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REVIEW

Nuclear Magnetic Resonance Spectroscopic Approaches for the Determination of Interglycosidic Linkage and Sequence in Oligosaccharides†

Pawan K. Agrawal* and Ashish K. Pathak

Central Institute of Medicinal and Aromatic Plants, Lucknow 226 015, India

The potential of various one- and two-dimensional nuclear magnetic resonance spectroscopic methods in deducing information about the interglycosidic linkage and sequence of monosaccharide residues in oligosaccharides is presented and the different techniques are evaluated.

Keywords: Oligosaccharides; interglycosidic linkage: ^{1}H and ^{13}C nuclear magnetic resonance; DIFNOE; delayed COSY; NOESY; ROESY; 3D TOCSY-ROESY; SINEPT; LR-HETCOR; HMBC.

INTRODUCTION

The carbohydrates are a ubiquitous class of biomolecules with a great array of diversity both in structure and in biological function. The latter includes their role as energy-rich metabolites and storage compounds, as structural components of a large number of natural products, and as cellular recognition and intercellular communication elements. A growing body of information indicates that the carbohydrates possess potent biological activities and that such oligosaccharides (OS) as well as compounds containing OS partial structures are widespread in nature (Agrawal *et al.*, 1985, 1991; Agrawal and Bansal, 1989; Agrawal, 1992; Boros and Stermitz, 1990; Chopin and Dellamonica, 1988; Hiller, 1987; Kennedy and White, 1983; Mahato and Nandy, 1991; McNeil *et al.*, 1979; Pfander and Stoll, 1991; Shibata, 1977; Srivastava and Kulshreshtha, 1989; Tschesche and Wulff, 1973).

The structural subunit of an OS generally consists of only a few residues, and may be composed of no more than five or six different monosaccharides. Moreover, in naturally occurring carbohydrates and carbohydrate-containing compounds, the *O*-glycosidic linkage is the main type of linkage connecting one monosaccharide residue (glycone) with another monosaccharide residue, or with a non-carbohydrate component (aglycone). The structural complexity arises from the variable linkage positions ((1→2), (1→3), (1→4), (1→6), etc.), the anomeric configuration (α- or β-) of glycosidic linkages, and also the ring size (pyranose or furanose form) of the monosaccharide units. OS structures are often branched and can have non-carbohydrate substituents such as acetate, phosphate etc. These features, together with unpredictable chemical characteristics associated with different glycosidic linkages and the existence of multiple substitution points, pose fundamental difficulties associated with structure determination and often require time-consuming further studies even after the monosaccharide composition has been determined.

The problem of structure determination of these biomolecules has been attacked using many techniques in past years, and the present state of the art continues to be confused owing to the lack of any single methodology which can be called upon for each new structural problem. Despite the fact that mass spectral data are quite informative (Dell *et al.*, 1994; Peter-Katalinic, 1994; Wolfender *et al.*, 1992), they do not provide an insight into the position and configuration of the interglycosidic linkages (IGL), or information concerning the precise location of the appended groups. Application of classical methods of structural analysis such as methylation (Geyer and Geyer, 1994) and consequent analysis of fragments obtained by chemical and enzymatic degradation studies (Hanisch, 1994; Jacob and Scadder, 1994), have been considered as complementary methods but cannot satisfy modern demands especially for analysis of small amounts of biological materials. Moreover, these degradative methods consume OS which is often difficult to isolate and could better be preserved for biological evaluation. In tackling a structure determination when only a limited amount of sample is available, nuclear magnetic resonance (NMR) spectroscopic methods are the best as these provide complete information concerning the covalent structure of, for example, an OS moiety without resort to any other method. However, this is not to say that standard protocols are available which, on the basis of NMR experiments, lead automatically to the structual analysis, but rather that there are a number of methods and techniques available which can be used to study the structure of an OS in solution.

The NMR approach to the solution of the above mentioned questions initially require unambiguous assignments of the ^{1}H and ^{13}C resonances. Information about the structural characteristics can then be obtained in several ways by identification of ^{1}H NMR resonances showing chemical shift perturbations due to monosaccharide substitution, by detection of scalar couplings through the

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* Author to whom correspondence should be addressed.

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glycosidic bonds between two protons in adjacent sugar residues, and by the investigation of the spatial neighbourhood of anomeric proton and linkage site protons of different residues by detecting nuclear Overhauser effect (NOE) or NOE in the rotating frame (ROE) cross peaks. Another possibility is to identify carbon resonances exhibiting glycosylation-induced shifts or the observation of a scalar coupling between an anomeric carbon and a proton of the neighbouring residue by means of selective insensitive nuclei enhanced by polarization transfer (SINEPT) or long-range heteronuclear correlation spectroscopy (LR-HETCOR).

It is the purpose of this article to describe the importance of the various NMR spectroscopic approaches in the sense of their applicability to structural studies on *O*-linked oligosaccharides derived from plant glycosides and oligosaccharides.

SOLVENT SYSTEM

Spectral interpretation and structure determination require the recording of the spectra of an OS in some deuterated solvent. One major factor dominates the acquisition of high-resolution ^{1}H NMR spectra of an OS and that is the possible interference by exchangeable protons (OH and NH). Therefore, preparation of aqueous solutions of OS compounds involves a prior exchange treatment with deuterium oxide (D_2O) and then the use of good quality D_2O (preferably 99.95 atom per cent) as solvent. Nevertheless a strong peak due to residual water (the HOD signal), as well as substantial side bands of the peak, are often obtained especially if the concentration of OS and OS-containing glycosides is less than 1 mM. The chemical shift of the HOD signal at room temperature is δ 4.8 and this shifts to higher field (δ 4.5) at 70°C. Sometimes, the ^{1}H NMR spectrum is not well resolved at ambient temperature and measurement of NMR spectral data at elevated temperature is a valuable aid. Deuterated dimethylsulphoxide (DMSO-d_6) and deuteriopyridine (C_5D_5N) are also good solvents for the dissolution of OS compounds and the use of mixed solvent systems sometimes helps in obtaining resolved spectral data.

Derivatized OS products, e.g. peracetate or fully methylated material, are readily handled in solutions in deuterated organic solvents such as deuterated chloroform ($CDCl_3$) and acetone-d_6. The chemical shifts are then referenced with respect to an internal standard such as TMS.

^{1}H NMR METHODS

In principle, ^{1}H NMR spectroscopy is well suited for providing structural information of monosaccharides, since vicinal coupling constants between ring protons can give essential information on the relative stereochemistry and anomeric configurations. This is because the ring conformation of a given monosaccharide remains essentially fixed on the NMR time scale, at least for pyranosides, when the monosaccharide is a constituent of an OS. However, ^{1}H NMR spectra are usually difficult to assign because most of the hydroxy-substituted protons have overlapping chemical shifts. Therefore, ^{1}H NMR spectra of OS compounds are surprisingly complex, due to extensive overlap of ring protons within a narrow spectral width (δ 3.0–4.2), and are often strongly coupled. A primary consequence of resonance overlap in an OS spectra is that only a limited amount of information can be ascertained from one-dimensional (1D) NMR studies. For this reason, analysis of ^{1}H NMR spectra of an OS was generally limited to chemical shifts and scalar couplings of well resolved resonances such as anomeric protons (H-1) (δ 4.4–5.6) and methyl resonances of 6-deoxy monosaccharide residues (δ 1.1–1.3). Information concerning assignments was, therefore, derived primarily from comparison with related structures. However, if completely novel structures are under investigation, model compounds are unlikely to be available and assignments of even an anomeric proton is difficult and additional assignments are needed for structural characterization.

The first task in using NMR to extract IGL information is to establish the glycosyl composition, i.e. the number and the structure of the constituent monosaccharide residues. As the anomeric proton resonances are usually well separated, they are commonly used as a spectral window in the analysis of ^{1}H NMR spectra of saccharides. Currently the most often employed assignment strategies invariably rely primarily upon one of the variants of the ^{1}H–^{1}H correlated spectroscopy (COSY) spectrum by following the correlation starting from the anomeric resonance which can be verified by a two-dimensional (2D) homonuclear Hartmann-Hahn (HOHAHA) spectrum. With the aid of multi-step 2D relayed coherence transfer (RCT or RELAY) and/or HOHAHA spectroscopy, the link from anomeric to other ring protons can be established. Various 1D and 2D derivatives of these experiments may be carried out by selective irradiation of each anomeric proton and these may be used to identify spin systems belonging to each monosaccharide residue and to establish its anomeric configuration (Agrawal, 1992; Agrawal *et al.*, 1994, 1995; Bax *et al.*, 1984; Bush, 1989; Dabrowski, 1987, 1989; Dabrowski *et al.*, 1987; Himmelsbach *et al.*, 1994; Homans, 1990; Inagaki *et al.*, 1989; Lerner and Bax, 1987; Patt, 1984; van Halbeek, 1994). After the identification of the spin systems belonging to individual monosaccharides residue has been made, the next question is to establish the IGL and the sequence between the monosaccharide units.

One-dimensional methods

These procedures simply involve a comparison of the observed 1D NMR spectrum with the spectra of a set of known structures thus enabling a structural identity to be made. The value of the technique is obviously directly related to the number of known structures available. The effect on the ^{1}H chemical shifts of glycosylation is typically a deshielding of the proton across the glycosidic bond as well as of the protons on the two neighbouring sites of the aglycone. The magnitude of this deshielding, normally 0.4–0.1 p.p.m. depends on the type of monosaccharide, the anomeric linkage and the conformation around the linkage. The main causes of this deshielding are the steric repulsion between hydrogens and the fixation of oxygen lone pairs close in space to the hydrogen in question. Since most of the resonances in the 1D spectrum are unresolved, identity between structures is determined by the characteristic sequence-dependent shifts of the reporter resonances. However, it is worthwhile to note that chemical shift perturbations due to monosaccharide substitution and detection are often additive to the first order. It is, therefore,

sometimes possible to determine the sequence of an OS which has partial homology to more than one structure in the database. Although straightforward, this method can be rather labour intensive for those with little experience of the NMR of OS compounds. Furthermore, as with all empirical observations, these effects should be interpreted with caution.

Glycosylation sites can be identified by comparison of ^{1}H NMR spectral data of the native and the peracetylated OS since acetylation of the free OH groups causes significant downfield shift (in the range 1–0.5 p.p.m.) of hydroxyl-substituted geminal protons, whereas α-resonance of the aglycone directly involved in the glycosidic linkage remains almost unaffected. This permits identification of the sites of glycosidic linkage as well as the identification of the terminal sugar residues (Achenbach *et al.*, 1994; Dabrowski, 1987; Egge *et al.*, 1984; Jiang *et al.*, 1991a). This strategy depends, however, on the successful chemical derivatization of the native glycoside to its peracetyl derivative and has not been widely employed to date.

Despite severe overlap of the majority of resonances, 1D NOE spectroscopy is quite successful since the anomeric proton signals of each residue are usually well separated from the rest of the signals and therefore proximity between glycone and aglycone proton can be observed. Selective irradiation of the anomeric-H reveals inter-residual contacts with aglycone-H and, in this way, all of the 1→2, 1→3, 1→4 and 1→6 combinations and both α- and β-linkages may be determined. In order to determine the sequence of monosaccharides, a series of 1D NOE experiments may be carried out in a sequential manner by presaturation of each of the proton resonances (Bae *et al.*, 1994; Chen *et al.*, 1993; Glaßgen *et al.*, 1992; Hamburger *et al.*, 1992; Inose *et al.*, 1991; Kitagawa *et al.*, 1993; Kuang *et al.*, 1991; Lutterbach *et al.*, 1993; Matsumoto *et al.*, 1994; Mizuno *et al.*, 1990a, b; Rockenbach *et al.*, 1992; Sakai *et al.*, 1994; Satoh *et al.*, 1994; Shoyama *et al.*, 1990; Siddiqui *et al.*, 1992; Tsurumi *et al.*, 1992; Yamamoto *et al.*, 1993a,b; Yoshida *et al.*, 1990). However, this strategy can be misleading when NOEs, correlated with an anomeric proton, appear at a chemical shift with two or more different hydrogens.

In NOE difference spectroscopy (DIFNOE), the anomeric proton is selectively saturated for a period of time followed by detection in the absence of irradiation. Subtraction of a spectrum recorded under identical conditions but with the decoupler set off-resonance yields the difference spectrum. Under the correct conditions this spectrum consists of the anomeric proton resonance of unit intensity together with resonances influenced by the NOE whose integrated intensity is dependent upon the usual factors. In principle, this method may be used to obtain needed information on residue linkage sites since NOE to and from the anomeric proton across a glycosidic linkage are invariably observed. Thus, this strategy involves irradiation at each anomeric proton signal followed by measurements of the differential NOE. Such a method has been utilized to determine the interglycosidic linkage in anthocyanins (Anderson *et al.*, 1991a, b; Brandt *et al.*, 1993; Johansen *et al.*, 1991; Kondo *et al.*, 1990; Lu *et al.*, 1991, 1992a, b; Odake *et al.*, 1992; Saito *et al.*, 1994; Toki *et al.*, 1994a, b; Trehara *et al.*, 1989, 1990a, b, c), flavonol glycosides (Bashir *et al.*, 1991; Pearce *et al.*, 1992; Satake *et al.*, 1993), pregnane glycosides (Schneider *et al.*, 1993), diterpenoid glycosides (Duc *et al.*, 1994), triterpenoid saponins (Qulad-Ali *et al.*, 1994; Warashina *et al.*, 1991, Yamamoto *et al.*, 1991, 1993a,b; 1994) and oligosaccharide multiesters (Miyase *et al.*, 1991, 1992; Powell 1990; Saitoh *et al.*, 1993a, b, 1994).

The ROE difference spectrum obtained following presaturation of the anomeric proton shows ROE across the glycosidic linkage of the aglycone and may thereby be used to identify the spin system of the aglycone. By utilizing this method, the interglycosidic linkage in glycosides of triterpenoids (Kakuno *et al.*, 1992; Nagao *et al.*, 1991a, b, c; Yamamoto *et al.*, 1994), oligosaccharide multiesters (Miyase *et al.*, 1991; Saitoh *et al.*, 1993a, b, 1994) and those of steroids (Warashina and Noro, 1994) have been determined. In terms of sensitivity per unit time, it is more efficient to employ the 1D NOE/ROE methods by selective irradiation of each anomeric proton provided these are sufficiently well resolved and do not overlap with aglycone-H in the case of glycosides. However, it is important to mention that in 1D ROESY sometimes ROE cross peaks can be found for monosaccharides which are not directly bonded but are close in space to the aglycone (Bader 1994a).

Two-dimensional methods

Since the protons across the glycosidic linkage are four bonds apart and therefore do not show any inter-residual ^{1}H–^{1}H spin–spin interactions in COSY and/or HOHAHA, these spectra are of great importance in delineating spin systems of individual monosaccharides.

Long-range ^{1}H–^{1}H couplings. Four-bond scalar couplings between anomeric-H and glycone-H ($^4J_{HCOCH}$) are usually so small and conformation dependent that they are not normally observable even in resolution enhanced spectra. However, they may, nonetheless, be detected, but not measured, since they are sufficient to cause a magnetization transfer between the protons mentioned when using long delays (typically 400 ms) before data acquisition as in the so called delayed COSY experiment (Massiot *et al.*, 1986). In this technique there is a loss of signal for large or medium-size molecules which is characterized by a short transverse relaxation time T_2. Structurally important long-range couplings, where thus observed, permit the establishment of IGLs and the sequence of the sugar chain (Batta and Liptak, 1984; Massiot *et al.*, 1988, 1990). Such long-range ^{1}H–^{1}H couplings can also be observed in COSY 45° (Ahmed *et al.*, 1994; Noorwala *et al.*, 1994), COSY 90° (Tommasi *et al.*, 1992), and double-quantum filtered (DQF)-COSY (Nishida *et al.*, 1991; Pancharoen *et al.*, 1994). However, in several instances, five-bond inter-residue couplings may also appear of comparable intensity to their four-bond counterparts and this may lead to ambiguity.

Nuclear Overhauser effect spectroscopy. The reliable method by which to obtain interaction information between an anomeric and aglyconic proton is by measurement of the inter-ring NOE connectivity transversing the glycosidic bond using 2D laboratory frame NOE spectroscopy (NOESY) or rotating frame NOE spectroscopy (ROESY). In such spectra, one observes intra-residual NOE contacts, useful in determining anomeric configuration and spatial relationships within the sugar residue, and inter-residual NOE/ROE contacts between the anomeric proton of a

particular sugar residue and proton(s) of another sugar, thus identifying the IGL. This method is advantageous over 1D NOE experiments as it does not require saturation of any anomeric-H and yields information concerning intra-residual and inter-residual NOE connectivity in a single experiment.

NOESY. Through-space dipolar interactions between the anomeric and the trans-glycosidic proton can be monitored in the form of NOE signals and serve as a basis for linkage and sequence analysis (Prestegard *et al.*, 1982). The added resolution introduced by the second dimension in the NOESY experiment often provides quantitative NOE data useful in defining glycosidic torsion angles. The genuine NOE coupling peaks can be identified by taking differences between the total correlation spectrum (TOCSY)/HOHAHA and NOESY data. This method has been widely employed in solving the structure of plant glycosides (Chen and Snyder, 1987, 1989; Ding *et al.*, 1993; Duc *et al.*, 1994; Fuijoka *et al.*, 1992; Ganbre *et al.*, 1991; Inada *et al.*, 1992; Kamel *et al.*, 1991; Kasai *et al.*, 1988, 1991; Kawashima *et al.*, 1991a, b; Kubo *et al.*, 1992a, b; Mizuno *et al.*, 1991, 1992; Nakamura *et al.*, 1994a, b; Nakanishi *et al.*, 1994; Nerdal and Andersen, 1991; Noda *et al.*, 1992a, b; 1994; Ohkima *et al.*, 1991; Ono *et al.*, 1990a, b; 1991, 1992, 1994; Pant *et al.*, 1988; Razanamahefa *et al.*, 1994; Sati *et al.*, 1989; Seaforth *et al.*, 1992; Serratice *et al.*, 1991; Thulborg *et al.*, 1994; Wong *et al.*, 1991; Yang, 1992; Yesilada and Houghton, 1991).

Despite its wide application, it is worthwhile mentioning that NOE depends on proton proximity rather than on bond connectivity, therefore care must be taken in assigning linkage positions based solely on NOE data. Several instances are documented where the strongest inter-residue NOE following saturation of the anomeric proton did not arise from the proton at the linkage position of the adjacent residue. For example, in the case of 3-glycosylated galactose, the aglycone proton at the linkage site and its equatorial neighbour (e.g. H-4) show NOE of comparable intensity, and sometimes the latter may be of greater intensity than the former.

The presence of NOESY cross-peaks depends on the conformation of the glycosidic linkage, and this experiment alone cannot provide a primary method for the determination of the inter-saccharide linkage for any new structure whose conformation is unknown; therefore it should be supplemented by other methods (Abeygunawardana *et al.*, 1990; Bush, 1989; Dabrowski, 1987, 1989). Ambiguity of such type can be resolved by other NMR spectroscopic approaches (*vide infra*).

ROESY. In cases when the product of spectrometer angular frequency and molecular rotational correlation time is equal to or exceeds unity NOE is negligible. When this product greatly exceeds one, as in the case of macromolecules, the NOE approaches -1 and specificity is lost due to spin diffusion (Bothner-By *et al.*, 1984). In these cases, and also where attempts to obtain reliable inter-residual NOE have been unsuccessful, the transient NOE experiment in a rotating frame provides a radical solution to this problem (Dabrowski *et al.*, 1989; Poppe and Dabrowski, 1989. The ROESY contains all NOE cross-peaks defining NOE as a function of molecular rotational correlation time, and this method has been applied by various workers to deduce IGLs and sequences for OS portions of several plant glycosides (Crespin *et al.*, 1993; Fuijoka *et al.*, 1992; Hartleb and Seifert, 1994; Himmelsbach *et al.*, 1994; Ikuta *et al.*, 1991; Jia *et al.*, 1994; Klimek *et al.*, 1992, Li *et al.*, 1994; Lin *et al.*, 1993; Lischewski *et al.*, 1992; Massiot *et al.*, 1990; Nagao *et al.*, 1993; Schneider *et al.*, 1993; Schroder and Haslinger, 1993a; Seifert *et al.*, 1991; Van Halbeek and Poppe, 1992). Since ^{1}H NMR assignments for individual sugar residues are well secured as being derived from through-bond connectivity observed in various homonuclear experiments, intra-residual NOE/ROE cross-peaks can be unambiguously identified and assigned. Thus, by searching for NOEs between each of the anomeric protons and relevant protons of the adjacent glycosidically-linked sugar residue, the IGLs can be established since there would be no inter-residual cross-peak for the reducing-end sugar residue. The absence of inter-residual NOE/ROE connectivity identifies the reducing end residue and, by following connectivities in a stepwise manner, sequence information can be obtained. Several workers have employed NOESY and ROESY together in deducing the structure of the glycosidic part of the glycoside (Li *et al.*, 1994; Perera *et al.*, 1993; Willker and Leibfritz, 1992).

ROESY/NOESY and TOCSY hybrid experiments. Hybrid experiments combining a ROESY/NOESY and a TOCSY step in either order (i.e. ROESY followed by TOCSY (ROTO) or TOCSY followed by ROESY (TORO)) contain both coherent and incoherent proton-polarization transfer steps in a single experiment and provide, in principle, identification of networks of coupled spins as well as of through-space proximity between adjacent spins. In such cases, sequences can be unambiguously determined by analysing the relayed NOE cross-peaks between the anomeric proton resonances of each pair of directly linked sugar residues. In the first step of the TORO experiment, H-1 magnetization is transferred by the TOCSY mechanism to further intra-residue protons of the same monosaccharide unit including the proton at the site of glycosylation. In the second step, the magnetization is transferred by the ROESY mechanism to the anomeric proton producing a cross peak in F_1 (horizontal connectivity) thereby determining the sequential order of the monosaccharide in the OS fragment. The unsymmetric cross-peak located in F_1 and F_2 defines the sequence in the direction towards the reducing end. Since resonances overlapped in one column may be well separated in the other, a careful analysis of the TORO spectrum can be extremely helpful in resolving possible ambiguities in assignments. It must be admitted, however, that such analysis requires some experience, as cancellation of ROESY by TOCSY cross-peaks (and *vice versa*) and the simultaneous occurrence of direct ROESY cross-peaks must be taken into account. A limitation of this method arises from the small coupling constants for protons of glycosylated residues (Dabrowski, 1989; Dabrowski *et al.*, 1987).

Homo-nuclear three-dimensional experiments

Complete information concerning ^{1}H NMR assignments for individual monosaccharide residues, their sequence and glycosidic linkage can be obtained by a three dimensional (3D) TOCSY-ROESY experiment (Schroder and Haslinger, 1993b, c). In an alternative 3D NOE-HOHAHA experiment, inter- and intra-residual NOEs are observed followed by the HOHAHA sequence, thus identifying the 'J network' of the aglycone residue (Uhrin *et al.*, 1993, 1994a). Implementation of the third dimension, however, limits possible digitization, and this could cause problems for highly crowded spectral regions. The low sensitivity, and

hence the high time requirement for data acquisition, is also an obstacle to the routine application of 3D NMR techniques despite the fact that OS compounds are ideal candidates for this approach in view of the small sweep-width which they span. In order to increase the resolution and thus avoid overlaps in the F_1 and F_2 dimensions, band selective pulses have been incorporated in 3D experiments. However, replacement of the first pulse by a selective Gaussian pulse, which only excites a particular non-overlapping ^{1}H NMR resonance (usually the anomeric-H atom), provides signals in the form of a 2D spectrum. The magnetization is transferred to other intra-residual protons of the same sugar residue by the TOCSY step and the magnetization is transferred through space by dipolar coupling to any proton of the neighbouring monosaccharide residue that is connected at the glycoside linkage position. By using this approach the information obtained is unequivocal.

The complete sequence analysis of a triterpenoid tetrasaccharide was achieved by TOCSY-ROESY pulse sequence, instead of a TOCSY-NOESY experiment, within 10 minutes (Schroder and Haslinger, 1993a). However, it is perhaps unlikely that routine use of 3D NMR will be necessary for the above mentioned purpose since complete assignments can usually be obtained with conventional 2D methods; however, a situation will certainly arise where additional spectral dispersion afforded by 3D techniques will be of value.

1D analogs of multidimensional (*n*D) experiments

The total acquisition time needed to perform one or more 2D experiments is in practice, often prohibitive and this has led to the development of experiments combining different polarization transfer mechanisms in a 1D manner, where only one spin is selectivity excited at the beginning of the pulse sequence. The 1D COSY in conjunction with multi-step RCT or 1D TOCSY can be used to assign all resonances of a particular carbohydrate residue which in combination with NOE transfer, gives the NOE at the resonance of the following carbohydrate residue (Haslinger *et al.*, 1990). The 1D NOESY or 1D ROESY difference experiments, in an analogous manner, yield information about the IGL (Haslinger *et al.*, 1990; Wessel *et al.*, 1991). The applicability of the 1D COSY technique for the detection of long-range coupling indicative of sequence has also been suggested (Uhrin *et al.*, 1994b; Uhrinova *et al.*, 1991). By using this approach the information obtained is equivocal, e.g., the final non-selective NOESY transfer in a 1D TOCSY-NOESY experiment gives rise to many signals depending on the NOE contacts of all of the protons for which magnetization had been created during the initial selective TOCSY transfer.

An obvious limitation of selective NMR techniques, especially their 1D versions, is the signal separation needed for their selective excitation by soft pulses. The improved digital resolution and the time-saving advantages make the application of 1D techniques in molecules of moderate complexity more attractive than their 2D counterparts. However, the essential requirement for the successful application of these experiments is the sufficient separation of resonances from each other to allow for selective irradiation (Poppe and van Halbeek , 1991). Truly selective experiments, which apply selection of the magnetization in both steps, retain all of the advantages of 1D experiments together with the unambiguity of the 3D approach.

CARBON-13 NMR METHODS

Unlike ^{1}H NMR spectra, line broadening and overlapping do not seriously affect ^{13}C NMR spectra owing to the much larger interval of chemical shifts covered. Moreover, ^{13}C chemical shifts are more sensitive to their chemical environments than are proton shifts. The number of protons coupled to a particular carbon can be determined easily by the use of spectral editing experiments. (Agrawal, 1992; Agrawal *et al.*, 1985; Bock *et al.*, 1984; Bock and Pedersen, 1983; Bock and Thoegersen, 1982; Broadbury and Jenkins, 1984; Gorin, 1981). Analysis of the ^{13}C NMR and long-range ^{13}C–^{1}H correlated spectrum of an OS, along with easily obtainable data on its monosaccharide composition, allows unambiguous conclusions to be drawn as to the type of glycosidic linkages present, their configurations and the nature of the adjacent monosaccharide units.

Glycosylation induced shift method

This method is based on the observation of a resolved resonance, and subsequent assignment, for each carbon atom in the OS, and on the fact that the ^{13}C NMR chemical shifts of the resonances from a monosaccharide residue within a large saccharide depend mainly upon the structure of the monosaccharide and upon the nature of the flanking sugar residues (Bock *et al.*, 1986; Kochetkov, 1990, Kochetkov *et al.*, 1984; Shaskov *et al.*, 1988). The assignment procedure was initially based on previously assigned spectra of model compounds containing fewer monosaccharide residues and these data formed the ground work for the sequence determination of the OS. The chemical shift of a resonance from a particular carbon atom of a particular sugar residue in an OS should be within ±0.3 p.p.m. of that of the resonance of the C atom of the monosaccharide.

In order to come to reliable conclusions concerning the primary structure of an OS from its ^{13}C NMR spectrum, it is obviously necessary to establish clear and explicit relationships between spectral parameters and elements of the structure of the OS. This means that the well known spectral characteristics of individual monosacchrides must be connected in some way with those of the same monosaccharide units of an OS chain. It is evident that chemical shifts of the carbon signal of a monosaccharide unit involved in a chain differ from those of a free monosaccharide due to glycosylation. Glycosylation induced shifts (GIS) can be represented as: $\delta_{GIS} = \delta_{\text{glycosylated monosaccharide}} - \delta_{\text{methylglycoside}}$. The most significant changes are associated with the carbons directly involved in the glycosidic linkage (C–α) and the adjacent carbons (C–β). In general, the C–α shifts to lower field by 4–10 p.p.m. whilst the C – β shifts to higher field by 1–4 ppm, with negligible changes in the chemical shifts of other carbon resonances of the aglycone residue (Fig. 1). Alternative substitution patterns can thus be unambiguously distinguished. These effects are the consequence of spatial proton-proton interactions which causes polarization of the C–H bond. The 1,3-interaction between H-1′ of the glycone and H-α of the aglycone causes a downfield shift of the corresponding C-1′ and C-α, and 1,4-interaction between

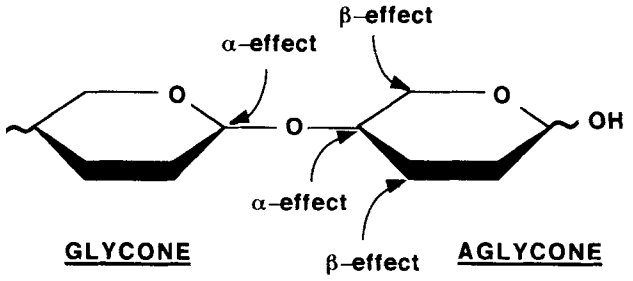


Figure 1. The effects of glycosylation on the chemical shifts of carbons involved in the glycosidic linkage.

H-1′ and H-β causes an upfield shift (Baumann *et al.*, 1988; Bock *et al.*, 1984, 1986; Broadbury and Jenkins, 1984; Kochetkov *et al.*, 1984; Kochetkov, 1990, Shaskov *et al.*, 1988).

Fundamentally important is the fact that the effects of glycosylation typical of a disaccharide residue remain practically unaltered when the disaccharide unit constitutes a fragment of a more complex OS. This means that the most pronounced effects are usually observed for the carbons in closest proximity to the glycosidic linkage, whereas more remote monosaccharide units have practically no effect on chemical shifts of carbon signals of a given monosaccharide unit. A subsequent elongation of the oligosaccharide chain has no further effect on chemical shifts of already glycosylated rings, and those patterns will obviously be recognizable in higher members of a series of related OS compounds. The GIS effects depend on the type of linkage and the stereochemistry of the monosaccharide residues connected.

Additivity of empirically derived ^{13}C GIS values usually holds with small deviations being attributed to conformation around the glycosidic bond, to spatial interaction of proton resonances, and to concentration and solvent composition. Thus, glycosylation effects are transferable parameters and can be employed in deducing the primary structure of an unbranched OS by employing average GIS values (Bhandari *et al.*, 1990; Kochetkov, 1990; Lipkind *et al.*, 1988; Mimaki *et al.*, 1992; Singh *et al.*, 1986, 1987; Uniyal *et al.*, 1990a, b; 1991). The establishment of the additive nature of the effects of glycosylation allowed the development of a computer-assisted approach to the structural analysis of linear, regular OS compounds on the basis of their ^{13}C NMR data. However, these effects become non-additive particularly for an OS with vicinally disubstituted branch point residues due to steric hindrance. Thus, whilst it is experimentally straightforward to record very accurate and reproducible chemical shifts, these depend on subtle differences in details of chemical bonding which are poorly understood. Therefore assignments based on apparently reasonable interpolations and extrapolations from chemical shifts of closely related structures have led to many erroneous assignments and repeated revisions of published assignments.

The chemical-shift analogy methods have been supplemented with a few *ad hoc* methods having a more sound theoretical footing such as distortionless enhancement by polarization transfer (DEPT) and the attached proton test (APT) which measures proton multiplicity. The value of this technique may, however, be limited by the presence of quaternary anomeric C-atoms as, for example, at the anomeric center of ketosides, since these exhibit a smaller GIS.

The ^{13}C NMR shifts for the terminal sugar residue exhibit a close resemblance to those reported for the respective methyl-*O*-glycoside, and thus sequence can also be predicted. However, this method requires assignment of preferably all, or at least most, of the ^{13}C NMR resonances.

Spectral assignment of an OS is usually accomplished by comparison with related compounds, and the best way to assign ^{13}C NMR resonances unambiguously is on the basis of assigned protons by various homonuclear techniques followed by a HETCOR experiment (under ^{13}C-detection mode) or ^{1}H-detected heteronuclear multiple-quantum or single-quantum coherence (HMQC or HSQC) experiments.

In case of ambiguity, HMQC-RELAY or HMQC-TOCSY experiments are useful at least for the assignments for adjacent carbon resonances (Pant *et al.* 1994). Assignments of the ^{13}C signal of each saccharide can be unequivocally achieved by tracing out the one-bond $^{1}H-^{13}C$ connectivities through the use of the HMQC spectrum (Martin and Crouch, 1991). Thus, from the completely assigned ^{13}C NMR data, the branched and/or linear nature of the sugar chain can be deduced by comparison of the ^{13}C shifts with those of reference methyl glycosides together with known *O*-glycosylation-induced shift data which will demonstrate observable ^{13}C shift difference between inner and terminal monosaccharide units.

The excitation of the anomeric proton under a 1D TOCSY-DEPT experiment with a long mixing time is of great value as it generates ^{13}C NMR sub-spectra of individual monosaccharide units, thus assignment of ^{13}C NMR chemical shifts may be performed unequivocally (Doss, 1992). Further, the identification of the glycosylated carbon can be determined by comparison with literature values of the monosaccharide.

Selective INEPT (SINEPT)

This technique allows magnetization transfer from a preselected proton to a ^{13}C nucleus, and can be used effectively to detect the presence of long-range $^{1}H-^{13}C$ couplings (Bax, 1984; Cordell, 1991; Cordell and Kinghorn, 1991). The critical factor in applying the SINEPT experiment for the determination of an IGL position is the size of the three-bond coupling constant $^{3}J_{HCOC}$. The magnitude of this coupling depends on the dihedral angle in a Karplus-type relationship. Typical values of $^{3}J_{HCOC}$ across the glycosidic linkage lie between 3.5 and 5.6 Hz and therefore SINEPT has been effectively utilized to detect in a direct and unambiguous manner the IGLs in glycosidic esters (Bashir *et al.*, 1993), a sweet triterpene glycoside (Suttisri *et al.*, 1993) and pregnane glycosides (Rasoanaivo *et al.*, 1991).

^{13}C NMR longitudinal relaxation times

The average ^{13}C NMR longitudinal relaxation times (T_1) of the carbons of each monosaccharide unit also provide information about the sequence of the sugar chain as longer T_1 times are generally associated with greater segmental motion. This means that a monosaccharide having a higher value of T_1 is most probably at the farthest position from the rigid non-sugar entity (aglycone), whereas monosaccharide units having shorter T_1 values are likely to be internal i.e. close to the aglycone in the case of a glycoside. This method, in conjunction with NOESY, has been used in

determining sugar sequence in saponins (Chen and Snyder, 1987; Li *et al.*, 1994; Yu *et al.*, 1994).

Heteronuclear methods

Two-dimensional one-bond $^{1}H-^{13}C$ chemical shift correlation experiments enable one to trace scalar connectivities between ^{1}H and ^{13}C atoms. Owing to the high resolving power of the ^{13}C nuclei, these methods are enjoying great popularity. The information for IGLs can be ascertained either by heteronuclear $^{13}C-\{^{1}H\}$-NOE or long-range $^{13}C-^{1}H$ $^{3}J_{COCH}$ connectivity.

$^{13}C-\{^{1}H\}$ NOE Spectroscopy

This method is based on the presence of a trans-glycosidic $^{1}H\{^{1}H\}$ NOE which is further transmitted to a directly bonded carbon atom through incoherent magnetization transfer and is thus indicative of the IGL as well as of the sequence. The three-spin effect results in an overall negative enhancement of the carbon involved in the glycosidic linkage. As the anomeric protons and the glycosylated carbons are in most cases shifted downfield from the bulk of the signal, selective saturation of the anomeric proton in one of the sugar units in a $^{13}C\{^{1}H\}$ NOE experiment traces the interglycosidically linked carbon.

The negative heteronuclear NOE observed on presaturation of anomeric-H to the aglycone-H measured either in the selective $^{13}C\{^{1}H\}$ NOE mode (one-dimensional approach) or the phase-sensitive 2D heteronuclear NOE mode (two-dimensional approach) is useful in determining IGLs as well as for OS sequencing (Batta and Kover, 1988; Kover and Batta, 1986, 1988, 1989). Despite the fact that intra-nuclear NOE signals are stronger than the indirect inter-unit NOE effects, the latter are still detectable but, because of its insensitivity, this approach does not find routine application.

Long-range $^{1}H-^{13}C$ connectivity

The long-range heteronuclear methods measure through-bond connectivity between two- and three-bond distant C and H atoms. The intra-residual couplings are helpful for assignment and for elimination of higher order effects in ^{1}H NMR spectra, and serve as a confirmatory proof of the assignments made for monosaccharide residues. Further, the inter-residual coupling across the glycosidic bond indicates the glycosylation pattern. Thus, this method relies on the fact that the anomeric aglycone carbon exhibits its three-bond coupling with glycone-H ($^{3}J_{COCH}$) or with the anomeric proton and the aglycone carbon ($^{3}J_{HCOC}$) across glycosidic linkage. The size of the three-bond coupling constant does depend on conformation but these couplings are invariably observed and hence long-range $^{1}H-^{13}C$ correlation spectroscopy can be used as an alternative method for the determination of IGL, and to yield sequence information in oligosaccharides (Batta and Liptak, 1984). For successful interpretation, a complete and definitive assignment of both the ^{1}H and ^{13}C resonances is an essential requirement. Various pulse sequences (such as FLOCK, COLOC, XCORFE etc) have been employed in order to observe these long-range heteronuclear couplings under the ^{13}C detection mode for deducing IGL as well as the sequence in flavonoid glycosides (Anderson *et al.*, 1991a, b; Hasler *et al.*, 1992; Hu *et al.*, 1992; Mizuno *et al.*, 1990), in saponins (Bader *et al.*, 1992; Ding *et al.*, 1992; Jiang *et al.*, 1994; Kawashima *et al.*, 1991a,b; Kubo *et al.*, 1992a,b; Mimaki and Sashida, 1990a,b; Nagao *et al.*, 1991a,b,c; Ohtani *et al.*, 1994; Orsini *et al.*, 1994; Reznicek *et al.*, 1992; Seaforth *et al.*, 1992; Xu *et al.*, 1992a,b), in glycoalkaloids (Chao *et al.*, 1990), in a bufanoloide glycoside (Krenn *et al.*, 1991), in mono- and di-terpenoid glycosides (Orihara *et al.*, 1991; Yahara *et al.*, 1991), in phenolic glycosides (Ikeya *et al.*, 1991; Noda *et al.*, 1992a,b) and in oligosaccharides (Enriquez, 1991; McIntyre and Vogel, 1989; Saitoh *et al.*, 1993a,b; 1994). The ^{13}C-detected methods for the determination of long-range heteronuclear connectivity require more sample, by at least an order of magnitude, than the one-bond connectivity experiment and, therefore, these have been largely replaced by inverse heteronuclear techniques.

Because heteronuclear multiple-bond correlatin (HMBC; Bax and Summers, 1986) is an inversely correlated ^{1}H-detected variant, it is very sensitive and reduces the requirement of OS sample to a few milligrams. This provides a valuable method for confirming ^{13}C and/or ^{1}H assignments of the constituent monosaccharides and for identifying an IGL linkage since the three-bond coupling between anomeric-C and/or anomeric-H with aglycone carbon at the IGL site is invariably observed. The intensities of the correlation peaks depend on the multiplicity of the respective proton resonance, i.e. broad multiplicity of the respective proton resonance leads to poor cross-peaks. Discrimination between intra- and inter-sacchride correlation (Abeygunawardana *et al.*, 1990; Reddy *et al.*, 1993) is made by reference to the established ^{1}H resonance assignments and by an iterative comparison of the one- and multiple-bond $^{1}H-^{13}C$ correlation maps which provide information in a sequential manner for the establishment of the IGL as well as monosaccharide sequence. This procedure has been widely employed by various workers (Ahmad *et al.*, 1993; Ayer and Miao, 1993; Bader *et al.*, 1994a,b; Callis *et al.*, 1994; Cui *et al.*, 1990; Dirsch *et al.*, 1994; Finger *et al.*, 1991; Himmelsbach *et al.*, 1994; Ikeshino *et al.*, 1994; Jia *et al.*, 1994; Jiang *et al.*, 1991a,b; Kadota *et al.*, 1990; Kakuno *et al.*, 1992; Kanmoto *et al.*, 1994; Katsuzaki *et al.*, 1994; Kondo *et al.*, 1992; Lacaille-Dubois *et al.*, 1993; Lavaud *et al.*, 1992; Lin *et al.*, 1994; Lou *et al.*, 1993; Massiot *et al.*, 1991a,b, 1992a,b; Mimaki *et al.*, 1992, 1993a,b; Miyase *et al.*, 1991, 1994; Nakamura *et al.*, 1994a,b; Noda *et al.*, 1992a,b; Pal *et al.*, 1994; Pant *et al.*, 1994; Powell *et al.*, 1990; Ridout *et al.*, 1994; Ripperger and Porzel, 1994; Saito *et al.*, 1990; Saitoh *et al.*, 1993a,b, 1994; Schneider *et al.*, 1993; Schopke *et al.*, 1994; Schroder and Haslinger, 1993a,b; Schwind *et al.*, 1990; Shoji *et al.*, 1994; Takeda *et al.*, 1993; Umehara *et al.*, 1994; Warashina and Noro, 1994; Xu *et al.*, 1992a,b; Yamamoto *et al.*, 1994; Yoshikawa *et al.*, 1993a,b).

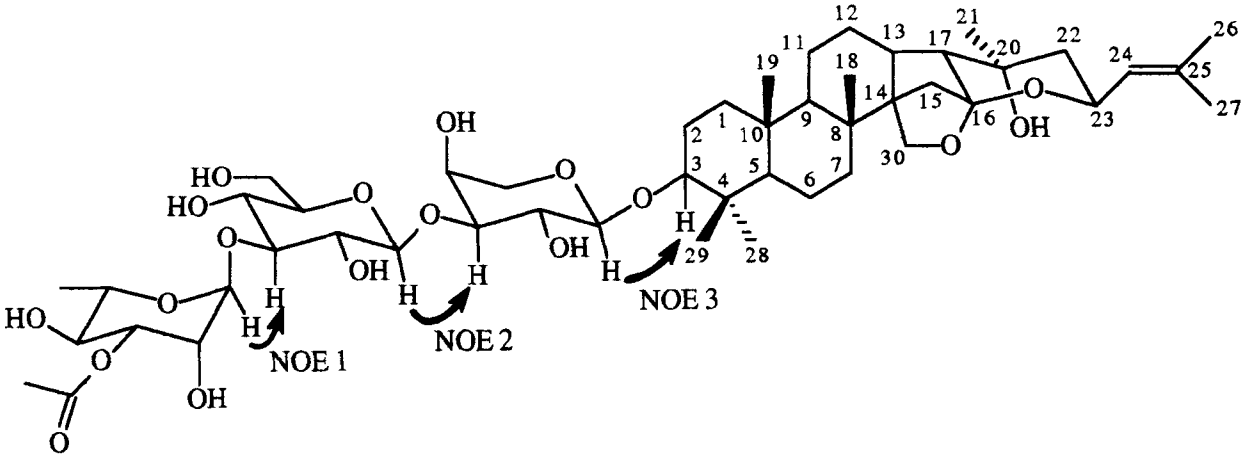
The sequence of monosaccharide residues in a sugar chain, including its linear or branched nature, can also be established by the careful analysis of the HMBC spectrum as there will be no inter-residual HMBC cross peaks for any ring carbons occupying terminal positions, while HMBC cross-peaks will be associated with internal monosaccharide residues.

The long-range heteronuclear single quantum correlation (LR-HSQC) spectrum can also give rise to observable long-range proton–carbon coupling cross-peaks indicative of an

IGL (Uhrin *et al.*, 1994b). Since the correlation is transmitted through bonds, heteronuclear methods provide linkage information of a more fundamental nature than that provided by the ^{1}H NOE/NOESY/ROESY method, which gives information only on proton proximity. Further, the three-bond coupling constant across the glycosidic linkage does not depend on conformation. Therefore, ^{13}C–^{1}H correlation either between the anomeric carbon and aglycone-H or aglycone carbon atom and anomeric-H provides unambiguous proof of the position of the IGL.

THE STRUCTURAL DETERMINATION OF ZIZYPHOISIDE C

The application of some of the above-mentioned techniques is now demonstrated using the triterpenoid saponin zizyphoiside C (**1**; Fig. 2) as an example (Li *et al.*, 1994). The ^{1}H NMR spectrum displayed nine methyl groups, one of which showed a doublet at δ 1.36 indicating an adjacent methine group, while the remaining eight were connected to



ZIZYPHOISIDE C (1)

Figure 2. The structure of zizyphoiside C showing NOE interactions (see also Fig. 5).

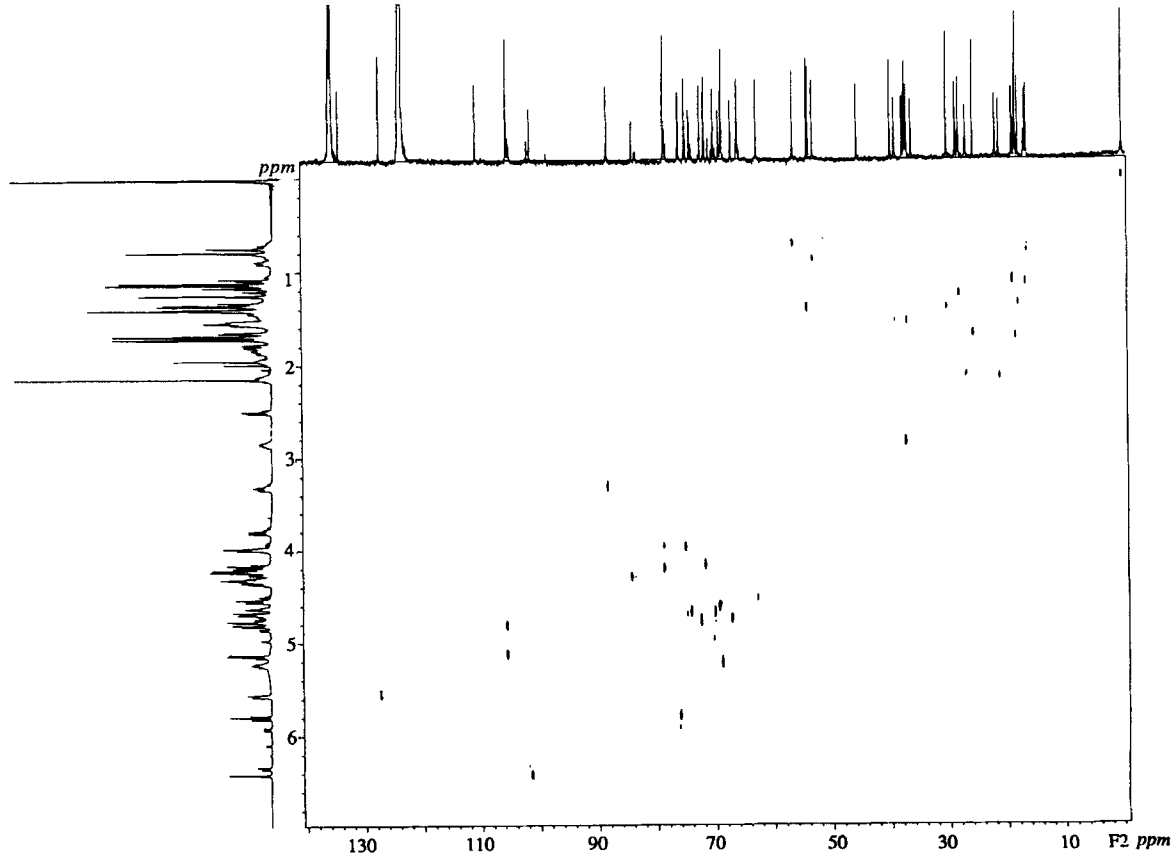


Figure 3. ^{13}C–^{1}H COSY spectrum of zizyphoiside C (**1**).

quaternary carbons. A singlet occurring at δ 2.15 was typical of an acetyl methyl group. There were eighteen proton resonances appearing between 3.9 and 6.4 p.p.m. which belonged to the saccharide as well as to the aglycone residue. In the anomeric region, there were three doublets at δ 4.83, 5.11 and 5.57, a triplet at δ 5.24 and a singlet at δ 6.40. The ^{13}C and DEPT experiments revealed the presence of $9\times CH_3$, $11\times CH_2$, $21\times CH$ and $8\times C$ atoms. There were two anomeric ^{13}C resonances at δ 105.5 and 101.3 the former of which was of almost double the intensity of the latter, and this is in accordance with the analysis of the ^{13}C–^1H correlation data (Fig. 3) as the ^{13}C signal at δ 105.3 showed cross-peaks with two anomeric proton doublet resonances at δ 4.83 and 5.11. The anomeric-C at δ 101.3 showed its one-bond connectivity to a singlet at δ 6.40. These spectral features indicated the presence of a trisaccharide moiety in zizyphoiside C.

The information concerning the discrimination of the ^1H NMR signals belonging to the sugar and the aglycone part was achieved by analysis of the ^1H–^1H COSY spectrum (Fig. 4). The proton coupling network of the individual monosaccharide residue was traced out by the combined application of COSY and TOCSY experiments. The ^1H NMR assignments were compared with the HETCOR data (Fig. 3) and this led to the assignment of the ^{13}C NMR resonances for the residue and subsequently the identification of the monosaccharide residues as rhamnopyranose, glucopyranose and arabinopyanose. The configuration at the anomeric carbon atoms of the sugars (β for glucose; α for the other two sugars) was established from the chemical shifts of the anomeric carbons as well as the multiplicities of the anomeric protons.

The nature of the sugar sequence was later established by the analysis of data from several experiments. The average ^{13}C NMR longitudinal relaxation times (T_1) of each of the methine carbons of each sugar unit revealed that rhamnose had the largest T_1 value (0.31 s), whereas arabinose had the shortest (0.25 s). This suggested that the rhamnose was probably the farthest away from the more rigid aglycone entity while the arabinose was the closest. The downfield ^{13}C NMR shifts of the C-3 resonances of all three monosaccharide residues (e.g., rhamnose–76.0 p.p.m. compared with the expected 72.3 p.p.m. shift in the reference compound; arabinose—74.90 p.p.m. compared with 70.8 p.p.m.; glucose—83.93 p.p.m. compared with 78.9 p.p.m.) indicated a (1→3) interglycosidic linkage (Table 1). Direct evidence for the sugar sequence and the linkage sites was further provided by observing the through-space coupling between two protons residing one on each side of the respective linkages. This was carried out by analysis of the ROESY spectra (Fig. 5). The anomeric-H (δ 4.83) of arabinose gave a ROESY crosspeak to H-3 of the triterpenoid moiety at δ 3.30 confirming its direct involvement in the sugar–aglycone linkage. The anomeric-H of

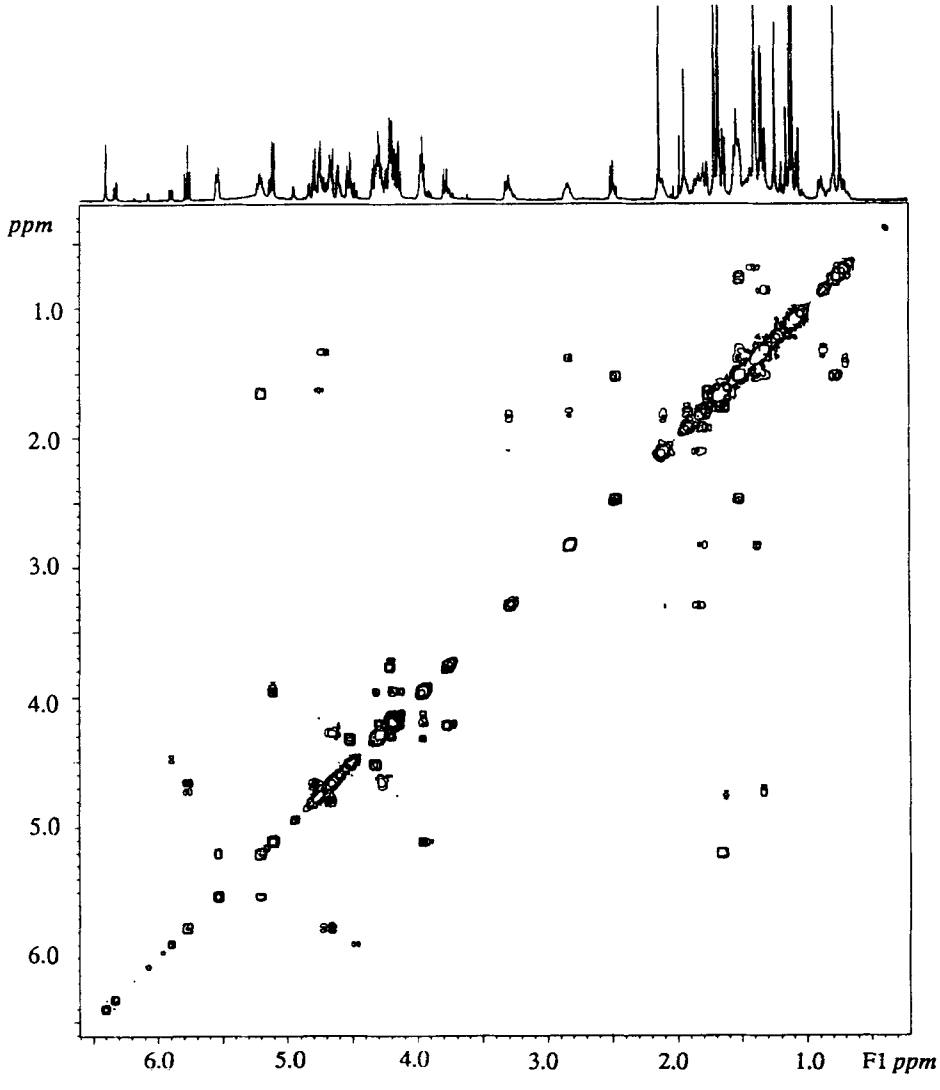


Figure 4. ^1H–^1H COSY spectrum of zizyphoiside C (**1**).

Table 1. ^1H and ^{13}C-NMR assignments of the sugar units of zizyphoiside C (1) and T_1 relaxation times of specific carbon nuclei

Compound	Zizyphoiside C $^{13}C^b$	Zizyphoiside C $^1H^b$	T_1^c	Reference sugars $^{13}C^b$	Reference sugars $^1H^b$	$\Delta^{13}C^a$
Arabinose						
1	105.3	(4.83, d)	0.25	104.6	(4.69)	+0.8
2	70.0	(4.76)	0.26	72.6	(4.66)	−2.6
3	74.9	(4.70)	0.25	70.8	(4.57)	+4.1
4	67.0	(4.76)	0.23	68.4	(4.49)	−1.4
5	66.0	(3.79, 4.25)		66.2	(3.54, 4.17)	−0.2
Glucose						
1	105.3	(5.11, d)	0.27	105.5	(5.01)	−0.2
2	78.6	(3.97)	0.23	75.0	(3.92)	+3.4
3	83.9	(4.32)	0.31	78.9	(4.11)	+5.0
4	71.6	(4.22)	0.28	71.5	(3.87)	+0.1
5	78.5	(4.22)	0.29	78.3	(4.03)	+0.2
Rhamnose						
1	101.3	(6.40, s)	0.35	101.8	(6.45)	−0.5
2	72.3	(4.59)	0.30	72.4	(4.76)	−0.1
3	76.0	(5.79)	0.31	72.3	(4.60)	+3.7
4	74.1	(4.18)	0.34	74.1	(4.20)	0.0
5	69.1	(4.61)	0.28	69.5	(4.71)	−0.4
6	17.9	(1.36)		18.8	(1.61)	−0.9

[a] Data represent the difference in ^{13}C-NMR chemical shift between the specific carbon in the sugar units of zizyphoiside C and the similar carbon in reference sugars.
[b] Chemical shift values are presented in p.p.m. with reference to TMS.
[c] Data are expressed in s.

rhamnose (δ 6.40) and glucose (δ 5.11) exhibited ROESY connectivity to H-3 of glucose (δ 4.32) and H-3 of rhamnose (δ 4.70), respectively. The appearance of H-3 of rhamnose at a significantly lower field position (δ 5.79) than expected indicated acetyl substitution at this position. Subsequently the structure of zizyphoiside C

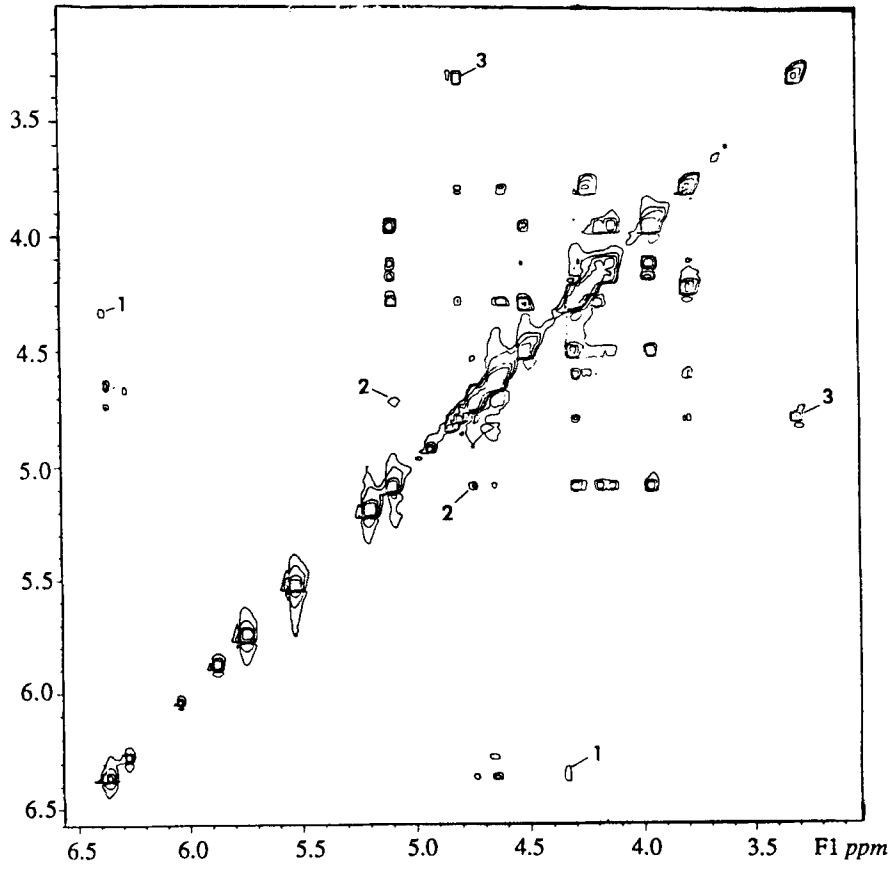


Figure 5. The ROESY spectrum of zizyphoiside C (**1**) showing three ^1H–^1H NOE responses numbered according to their corresponding glycosidic bonding locations as shown in Fig. 2.

was established as 3-*O*-acetyl-α-L-rhamnopyranosyl (1→3)-β-D-glucopyranosyl (1→3)-α-L-arabinopyranosyl (1→3) jujobogenin (**1**).

CONCLUSION

Initial information concerning monosaccharide composition and anomeric configuration can be obtained by through-bond connectivity analysis using various variants of ^1H–^1H shift correlated spectroscopic techniques such as COSY, DQF-COSY, TQF-COSY. The complete '*J*-network' of the individual monosaccharides can also be determined by multi-step RCT or HOHAHA spectral data, if there is at least one resonance in the spin system, such as the anomeric proton, which is well isolated and which has a reasonably large coupling to its neighbouring spin. Therefore, a slice taken through later spectra at each anomeric proton along the diagonal yields ^1H sub-spectra containing all scalar-coupled protons within that sugar residue. However, the distribution of magnetization around the spin system can be impeded by small couplings such as typically found between H_4 and H_5 of galactosyl residues. In order to circumvent the bottleneck of a small coupling, 1D and 2D versions of the relayed HOHAHA pulse sequence are of intrinsic value (Inagaki *et al.*, 1989).

Once the unambiguous ^1H NMR assignments are evident, the sequence of the carbohydrate chain and the position of the sugar connectivity can be subsequently established by the observation of inter-residue effects from dipolar (through-space) connectivities detected by 1D inter-glycosidic $\{^1H, ^1H\}$ NOE effects, 1D NOE or ROE, or by laboratory frame NOESY or rotating frame ROESY experiments. These approaches rely on proton NMR, and are often complicated because of severe spectral overlap. Additionally, the effects on which these methods are based (i.e. the NOE effect across the glycosidic linkage and the $^4J_{HCOCH}$ scalar coupling) are strongly conformation dependent and therefore not always unambiguous. Hybrid methods combining TOCSY and ROESY in either manner, contain both coherent and incoherent proton-polarization transfer steps in a single experiment and provide, in principle, identification of networks of coupled spins as well as through-space proximity between adjacent spins. If a minute amount of material only is available, observation of other nuclei such as ^{13}C is not practical on grounds of sensitivity and one should rely on homonuclear methods. However, in order to avoid risk of misinterpretation, one should supplement dipolar inter-residual connectivity observed in NOESY/ROESY spectra with other spectral evidence such as consideration of ^{13}C NMR GIS effects or long-range ^1H–^{13}C connectivity.

One-dimensional analogues of multi-dimensional methods, which combine different polarization-transfer mechanisms, are also valuable supplements to their multi-dimensional counterparts. Since they contain all the advantages of 1D experiments and the unambiguity of a

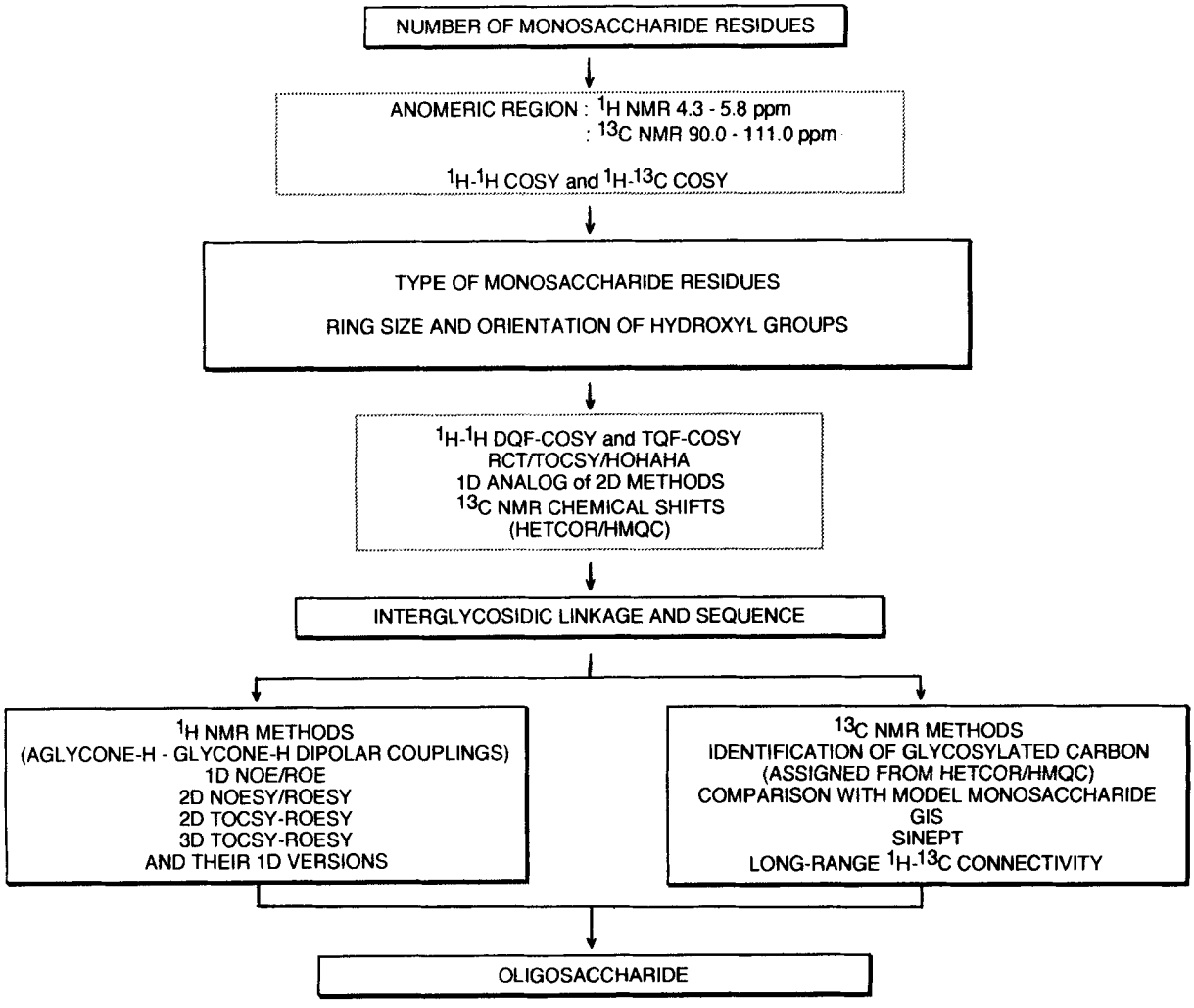


Figure 6. An outline of the NMR spectroscopic approaches for deducing molecular structure of an oligosaccharide.

multi-dimensional experiment, they are the method of choice when only a few assignments remain questionable after standard methods of 2D NMR spectroscopy have been applied. Thus, concerted application of homo- and hetero-nuclear NMR approaches can lead to the unambiguous ^{1}H and ^{13}C signal assignments for an OS moiety, and to the tracing of either (a) inter-sugar NOE or ROE cross peaks, or (b) long-range $^{3}J_{HCOH}$ coupling between the anomeric proton of one sugar unit with the linkage-site C-atom of the adjacent unit, and this allows the *de novo* sequencing of the OS. However, it may be mentioned that a great deal of experience is required in order to utilize the *ab initio* method effectively, particularly for heterogeneous samples.

There may be several other NMR experimental procedures which are applicable to the determination of the above information but, as revealed by trends observed in the literature as well as by our own experience in the field, the above homo- and hetero-nuclear correlated NMR chemical shift methods (Fig. 6) are efficient and reliable (in principle) to solve most of the oligosaccharide structural problems. It is important to mention that there is no single method which is applicable to all problems and therefore for unambiguous structural analysis one should verify the information derived from one experiment using another method, even though these experiments require a high-field NMR instrument and significant measuring time.

The use of magnetic field gradients offers the spectroscopist an operationally simple means of increasing the acquisition speed of 2D and 3D NMR experiments by at least ten-fold thus effectively reducing the measurement time from several hours to less than 10 min for certain 2D experiments (Carpenter *et al.*, 1993; Hurd and John, 1991). However, this facility is available only to a few research groups at present.

For OS compounds with many identical subunits, it is also possible that none of the above methods will yield complete assignments without derivatization or isotopic labelling. Because of the non-invasive nature of the NMR method, the sample can be recovered unadulterated after NMR measurements and the recovered sample can be examined by other methods such as methylation analysis, gas chromatography–mass spectrometry etc. in order to provide additional data to support the incomplete data available from the NMR experiments. Therefore, these methods can be considered as the primary method for the determination of the complete covalent structure of the OS and can be applied to samples available in only a few milligrams in their native form. It would also be worthwhile to investigate complete sets of ^{1}H and ^{13}C signals for OS compounds to monitor the influence on chemical shift not only of direct glycosylation but also of long-range effects between residues not directly connected.

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