previously given[23]. The differences are due to different methods of spectral referrencing. They show the limits of applicability of additivity rules if literature data must be used, and stress the need for uniform measuring conditions if the use of additivity rule is contemplated. Our data, which were obtained under such uniform conditions and which were assigned without involving any use of additivity rule, permit a limited test of the empirical assignment procedure frequently employed in NMR spectroscopy of oligo- and polysaccharides. The procedure assigns the shifts on the basis of comparison with the (assigned) spectra of similar compounds taking into account possible substituent effects[22]. Accordingly, the chemical shifts of unprimed carbons (C) in *2* and of single prime carbons (C′) in *1* should be equal to the shifts of the corresponding carbon atoms in disaccharide *4*, the chemical shifts of carbon atoms of arabinofuranosyl units (C″) should be (in *1–3*) essentially the same as in the monosaccharide *6 etc.* A brief inspection of Table I confirms that these expectations are fullfilled for most of the carbon atoms within ±0·3 ppm; possible exceptions are branching sites in *1*. In fact, the assignment procedure using models *4–6* leads to correct assignments for compounds *1–3* if our uniform data are used. Literature non-uniform data, however, lead to ambiguous assignments since the errors are larger than the shift differences.

CONCLUSION

The proposed combination of homo- and heteronuclear shift correlated 2 D NMR is an effective method for spectral analysis and assignment. The assignment procedure is greatly facilitated if assignment of some of the signals is known from other sources and if an advantage is taken of characteristic homonuclear cross-peak patterns. The results obtained for the studied tri- and disaccharides agree with the empirical assignment rule, if the model compounds are measured under the same conditions.

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