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Carbohydrate chemistry: synthetic and structural challenges towards the end of the 20th century*

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Abstract: Carbohydrate chemistry has acquired considerable interest in many different facets during the last decades of the century. Particularly, the synthesis of complex carbohydrates in the form of large oligosaccharides has paved the way for a better appreciation of the function of carbohydrates in biological systems. The impressive advances associated with the structural determination and analysis of oligosaccharides has furthermore contributed to the increased focus on the role of carbohydrates particularly in the form of glycoproteins but also as signal molecules in general.

The primary goal of this work is to discuss the advancement of structural assessment of large oligosaccharides as typified by the structural determination of the 22-mer saccharide from the LPS of *Salmonella enterica* ssp. *typhimurium* strain 1135 using very high field strength NMR spectroscopy. The challenges of synthesizing and analysing the structure of glycopeptides will also be discussed. Finally, the latest developments in glycopeptide libraries generated by solid-phase combinatorial chemistry will be presented in addition to examples of new techniques where the increased sensitivity using nanoprobe technology in combination with MALDI-TOF MS spectroscopy was used in the structural assignment of such complex glycopeptides; a methodology of invaluable importance for the analysis of glycopeptides on single beads.

INTRODUCTION

Carbohydrate chemistry has witnessed a rapid evolution during the last decades of this century not only due to the increasing evidence for the importance of carbohydrates in biological systems [1,2] but also due to the recognition both in public and in the scientific community, that the complex carbohydrates have many beneficial effects in food, such as health. The present review will focus on the developments in structural analysis and synthesis of complex carbohydrates particularly from the author's laboratory. However, some of the seminal contributions which had been documented during the last decades and which have been instrumental to the development of carbohydrate chemistry in this century will also be summarised.

Structural analysis

The introduction of methylation analysis by the Stockholm group in the 1970s [3] suddenly made it possible to determine the structures of bacterial polysaccharides; a technique later applied in structural assessment of all types of complex oligosaccharides. Combinations of this methodology with the rapidly advancing instrumental developments of GC-MS and particularly NMR spectroscopy both ^{1}H-NMR but especially Fourier transform ^{13}C-NMR spectroscopy made it possible to make a complete structural analysis of microgram amounts of such complex carbohydrates. At the end of the century the analysis of

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oligosaccharides with up to about 22 monosaccharides is possible with the use of high field NMR spectrometers combined with MALD-TOF MS spectroscopy and nano-ES technology as shown in Fig. 1 and discussed below.

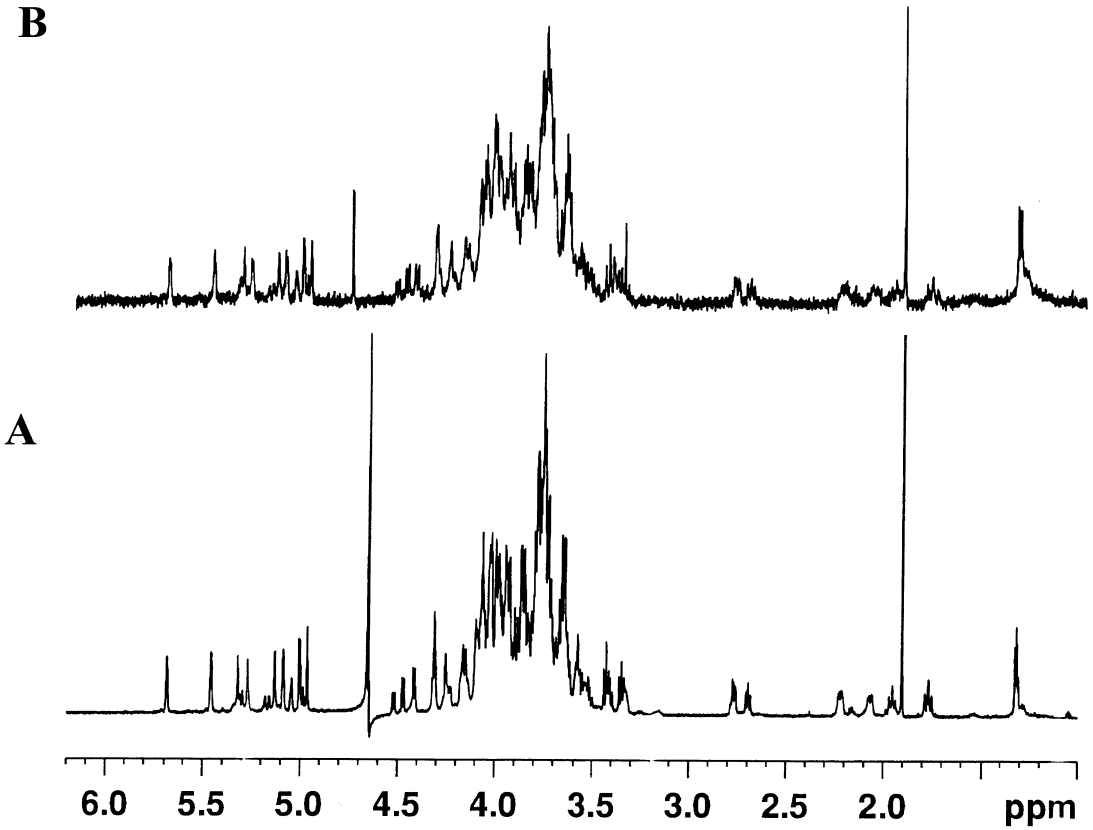

Fig. 1 (A) 750 MHz NMR spectrum, 32 scan at 37 °C, of an 22-mer oligosaccharide, 2.0 mg in 600 μL, from *Salmonella enterica* ssp. *Typhimurium* 1135 LPS. (B) NMR spectrum of the same compound 0.14 mg in 40 μL in a nanoprobe operating at 500 MHz, 32 scan at 25 °C.

Finally, the PCR reaction [4] which amplifies signals from smaller pieces of DNA (oligonucleotides) is probably the single most important reaction developed in this century with respect to general use and impact of our lives and the progress of science in many aspects.

Synthesis of carbohydrates

The synthesis of complex oligosaccharides presents a major achievement which also exploded from the middle of the 1970s starting with the chemical synthesis of the blood group terminants [5] and later very impressive accomplishments with total synthesis of carbohydrate containing material products like calicheamicin [6] and palytoxin [7]. These development would not have been possible without the above mentioned structural assignment tools but also the rapid development of chromatographic methods, like silica gel TLC and HPLC which were instrumental for such achievements both as a routine analysis and purification of intermediates.

Biological functions and conformational analysis

The ability to synthesise, isolate and characterise complex carbohydrates from biological systems paved the way for a deeper understanding of the function of these molecules in their natural environment. [1,2] The recognition of the key–polar interactions [8] presented by specific hydroxyl groups in protein–carbohydrate interaction was a major step forward primarily promoted by the chemical synthesis of analogues (particularly deoxy-derivatives) [9] of biologically important oligosaccharides. This polar-gate

theory was further substantiated by the development of conformational analysis and the realisation of the chair conformation of pyranosidic sugars. The important consequences of the anomeric-and exo-anomeric-effect [10] of oligosaccharides on the overall shape of oligosaccharides either in solution or as complexes with their protein receptors has also contributed significantly to the understanding of the biological function of carbohydrates. Furthermore, from analysis of the preferred three-dimensional structures, it was realised that the hydrophilic carbohydrate molecules could present hydrophobic surface areas often spanning over several monosaccharide residues. [11].

Also the realisation of the double-helix structure of the genetic material, the DNA, has had very important consequences for the understanding and subsequent manipulation and control of these important molecules [12].

Even though such impressive progress has been made, only relatively few carbohydrate molecules have made it to the drug market in agreement with the general 'rule of thumb' in medicinal chemistry, that drugs must have molecular weight below approximately 600. Most impressive has been the development of antibiotics and polysaccharide based vaccines. However, the most effective drug for treatment of HIV infected patients on the market [13], AZT, must be considered the carbohydrate based drug-compound produced in probably the largest amount (about 100 tonnes/year).

Glycopeptides

The glycosylation of proteins and lipids is ubiquitous in Nature and is of vital importance to the proper functioning of biological systems. It is well-established that glycoconjugates on cell surfaces play a vital role in a wide variety of biological phenomena including immune response, intercellular recognition, cellular adhesion, intracellular targeting, cell growth regulation, cancer cell metastasis, and inflammation [1,2]. As a consequence, there has been a tremendous impetus to develop carbohydrate-based therapeutics [14]. However, the isolation, purification, structural characterisation and subsequent synthesis of complex oligosaccharides, which are responsible for many important biological interactions, represents each a major research project which requires special skills and expertise, particularly when labile residues like sialic acid are present in the parent structure. Chemical synthesis of such complex oligosaccharides presents a serious challenge and the organic synthesis of about 100 mg of an oligosaccharide larger than a pentasaccharide is a major achievement. In many cases, only a few residues at the nonreducing end of a complex glycan are necessary for the interaction to the receptor. Therefore, the use of simplified synthetic molecules, glycopeptides, which can be rapidly generated and can mimic the natural ligand, can give important information about the nature and topology of the ligand-receptor interaction [15].

Glycopeptide mimics of complex oligosaccharides

It has previously been demonstrated that synthetic glycopeptides can mimic the interaction of a complex oligosaccharide and its receptor in biological assays [16–26]. These results are in some cases, most likely due to a topological similarity of the two types of compounds. In one example, linear tripeptides bearing two 6′-O-phosphorylated mannose disaccharide's linked α(1→2) were found to be 10-fold tighter binders of the mannose-6-phosphate receptor (MPR) than one of its natural ligands, a branched mannose pentasaccharide [16]. However, when cyclic peptides were used to present 6-*O*-phosphorylated α-D-Man-1→2-α-D-Man ligands to the MPR, the binding affinity was much reduced compared to their linear analogs [17].

Glycopeptide mimetics of SLex for the inhibition of selectin binding have also been developed. Surprisingly, these glycopeptide ligands show increased binding compared to the original SLex ligand, probably due to favourable interaction of the peptide scaffold with the receptor. A trivalent SLex tetrasaccharide derivative attached to a rigid cyclic heptapeptide scaffold showed a 2–3-fold (on a per mole of SLex basis) enhanced inhibition capability [18], while linear displays of SLex on different scaffolds showed no increase in selectin binding [19,20]. The best results come from a series of glycopeptide mimetics containing Fuc, Man or Gal residues which demonstrated up to 40-fold increase in potency over the parent oligosaccharide and were much more facile to prepare [21–25].

Glycopeptide synthetic strategies

In the chemical synthesis of glycopeptides it has been demonstrated that the most versatile methodology for chemical assembly is the use of appropriately glycosylated amino acid building blocks in solid phase peptide synthesis as outlined in Fig. 2B [27].

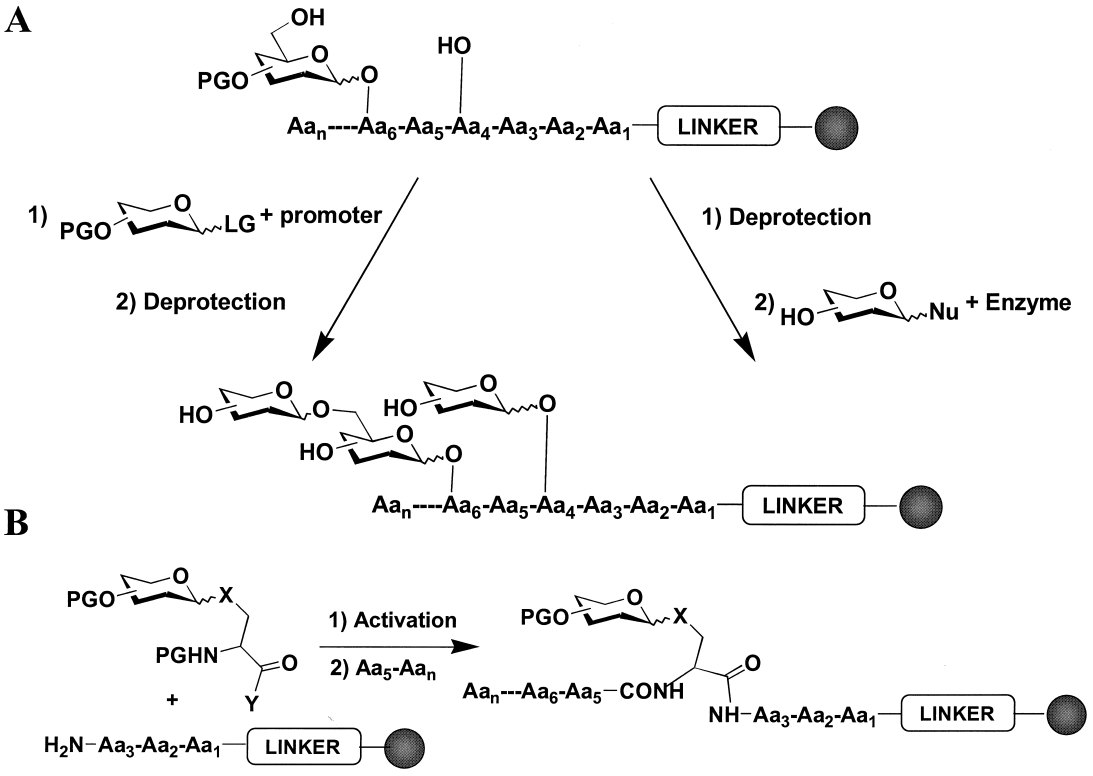

Fig. 2 Strategies for glycopeptide synthesis. (A) Chemical or enzymatic glycosylation of peptide or glycopeptide; Nu = nucleotide sugar. (B) The building block approach (X = O, C, S, N; Y = suitable leaving group).

Synthesis of O-linked mucin core oligosaccharides

A variety of different schemes have been presented for the synthesis *O*-linked mucin core oligosaccharides all characterised by the presence of a GalNAc residue α-linked to Ser or Thr. These are predominantly based on the introduction of $GalN_3$ onto a Fmoc-protected amino acid derivative, followed by elongation of the glycan structure [28–30] or alternatively the use an oligosaccharide building block with a $GalN_3$ reducing end residue as a glycosyl donor [31,32]. The azido protecting groups often employed as a nonparticipation group in α-glycosylation reactions are readily reduced with DTT [30] or thioacetic acid [33] on the solid phase and can even be used for solid phase peptide and glycopeptide synthesis employing α-azido acids [34,35]. Alternatively, the use of the β-directing 2-*N*-dithiasuccionyl protecting group for amino sugar glycosylation reactions which allowed mild thiolytic reduction with DTT or even selectively in the presence of azides with propanedithiol on the solid phase [30,36–38] to liberate the amino functions afforded a range of the GlcNAc containing mucin core glycosyl amino acids as Fmoc-Ser/Thr-OPfp esters activated and ready to use directly in multiple glycopeptide assembly. In solution at the building block stage, both the azido- and Dts-functionalities can most conveniently be converted to *N*-acetates by reduction with Zn in the presence of acetic anhydride [28].

Synthesis of N-linked oligosaccharides

The synthesis of *N*-linked glycosyl building blocks has been predominantly achieved by coupling of the glycosylamine to aspartic acid. However, the use of the glycosyl azides as precursors for glycosylamines

has proven to be a very versatile approach [39,40]. The release of complex glycans by chemical means followed by transformation into building blocks have provided a novel method of preparing large amounts of pure *N*-linked glycopeptides with a single glycoform [41]. Recently, complex *N*-linked glycopeptides have been prepared chemo-enzymatically or on solid phase *via* glycal assembly [42].

Solid phase glycosylation of peptides

Solid phase glycosylation reactions have been highlighted in the recent years particularly by use of the highly reactive glycosylsulfoxides or trichloroacteimidates which can both be used under conditions of homogeneous catalysis. Glycosylation of saccharides linked to solid phase either directly or as a glycopeptide has been achieved and can be quantitative especially on nonpolar resins [43]. However, polar resins are required for solid phase bioassays and methods were developed for glycosylation on a range of polar resins [36]. Direct peptide glycosylation on solid phase has the potential to generate libraries of template glycopeptides; however, for unresolved reasons, direct glycosylation of peptides have proven much more difficult to achieve. Quantitative peptide glycosylation was achieved on a novel PEG-based resin containing only ether bonds [44] and a small model library comprising four compounds was generated by two consecutive glycosylation reactions (Fig. 3) [45,46].

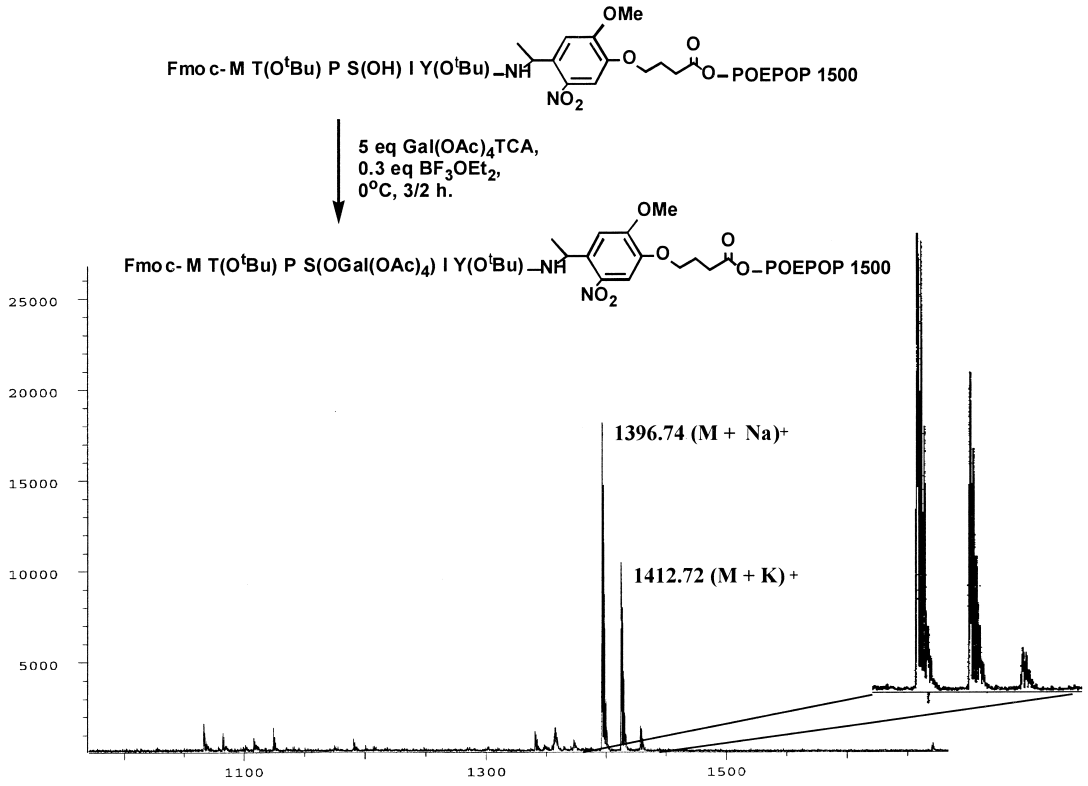

Fig. 3 Monitoring of glycosylation on solid phase using a photo-cleavable linker and MALDI-TOF-MS analysis of the products on single beads.

Glycopeptide libraries: synthesis and analysis

The glycopeptide building block approach used in portion mixing library synthesis allows the expedient formation of numerous glycopeptides as putative ligands in protein binding assays. However, rapid and unambiguous analysis of modified peptides on solid phase remains challenging. One successful glycopeptide library synthesis has been developed and includes an analysis based on *ladder synthesis*, an analytical technique which involves capping a small portion of the growing oligomer during synthesis [47]. The 'ladder' of peptide fragments is generated by an *encoded in-situ capping* methodology that

allows immediate distinction between glycopeptide residues of identical masses and features rapid *on-bead* MALDI-TOF mass analysis facilitated by use of a photolabile linker. Glycopeptide ligands were identified for the C-type lectin from *Lathyrus odoratus* by screening the fluorescent labelled protein in a solid phase binding assay of the PEG resin-bound glycopeptide library [48].

Glycopeptides interacting with MHC class-II

The glycopeptide building block methodology has also been used to synthesise a long series of glycopeptides related to the nonimmunogenic peptide fragment from haemoglobin 86–96, which has been demonstrated to bind strongly to MHC II, but not been able to trigger T-cell proliferation [49]. A systematic scan of this structure with glycosylated amino acid building blocks (GalNAc-α-Ser/Thr) revealed that particularly position 92 with an asparagine residue could be substituted by glycosylated amino acids and at the same time be able to stimulate T-cell proliferation [50]. Several such peptides were therefore synthesised in order to map out the size and specificity of these interactions [51]. It was shown that particularly glycopeptides substituted with T-and Tn-antigens were active, whereas larger structures or different monosaccharide structures rendered this stimulation absent [52,53].

Structural determination of complex carbohydrates from *Salmonella enterica ssp. typhimurium* 1135

The structures of O-antigens and more recently of the corresponding core structure of many *Salmonella* strains have been determined over the years as mentioned above. The structure of the intact O-antigen and core for the *Salmonella enterica* ssp. *typhimurium* 1135 described below is the most recent example from the authors laboratory of how such complex oligosaccharides structure can be determined by NMR spectroscopy [54]. This study has utilised high field NMR combined with modern mass spectrometric methods. The emphasis here is mainly on the NMR and conformational aspects. The sample investigated by NMR (Fig. 1) contains as the largest structure, a 22-mer oligosaccharide, shown in Fig. 4, consisting of three repeats of the O-antigenic trisaccharide (as the 3,6-dideoxy sugar is cleaved upon work up of the sample), an 11-mer core and two residues from the lipid A part. The structure of the lipid A and core parts have previously been described [55] and these results turned out to be very useful as reference data for the present study.

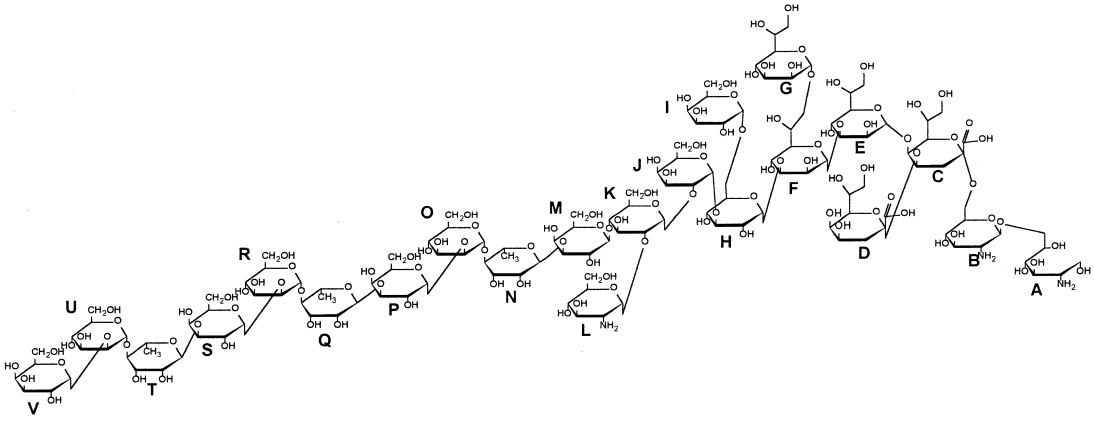

Fig. 4 Structure of 22-mer oligosaccharide from *Salmonella enterica* ssp. *typhimurium* 1135.

The NMR study was carried out mainly using traditional techniques, such as DQF-COSY, NOESY and TOCSY and a few newer, but well established, techniques such as HSQC-TOCSY and one-dimensional versions of NOESY and TOCSY experiments, all using pulsed field gradients. The key to the successful structure elucidation is the availability of a high field spectrometer operating at a proton frequency of 750 MHz. It should be emphasised here that in order to utilise the dispersion of the high field it is necessary obtain high quality spectra, i.e. good signal to noise ratio and high digital resolution in both

dimensions. These require long experimental measuring time, which is a limiting factor on most high field instruments.

The NMR structural assignment proceeds by a standard protocol starting by assigning the spin systems of the single oligosaccharides followed by establishing the linkage positions mainly by through space correlation in the NOESY spectra. In order to perform this task most efficiently in practice, good software tools allowing for overlay of any combination of spectra interactively. The program mainly used in this work is PRONTO, a software package originally developed for protein NMR studies in the Carlsberg Laboratory [56] and now continuously being expanded with new features, e.g. for measuring heteronuclear coupling constants.

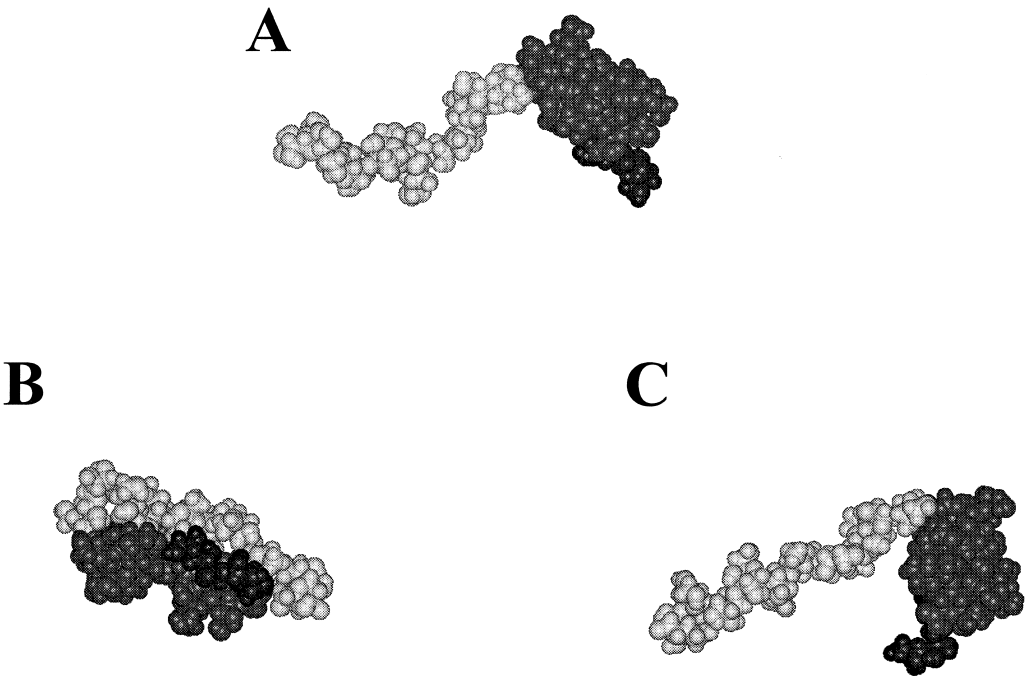

Fig. 5 (A) Energy minimised starting structure of a 22-mer oligosaccharide from *Salmonella enterica* ssp. *typhimurium* 1135. (B) Selected structure from MD *in vacuo*. (C) Selected structure from MD in water.

Determinations of the linkage positions from the NOESY spectra are confirmed by gradient HMBC experiments and evaluation of the assigned chemical shifts. These contain much information like the ^{13}C chemical shift changes upon glycosylation, the so-called glycosylation shifts proposed by Bock *et al.* [57]. Analyses of the ^{1}H chemical shifts are not quite as straightforward. However, this is certainly changing due to the recent efforts in developing software tools for chemical shift calculations based on quantum mechanical models [58]. Earlier programs based on empirical correlations have been used successfully in three-dimensional studies mainly of proteins [59]. However, the application of such programs to the carbohydrate field will have to also take into account the local flexibility of the glycosidic linkage in order to give meaningful results.

It is well known that the use of NOESY contacts in complex oligosaccharides for secondary structure determination relies critically on certain assumptions about the conformational behaviour. The conformational analysis of the proposed secondary structure is therefore critically evaluated based on the obtained NMR data and primarily on the NOESY intensities. In the current example, molecular dynamics of the proposed structure was carried out using the AMBER force field with the modifications for carbohydrates published by Homans [60]. The initial calculation was a molecular dynamics simulations (MD, 500 ps) and energy minimisations *in vacuo* using a dielectric constant of 80 to mimic water. Under these conditions it is possible to compare the observed NOEs and short distances with the

calculated model. It is however, clear that simple dynamics *in vacuo*, produces compact structures, which fulfil these short range interactions (Fig. 5).

The many NOEs observed between adjacent residues and also a few 'long range' NOEs, e.g. between residues L and H comply well with such a structure. The compact structure is also observed if the calculation is done with inclusion of the observed NOEs, as distance restraints by a standard grouping of the NOE intensities into weak, medium and strong. This calculation still produces a fairly compact structure as above. The next step forward was therefore to perform as full molecular dynamic simulation in a water box. The size of the extended oligosaccharide requires that the water box is fairly large with a side length of 80 Å. The computer time required to perform such a 190 pico second dynamic was approximately 24 days. It would be desirable to perform much longer dynamic calculations, but this short run still indicated that the compact structures obtained *in vacuo* is an artefact of not including water. In this new ensemble of structures, the NOEs observed also compared well to short distanced sampled by the structures. The relative large overall flexibility of the oligosaccharide in this study should be seen in light of fairly restricted conformations sampled for each glycosidic linkage adding up to an overall large sampling of the conformational space.

Carbohydrate chemistry in the 21st century

In spite of the impressive progress of carbohydrate chemistry during the last 50 years, several challenges are still waiting to be solved and only so will carbohydrates become as central to the development of biological sciences as protein and DNA has been for the last decades. Therefore the following issues must be addressed.

Glycopeptide synthesis

The area of glycopeptide synthesis has evolved into a high level of sophistication amenable to automated procedures. It has clearly been demonstrated above that access to synthetic glycopeptide structures in relatively large amounts made it possible to study protein–carbohydrate interactions in greater detail and more expediently than using a traditional approach. However, the use of glycopeptides as true oligosaccharide mimics identified by combinatorial approaches still remains to be optimised.

Structural analysis

More focus on automated carbohydrate analysis with higher sensitivity will be important so that the analysis of carbohydrate structures on picomolar or less will be as simple as Edman degradation or DNA sequence analysis. It is expected that both NMR and MS spectroscopy will experience higher and higher sensitivity with the development of nanoprobe technology and cryo-probe design. However, both of these techniques as powerful as they are still require skilled scientists to deduce the structures from the data acquired.

Synthesis

The synthesis of complex oligosaccharides is a field which is lagging behind in technological development achieved for peptide or protein and DNA synthesis, both of which are highly automated today. The many different choices of promoters for glycoside formation published indicate that the assembly of oligosaccharides is still a virgin field open to important discoveries, particularly in order to construct the molecules on solid support as efficiently as demonstrated for the synthesis of peptides and DNA fragments. Furthermore, the next century will experience more work on carbohydrates used as templates for drug developments based on their multifunctionality and low toxicity and biological compatibility. Alternatively, carbohydrate mimics will be potential drug candidates in order to overcome the problem of the synthesis of complex oligosaccharides.

Biological functions

The many different glycoforms experimentally observed on intact glycoproteins should also be adequately explained in order to take full advantage of understanding the signalling of carbohydrate structures in biological systems. One particular field of interest to study will therefore be specific enzyme

inhibitors or access to gene knock-out animals where the detailed answer to the lack of a given transferase action can be analysed for the consequences in living cells or animals.

Theoretical aspects

The impressive advances in the computational area will undoubtedly continue and more theoretically oriented carbohydrate chemists should therefore address the issues of understanding the behaviour of complex carbohydrates in aqueous environments and their interactions with proteins receptors and the forces involved in these intricate recognition phenomena. Likewise the *ab-initio* prediction of DNA or RNA structures and the consequences for genetic specificity would probably require a better description of the assembly of the furanosidic structures forming the backbone of DNA and RNA.

Drugs

As a result of the above advances a hope for carbohydrate based vaccines towards a cure for different forms of cancer or eventually HIV can be envisaged. This will, however, require a very focussed activity within these areas of research with access to adequate resources.

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