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Aromatic–Carbohydrate Interactions: An NMR and Computational Study of Model Systems

Sophie Vandenbussche,[a] Dolores Díaz,[b] María Carmen Fernández-Alonso,[b] Weidong Pan,[c] Stéphane P. Vincent,[c] Gabriel Cuevas,[d] Francisco Javier Cañada,[b] Jesús Jiménez-Barbero,*[b] and Kristin Bartik*[a]

Abstract: The interactions of simple carbohydrates with aromatic moieties have been investigated experimentally by NMR spectroscopy. The analysis of the changes in the chemical shifts of the sugar proton signals induced upon addition of aromatic entities has been interpreted in terms of interaction geometries. Phenol and aromatic amino acids (phenylalanine, tyrosine, tryptophan) have been used. The observed sugar–aromatic interactions depend on the chemical nature of the sugar, and thus on the stereochemistries of the different carbon atoms, and also on the solvent. A preliminary study of the solvation state of a model monosaccharide (methyl β-galactopyranoside) in aqueous solution, both alone and in the presence of benzene and phenol, has also been carried out by monitoring of intermolecular homonuclear solvent–sugar and aromatic–sugar NOEs. These experimental results have been compared with those obtained by density functional theory methods and molecular mechanics calculations.

Keywords: carbohydrates · molecular modeling · molecular recognition · NMR spectroscopy · protein–carbohydrate interactions

Introduction

Understanding of molecular recognition processes is of paramount importance for the life sciences and for the elucidation, at the molecular level, of the events involved at the heart of biological phenomena. Many of these are mediated by interactions between proteins and the carbohydrates present on the surfaces of cells.[1] Proteins that recognize carbohydrates are found in a wide variety of organisms, from viruses and bacteria to plants and animals, and include enzymes, glycoproteins (antibodies, for example), and lectins. These protein–carbohydrate interactions are highly selective—lectins can distinguish between carbohydrates that differ only in the stereochemistry at one carbon atom[2]—and are characterized by affinity constants typically of the order of $10^3\,M^{-1}$ for monosaccharides and up to $10^7\,M^{-1}$, or even higher, for complex carbohydrates. Hydrogen-bonding and nonpolar interactions[3,4] play important roles in the binding process. In the last few years, the presence of aromatic rings in the binding sites of lectins has been highlighted as essential for recognition of neutral sugars, especially of the Gal/GalNAc and Glc/GlcNAc families.[5–14] The importance of these aromatic amino acids has been confirmed by site-directed mutagenesis.[5,6] For the molecular recognition process, both the structure and the conformation of the carbohydrate,[9] as well as the nature and orientation of the aromatic rings, are of importance.[10,11] This so-called "stacking interaction" between the carbohydrate and aromatic amino acid side chains is also referred to as a "CH–π inter-

[a] Ir. S. Vandenbussche,[+] Prof. Dr. K. Bartik
Molecular & Biomolecular Engineering
Université Libre de Bruxelles, Brussels (Belgium)
Fax: (+32) 26503606
E-mail: kbartik@ulb.ac.be

[b] Dr. D. Díaz,[+] Dr. M. C. Fernández-Alonso,[+] Dr. F. J. Cañada, Prof. Dr. J. Jiménez-Barbero
Centro de Investigaciones Biológicas, CSIC
Ramiro de Maeztu 9, 28040 Madrid (Spain)
Fax: (+34) 915-360-432
E-mail: jjbarbero@cib.csic.es

[c] Dr. W. Pan, Prof. Dr. S. P. Vincent
Laboratoire de chimie bio-organique
Facultés Universitaires ND de la Paix
Namur (Belgium)

[d] Dr. G. Cuevas
Instituto de Química
Universidad Nacional Autónoma de México
Ap Postal 70213
04510, Coyoacán, Circuito Exterior
México DF (México)

[+] Contributed equally to this work.

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action",[5,12–14] and has also been shown to occur in the gas phase.[15]

Sometimes regarded as a hydrogen bond, the CH–π interaction is much weaker than the "classical" hydrogen bond (ca. 30% according to QM calculations).[16–18] In the computed benzene–methane and benzene–chloromethane systems, the most stable configuration is always the one in which the CH bond is perpendicular to the benzene ring plane, although the interaction energy does not seem to be much modified by the position of the hydrogen over the aromatic ring (i.e., above the center of the aromatic cycle or at the edge of the cycle).[17–19]

Ab initio HF, MP2, and CCSD(T) calculations undertaken on several simple systems (benzene–methane, -ethane, and others[16,17]) have suggested that the dispersion interaction between the aromatic entity and the carbon atom of the CH bond is largely responsible for the CH–π interaction, with the electron correlation greatly enhancing the calculated binding energy.[17] The electrostatic contribution increases when the acidity of the hydrogen increases.[17] The presence of an electrostatic contribution to the attraction might be responsible for the directionality of the CH–π bond.

Ab initio studies on CH–π interactions in sugar–protein complexes have also been reported.[14,19–22] An extensive study of protein–carbohydrate complexes obtained from six high-resolution (1.3 Å or better) X-ray structures has been undertaken by Spiwok et al.[14] The estimated interaction energies fall between −11.7 and −26.8 kJ mol^{-1}, except for one complex in which the higher interaction energy (−51.4 kJ mol^{-1}) was explained by the additional presence of a classical H-bond between a hydroxyl group of the carbohydrate and the hydroxyl group of a tyrosine. Within these complexes, a wide range of angles was observed between the donor C–H bond and the aromatic plane (31° to nearly perpendicular). The perpendicular distances calculated between the hydrogen atom and the plane of the aromatic ring ranged between 2.6 and 3.3 Å, fairly similar to those found in the experimentally determined X-ray structures.[23] In comparison, for the benzene–methane and benzene–methanol complexes, the distances between the hydrogen atom and the aromatic cycle were only 2.7 and 2.5 Å, respectively.[17]

The group in Madrid has previously performed calculations (at the MP2/6–31G(d,p) level with counterpoise correction) relating to the complex formed by β-D-fucose (6-deoxy-β-D-galactose) and benzene, and modeled the geometry of the interaction.[24] The interaction energy was −10.9 kJ mol^{-1}, falling in the lower limit of the interaction energies obtained by Spiwok et al.,[14] but of the same order of magnitude as that computed for the methanol–benzene complex[17] (taking the known 20% overestimation of MP2 calculations relative to CCSD(T) ones into account[16]).

In an application of all this knowledge, a synthetic lectin has very recently been chemically prepared[25] through the combination of different simple molecular fragments that simultaneously allow hydrogen bonds and stacking interactions with the target saccharides to be established. Millimolar affinities were obtained with synthetic receptors for the first time.

On this basis, in order to allow better understanding of CH–π interactions, it seems interesting to study—not only theoretically but also experimentally—simple model systems. Here we present a methodology for exploration of carbohydrate–aromatic interactions from the structural viewpoint.

NMR is a particularly interesting tool for studying geometrical features and molecular interactions. Provided that a CH–π interaction takes place, the chemical shifts of the protons involved in the interaction should be modified according to their spatial positions in the field created by the aromatic ring current. Here we report an NMR study of the interactions between different D-monosaccharides (the α- and β-methyl anomers of glucopyranose, galactopyranose, and ribofuranose, as well as α-methyl mannopyranose) and several aromatic entities (phenol and the L-amino acids phenylalanine, tyrosine, and tryptophan), in an attempt to generalize and to extend the previously obtained conclusions. Measurements of the chemical shift variations of the sugar protons in the absence of each aromatic moiety and in the presence of excesses (10–20 molar equivalents) have been undertaken. Titration experiments have been performed for some of these systems.

We have also undertaken the analysis of the possible changes in the solvation sphere of one simple carbohydrate (methyl β-D-galactopyranoside) upon addition of phenol, through intermolecular homonuclear NOEs. More precisely, we have investigated whether intermolecular sugar–water and sugar–aromatic NOEs can provide experimental information relating to the existence of the complex and to the solvation of this carbohydrate in the absence and in the presence of aromatic compounds.

All the observed data indicate that, depending on the chemical nature of the sugar, aromatic–carbohydrate interactions indeed take place, and can be easily monitored by NMR. These NMR data have also been interpreted in terms of geometrical models with the aid of molecular mechanics calculations.

Results and Discussion

The most commonly used reference compound for NMR studies of biological and bioorganic molecules in water is 2,2-dimethyl-2-silapentane-5-sulfonate (DSS); the chemical shift of the methyl protons is set to zero. An alternative reference is sodium 3-trimethylsilylpropionate (TSP), with one CH_2 group fewer than DSS, and thus less prone to provide interactions with the solutes.[26] Initial experiments demonstrated that minor, but appreciable, changes in the chemical shift of the CH_2 adjacent to the silicon atom took place when a 0.1 mM concentration of DSS in D_2O was mixed with a 220 mM concentration of phenol. In view of these observations, and although the employed DSS concentration

for reference purposes is one order of magnitude lower (ca. 10 μM), it was obvious that DSS was not appropriate for use as an internal reference for the aromatic interaction experiments. Significantly smaller, but still observable, variations took place for TSP. Different referencing methods were employed in the two labs. In Brussels, experiments were undertaken with an external reference in a spherical insert. The bulk magnetic susceptibility varies with the solvent, so a spherical insert, which has a shape factor of zero, was therefore essential.[27] In Madrid, TSP was employed as an internal standard at a 10 μM concentration (see Experimental Section).

The interaction of methyl glycosides with phenol—chemical shift perturbation data: Table 1 shows the variations in the ^{1}H NMR resonance frequencies (Hz) of the signals of a variety of methyl glycosides in the presence of variable amounts of phenol. Shielding of all the resonance signals is observed. In order to allow easy comparison of the results obtained in the two laboratories, the sugar proton that experienced the smallest shielding was used as an internal reference (its chemical shift variation was set to zero). The experimental errors in $\Delta\nu$ reported in Table 1 are smaller than 3 Hz (calculated on the basis of the spectral resolution and with comparison of results obtained from several experiments on the same system). No variations in the chemical shifts of methyl β-galactopyranoside were observed when the experiments with an excess of phenol were conducted in DMSO or acetonitrile, highlighting the importance of water for the interaction to take place.

Analysis of the data in Table 1 clearly shows that the differences in the chemical shift variations of the protons of a sugar depend on the chemical nature of the sugar. Negligible differences in the variations were observed between the various protons of methyl α-mannopyranoside. Larger differences were noted between the protons of methyl α-glucopyranoside, and even larger ones between the protons of its β-epimer. The differences in the proton chemical shift variations are similar for the methyl α- and β-ribofuranosides.

The differences in the chemical shift variations of the protons of the methyl α- and β-galactopyranosides were significantly larger than for the other sugars, well beyond the maximum estimated experimental error. In both sugars H2 is the proton that experiences the least shielding, followed by one of the H6 atoms and the *O*-methyl protons. H3, H4, and H5 for the α-methyl derivative, and H1, H3, H4, and H5 for

Table 1. Shielding (in Hz) of the protons of methyl pyranosides and furanosides upon addition of phenol or an aromatic amino acid.

Sugar	Aromatic moiety	Chemical shift variation [$\Delta\delta$ (Hz)]							
		H1	H2	H3	H4	H5	H6A	H6B	OMe
methyl α-galactopyranoside	phenol[a]	−12	−10	−17	−24	−26	−12	−16	−12
	phenol[b]	−2	0	−7	−14	−16	−2	−6	−2
	phenol[c]	−5	0	−15	−14	−15	−6	−7	−3
	L-Phe[d]	–	0	−1	–	−3	−1	−1	0
	L-Phe[e]	−5	0	−5	−4	−7	−2	−2	−1
	L-Trp[f]	–	−5	−8	−10	−11	−12	0	−4
	calcd $\Delta\delta$[g]	−0.2	−0.2	−0.5	−0.6	−2.7	–	–	–
methyl β-galactopyranoside	phenol[a]	−21	−7	−20	−20	−28	−15	−12	−10
	phenol[b]	−14	0	−13	−13	−21	−8	−5	−3
	phenol[c]	−14	0	−12	−12	−19	−9	−6	−4
	L-Phe[d]	−2	0	−1	−2	−3	−2	−1	0
	L-Trp[f]	−6	0	−7	−6	−8	−2	−1	0
	L-Tyr[h]	−4	0	−5	−4	−7	−2	−2	−1
	calcd $\Delta\delta$[g]	−1.9	−0.3	−0.5	−0.4	−1.7	–	–	–
methyl α-glucopyranoside	phenol[a]	−13	−11	−5	−9	−11	−17	−11	−11
	phenol[b]	−8	−6	0	−4	−6	−12	−6	−6
	phenol[c]	−10	−5	0	−3	−5	−6	−5	−4
	L-Phe[d]	–	−2	0	−1	−1	−2	−2	−1
	L-Trp[f]	–	−2	0	−1	−1	−1	−1	−1
methyl β-glucopyranoside	phenol[a]	−20	−7	−9	−8	−20	−12	−11	−11
	phenol[b]	−13	0	−2	−1	−13	−5	−5	−4
	phenol[c]	−12	0	−3	−2	−11	−5	−3	−4
	L-Phe[d]	−2	–	0	−1	−2	−1	−1	−1
	L-Trp[f]	−4	0	–	−1	–	−1	−1	0
	calcd $\Delta\delta$[g]	−2.9	−0.3	−0.4	−0.2	−0.6	–	–	–
methyl α-mannopyranoside	phenol[b]	−5	−4	0	−3	−1	−3	−1	−7
methyl α-ribofuranoside	phenol[a]	−24	−22	−20	−16	−19, −20	–	–	−16
	phenol[b]	−10	−8	−7	−3	−6, −7	–	–	0
methyl β-ribofuranoside	phenol[a]	−21	−20	−21	−15	−20, −21	–	–	−21
	phenol[b]	−6	−5, −6	0	−5	−6	–	–	−6
	L-Phe[d]	–	–	−1,	–	−1, –	–	–	0

[a] Absolute shielding (Hz) measured for the resonance signals of the sugars (10 mM) upon addition of a 20-fold excess of phenol, as determined at 600 MHz and 298 K, pH 5–6. DSS is used as external standard.All other data shown were obtained by using the least shielded proton as reference (its chemical shift variation is set to zero); [b] relative shielding in the same conditions as above; [c] sugars 15 mM, addition of a 15-fold excess of phenol, 500 MHz, 298 K, pH 5–6; [d] sugars 5 mM, addition of a 20-fold excess of L-Phe, 600 MHz, 298 K, pH 5–6; [e] sugars 15 mM, addition of an 11-fold excess of L-Phe, 500 MHz, 298 K, pH 6–7; [f] sugars 4 mM, addition of a sevenfold excess of L-Trp, 500 MHz, 298 K, pH 6–7; [g] estimated shielding (ppm) due to the presence of an aromatic moiety, calculated by the modified Bovey–Johnson equation[28] for the protons of both anomers of methyl galactopyranoside and for methyl β-glucopyranoside, according to the geometry calculated by molecular mechanics calculations. In the case of methyl β-galactopyranoside the shielding calculated for the so-called 1, 3, 5 and 3, 4, 5 arrangements (see text) was 50:50 averaged. The estimated proportion of complex was deduced from the averaged ratio of the experimentally observed absolute shifts at 600 MHz and 298 K to those estimated theoretically; [h] methyl β-galactopyranoside 4 mM, addition of an eightfold excess of L-Tyr, 500 MHz, 298 K, pH 11–12. Although this pH is not physiologically relevant, and is above the pK for the different tyrosine acid/basic groups, the data are given for purposes of comparison.

the β-epimer showed the largest chemical shift variations. As an example, Figure 1 shows the ^{1}H NMR spectra of methyl α- and β-galactopyranosides in water and in the presence of phenol. As can be seen, a remarkable effect on the chemical shifts of the sugar protons is revealed. The H4 and H5 resonance signals undergo the largest chemical shift variation. An upfield shift of the H3 signals is also measured. For the α-epimer, the H1 chemical shift does not show a significant shielding, in contrast with the observations for the β-epimer, in which H1 showed remarkable changes.

Titration experiments were undertaken in order to try to quantify the interactions of phenol with methyl β-galactopyranoside (10 mM) and methyl α-glucopyranoside (10 mM). As an example, Figure S1 in the Supporting Information shows the behavior of the chemical shift of the H3 proton of methyl β-galactopyranoside on phenol addition up to a 55:1 molar ratio. A 1:1 model under fast-exchange conditions was fitted to the experimental data by use of a nonlinear equation for the analysis, yielding a value of about 1 M^{-1} for the binding constant. Similar behavior was observed for all the non-exchangeable protons of both sugars. However, since no plateau value was reached during the titrations, the affinity constant values should be regarded as qualitative, as a higher limit. Analogous behavior was observed when both methyl ribofuranosides were titrated with phenol.

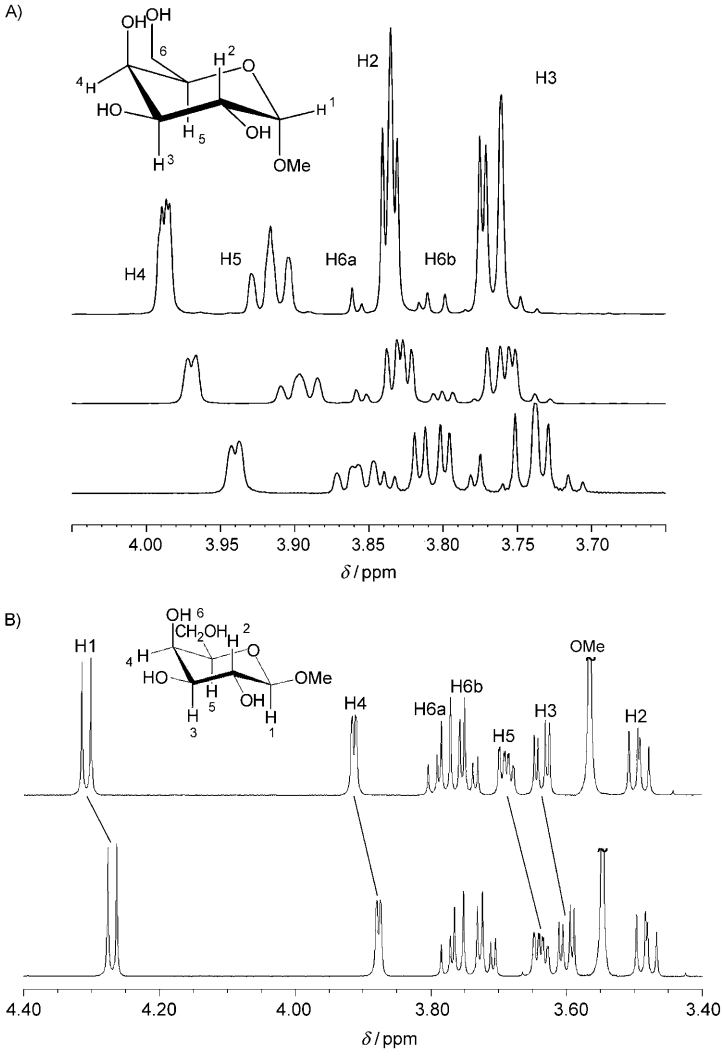

Figure 1. A) 500 MHz ^{1}H NMR spectra (D_2O, 298 K) of a methyl α-galactopyranoside solution (a, above) in the presence of 0.75 equivalent of phenol (b, middle panel), and in the presence of 6.5 equivalent of phenol (c, below). B) 600 MHz ^{1}H NMR spectra (D_2O, 298 K) of a 10 mM solution of methyl β-galactopyranoside (above) and upon addition of 21.2 molar equivalent in phenol (below).

The interaction of methyl glycosides with aromatic amino acids—chemical shift perturbation data: In a further step, the interactions between sugars and aromatic amino acids were studied. Obviously, the CH–π interactions that occur in the active sites of lectins are mediated by the lateral chains of the aromatic amino acids, and so we decided to investigate the interactions of methyl α- and β-galactopyranoside and methyl α- and β-glucopyranoside with the naturally occurring aromatic amino acids L-phenylalanine (Phe), L-tyrosine (Tyr), and L-tryptophan (Trp). The NMR experiments were carried out at different pH values, and the results were found to be independent of pH. Table 1 shows the variation in the resonance frequencies of the different sugars in the presence of Phe, Tyr, and Trp. The most significant differences between the chemical shift variations are observed for the methyl galactopyranoside epimers. For the methyl β-epimer, for instance, although the observed trend is similar for the three aromatic moieties, the major effect on the sugar resonances occurs when Trp is added to the monosaccharide solution. Because only lower aromatic/sugar ratios were accessible, because of the solubility limit of the aromatic amino acids, the observed chemical shift variations are smaller than those observed in the presence of phenol.

Because the CH–π interactions are probably a consequence of the dispersion of the electronic density of π-molecular orbitals of the aromatic rings interacting with the hydrophobic faces of carbohydrates, we also examined a mixture of methyl α-galactopyranoside and hexafluorobenzene, a strongly deactivated aromatic ring because of the electron-withdrawing capacity of the six fluorine atoms. The NMR spectrum of the methyl α-galactopyranoside/hexafluorobenzene mixture did not show any significant differences from the spectrum of the sugar in the absence of the aromatic moiety. The data clearly indicate that π-electron-rich aromatic rings seem to be required in order to establish stabilizing CH–π interactions with the carbohydrates.[4]

All the experimental data presented above suggest that three C–H vectors pointing into the same spatial region are required in order to produce significant deviation in the observed NMR chemical shifts. This is clearly highlighted by the data pertaining to the methyl α- and β-galactopyranosides. Phenol—and also the aromatic amino acids—interacts with the pyranose chairs in a specific (although weak) manner, by establishing CH–π interactions. Indeed, for the interaction to take place, three C–H vectors of the sugar with suitable orientations need to exist.

The chemical shifts of the sugar protons were shifted upfield in the presence of the studied aromatic moieties. These observed perturbations are the result of the effect of aromatic ring currents[28] and consequently yield information on the geometry of interaction (see below).

Further demonstration of the existence of sugar–aromatic complexes—intermolecular solvent–sugar and aromatic–sugar NOEs: Several studies have previously underlined the importance of the solvent for the formation of carbohydrate–protein complexes.[29,30] Consequently, a preliminary examination of the solvation state of the sugars in the presence of phenol or benzene was undertaken. We expected that the solvation spheres of carbohydrates would be modified somehow when the aromatic rings interacted with the hydrophobic faces of the sugars. Examples of NMR-based studies of the solvation of small organic molecules, based on the detection of intermolecular NOEs between solvent and solute, have been reported.[31,32] In particular, the solvent compositions of the first solvation shells of carbohydrates in binary mixtures have been studied by detection of intermolecular NOEs from the solvent to the solute[31b] or vice versa.[33]

To identify interactions between water and the CH groups of methyl β-galactopyranoside, 1D-DPFGSE-NOE experiments[34] were performed on samples of the sugar with and without phenol (phenol/methyl β-galactopyranoside molar ratio 20:1). A semiquantitative analysis of the NOE build-up curves for the different protons of the free sugar in water, by the initial build-up rate method,[35] showed that the slopes of the curves at short mixing times were basically identical, suggesting that methyl β-galactopyranoside is essentially solvated in an isotropical manner when dissolved in water. In contrast, strikingly different results were deduced for the same sugar protons (Figure 2 and Figure S2 in the Supporting Information) when the sugar was dissolved in a water/phenol mixture. Indeed, H1, H2, and H6 seem to be more water-exposed than H3, H4, and H5, indicating a certain degree of protection from the solvent for the latter set of protons. Although these preliminary data should be considered with caution, they suggest the formation of a sugar/aromatic complex, indirectly probing the existence of the CH–π interaction, which partially protects H3, H4, and H5 from water. This result is also consistent with the reported preference for phenol to be surrounded by the more hydrophobic cosolvent when dissolved in aqueous binary mixtures.[31b]

If the measurement of intermolecular NOE is a valid approach for extracting information about weak interactions (i.e., solvent–solute interactions), one might also consider the detection of sugar/aromatic interactions (i.e., solute–solute interactions) when the complex is formed. Thus, the search for intermolecular NOEs between methyl β-galactopyranoside (as model compound, given the lack of overlapping of the key NMR resonance signals) and benzene was attempted. Intermolecular NOEs between two solutes of low molecular weight are usually too small to be detected, unless a large binding constant exists, which is not the case for the system under study. As a result, no sugar–aromatic NOEs were detected under standard conditions, with sugar concentrations of 10–30 mM and a 10–20-fold excess of phenol. However, as shown above, solvent–solute interactions, which are intrinsically weak, became measurable thanks to the convergent addition of the single contributions of the huge number of solvent molecules, which greatly exceed the number of sugar molecules.[31d] A sample containing a 400 mM concentration of sugar in water saturated with benzene (solubility ca. 22 mM) was prepared. Different sugar protons were inverted and the benzene signal was observed. Intermolecular NOEs were indeed observed (Figure S3 in the Supporting Information), especially when H1 or H4 of the sugar ring were inverted, while no NOE was observed when H2 was the target-inverted proton. These observations also point towards the existence of sugar–aromatic complexes. The experimentally measured NOE build-up curve for the methyl β-galactopyranoside/benzene pair is shown in Figure S4 in the Supporting Information.

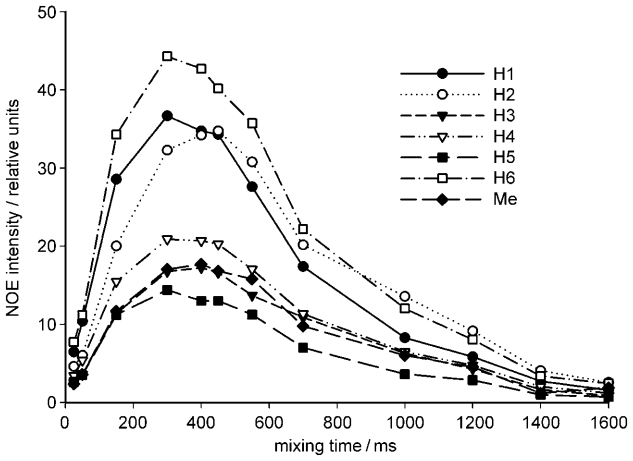

Figure 2. Build-up curves of the experimentally determined intermolecular homonuclear NOE enhancements measured between water and protons of methyl β-galactopyranoside (10 mM), as a function of mixing time in a water/phenol mixture at 20:1 phenol/sugar molar ratio. Different slopes are evident for the different protons, in contrast to the observations in the absence of phenol.

A 3D model—theoretical calculations: As additional support to verify the existence of stabilizing CH–π interactions, density functional and molecular mechanics calculations were carried out on simple sugar–aromatic systems, as described in our previous study of the fucose/benzene complex.[24,36] Studies on the complex formed by a β-fucopyranoside (Fuc) residue interacting with the aromatic ring of tryptophan (Trp) were performed. For purposes of comparison, and also to judge the influence of deactivation of the aromatic ring, calculations for the interaction of a simple sugar (methyl 2,3,4-tri-*O*-methyl α-fucopyranoside, FucMe4) with difluorobenzene ($C_6H_4F_2$), and hexafluorobenzene (C_6F_6) were also performed.

DFT calculations: Firstly, the previously studied Fuc/benzene system[24] was modified as necessary, for preparing Fuc/Trp, FucMe4/$C_6H_4F_2$, and FucMe4/C_6F_6. In the cases of the last two complexes it was necessary to substitute the different hydroxyl groups of the sugar to avoid hydrogen bonding of the hydroxyl groups with the fluorine atoms.

Calculations were performed by density functional theory. Although it is well known that the hybrid B3LYP method[37] is not fully adequate for study of long-distance interactions, it was used to obtain approximations of the geometries of the different complexes. It has been reported that its consideration of the dispersion term is somehow deficient,[38] although recent developments may overcome these drawbacks.[39]

The obtained geometry for the basic Fuc/Trp complex (Figure 3A) shows that the hydrogen atoms at the 3-, 4-, and 5-positions are those closest to the aromatic ring, as experimentally deduced for a variety of sugar–aromatic complexes,[23] including those described above, while H1 is the furthest away. H3 is at 2.95 Å from C′3, the equatorially oriented H4 is at 3.07 Å from the nitrogen atom, whereas H5 is at 3.15 Å from the bridging C′7 atom.

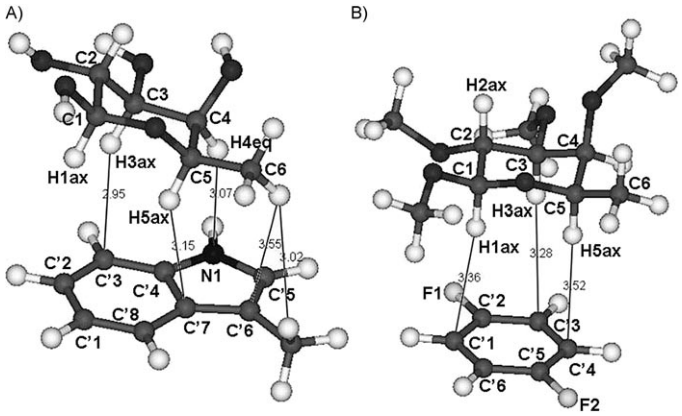

Figure 3. Stationary states of the supramolecular complexes formed from Fuc/Trp (A), and FucMe4/$C_6H_4F_2$ (B). The key distances (in Å) from CH groups of the sugar to the aromatic ring atoms are shown. Calculations were performed at the B3LYP/6-31G(d,p) level. According to the notation, the ring carbon atoms from the aromatic are denoted by primes (C1′–C6′), while the sugar atoms keep their regular numbering.

A different situation is observed for the FucMe4/$C_6H_4F_2$ complex. In this case, proximity between the axially oriented protons at the 1-, 3-, and 5-positions of the sugar ring and the aromatic ring is observed (Figure 3B) but the obtained distances are clearly longer. The sugar H1 is located at 3.36 Å from carbon C′1, whereas H3 and H5 are at 3.28 Å and 3.52 Å from C′3 and C′4, respectively. The obtained distances are also longer than those previously calculated for the Fuc/benzene complex—3.21, 3.09, and 3.28 Å, respectively—at the same theory level. In principle, this could be explained by the deactivating role of the fluorine atoms, which was confirmed when the geometry of the FucMe4/C_6F_6 complex was calculated. In this case, the presence of the six fluorine atoms attached to the aromatic moiety produced a strong modification of the electronic density of the ring, thus precluding the existence of the CH–π interaction. The sugar moiety was displaced from the ring, and C–H···F interactions were observed (see Figure S5 in the Supporting Information).

Interaction energies were calculated by consideration of the BSSE correction. For the Fuc/Trp complex, the minimum-energy conformer was obtained with a stabilizing interaction energy of $-1.90\,kJ\,mol^{-1}$. For the complex FucMe4/$C_6H_4F_2$, a transition state was obtained, characterized by one imaginary frequency (−17.90) and associated with the motion of approximation of the F1 atom to the C3 methoxyl group, with no change in the 1,3,5-type sugar–aromatic interaction. It could be expected that the geometry of the minimum associated with this transition state should not change from the reported one (and the same with regard to the energy). Interestingly, in this case, the interaction energy was increased to $+2.72\,kJ\,mol^{-1}$, corresponding to an endothermic process and thus supporting a destabilizing role of the fluorine atoms. A similar trend has been experimentally observed for mutant hevein domains, with non-natural fluorinated aromatic amino acid residues, interacting with oligosaccharides.[4] The increment is even bigger when the FucMe4/C_6F_6 complex is considered. In this case, the interaction energy was strongly unfavorable, at $+43.85\,kJ\,mol^{-1}$.

AMBER* molecular mechanics calculations: In a further step, we decided to resort to molecular mechanics calculations, which are much less computationally time-consuming (than the much more time-consuming MP2 used previously[24]) and because dispersion of electronic density and van der Waals forces seem to be the key factors responsible for the interactions. The corresponding terms are properly parameterized in the force fields currently used to deal with biomolecules, such as AMBER*,[40] as integrated in the MAESTRO package.[41] Furthermore, long-distance interactions are well described by Newtonian Mechanics, employed in Molecular Mechanics. The results indicate that the distances are significantly smaller than those observed in the geometries obtained at the B3LYP level, which is in agreement with the observations relating to the Fuc/Ben complex at the MP2/6–31G(d,p) level of calculation[24] (see Table 2). For the interaction between methyl β-galactopyranoside and benzene, clear minimum-energy geometries (with very similar energy values) were obtained for the two possible geometrical arrangements, for which either H1, H3, and H5 (Figure 4B) or H3, H4, H5 (Figure 4C) interact with the benzene ring. In fact, this last geometry was the only one provided when the calculations were performed for the benzene/methyl α-galactopyranoside complex (Figure 4A). According to the calculations, two geometries are also possible for the methyl β-glucopyranoside complex, but in this case, the interaction may take place through the lower or the upper face of the pyranose ring, interacting either with H1, H3, and H5 or with H2, H4, and one of the H6 protons. The two possible arrangements are shown in Figure 4D.

Table 2. Calculated distances (Å) between selected protons of the sugar moiety (1,3,5 and 3,4,5 CH-arrangements) and the aromatic ring carbons.

Distance	FucMe4/$C_6H_4F_2$		Fuc/C_6H_6[a]		
	B3LYP	AMBER*	B3LYP	AMBER*	MP2
$H1_{ax}$–C′1	3.363	3.041	3.212	3.641	3.150
$H1_{ax}$–C′5	3.997	2.906	3.639	4.882	4.556
$H1_{ax}$–C′6	3.572	2.938	4.176	4.269	3.896
$H3_{ax}$–C′2	3.429	2.982	3.294	2.916	2.894
$H3_{ax}$–C′3	3.279	2.961	3.087	2.956	2.896
$H5_{ax}$–C′4	3.516	3.310	3.396	3.042	2.793
$H5_{ax}$–C′5	3.626	4.060	3.280	2.905	2.735
$H4_{eq}$–C′4	4.831	5.308	4.207	3.021	3.229

[a] For purposes of comparison, distances for the Fuc/benzene complex from ref [24] are included. 6-Deoxygalactose (fucose) was used to circumvent the possibility of rotamers around the C5–C6 bond.

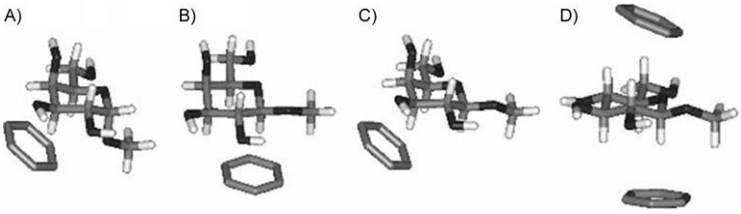

Figure 4. Minimum-energy geometries obtained by molecular mechanics calculations for (from left to right) methyl α-galactopyranoside/benzene (A), methyl β-galactopyranoside/benzene with the aromatic ring interacting with H1, H3, and H5 (B), methyl β-galactopyranoside/benzene with the aromatic ring interacting with H3, H4, and H5 (C), and methyl β-glucopyranoside with two benzene moieties (D). Calculations were performed with AMBER*, as implemented in the MAESTRO package.

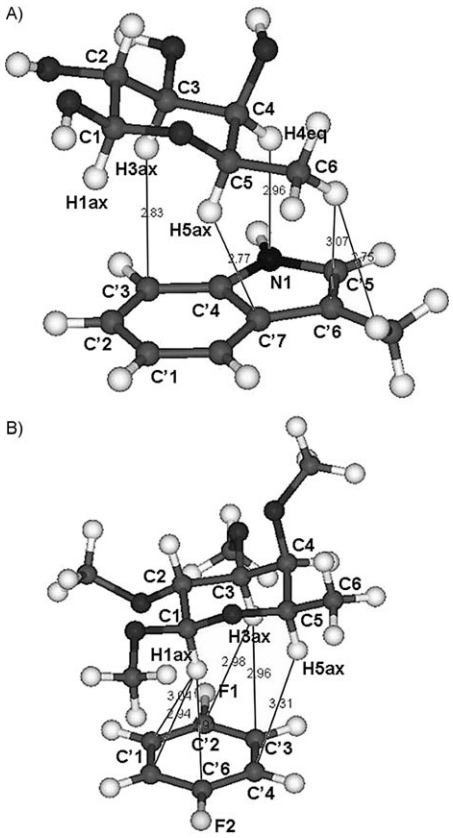

Figure 5. Minimum-energy geometry structures of the supramolecular complexes made up by Fuc/Trp (A) and FucMe4/$C_6H_4F_2$ (B). The key distances (in Å) from CH groups of the sugar to the aromatic ring are shown. Molecular Mechanics calculations were performed with the AMBER* force field. The ring carbon atoms from the aromatic are denoted by primes (C1′–C6′), while the sugar atoms keep their regular numbering.

For less simple aromatic systems, such as the Fuc/Trp complex (Figure 5A), the sugar–aromatic distances are clearly shorter than those obtained by the B3LYP calculations. The H3–C′3 atom pair is separated by 2.83 Å, $H4_{eq}$–N1 and H5–C′7 by only 2.96 and 2.77 Å, respectively. The AMBER* energy estimation for this stable complex is $-23.28\ kJ\,mol^{-1}$. In the case of the FucMe4/$C_6H_4F_2$ complex (Figure 5B), H1 is located 2.91 Å from the closest aromatic carbon atom, whereas H3 is 2.96 and 2.98 Å from C′3 and C′2, respectively. H5 axial is the furthest away, at 3.31 Å from C′4. The AMBER-based interaction energy for this complex was heavily positive, at $+128.87\ kJ\,mol^{-1}$.

The geometries obtained from the molecular mechanics calculations for the methyl α- and β-galactopyranosides were used to calculate, with the aid of the MOLMOL program, the expected ring current shifts from the Johnson and Bovey equation.[28a] Johnson and Bovey calculated the shielding increment in the space region around benzene using a current loop model, and in the program the equation takes account of the fact that the ring current is different for each amino acid.[42] Calculations were performed with the parameters for tyrosine. A comparison between the estimated and calculated data is given in Table 1. The computed data are in agreement with the experimental observations: the experimentally most shielded protons in the NMR spectra are those that are also predicted to experience the maximum shielding. If the weakness of the complexes, as well as the semiquantitative character of the model, are taken into consideration, the agreement can be considered as satisfactory. If the calculated shieldings are compared with those observed experimentally, the estimated proportion of complex in solution for the two methyl galactopyranoside samples containing a 1:20 sugar/phenol ratio should be around 10%.

Conclusion

The study of the interactions between carbohydrates and proteins is a field of current interest. Aromatic amino acids are nearly always involved in these interactions. In order to gain insight relating to this experimental observation, we have studied model systems using different monosaccharides and different aromatic moieties. We showed that, depending on the nature of the sugar and the stereochemistry of the asymmetric carbon atoms, simple carbohydrates may interact with aromatic moieties, including aromatic amino acids, although with rather low affinity constants (below $1\ M^{-1}$). A hydrophobic component to this interaction is detected, since

the interaction cannot be detected in other polar solvents (DMSO, acetonitrile). Provided that three sugar protons point towards the same spatial region, the CH–π interaction indeed exists and can be detected by simple NMR experiments in water. Both theoretical calculations and experimental NMR data support this interaction model. In fact, the corresponding sugar ring protons experience shielding due to a relative orientation that places these protons above the aromatic system. As it is well known that carbohydrate-binding proteins have high selectivities and high affinities for their carbohydrates ($10^3\,M^{-1}$ to $10^7\,M^{-1}$, depending on the complexity of the carbohydrate), these results show that more than one type of interaction is necessary in order to achieve a high affinity constant and to observe appropriate selectivity towards the distinct carbohydrates. Spatial organization of multiple aromatic entities may be responsible for the selectivity of proteins towards carbohydrates, together with additional interactions (i.e., hydrogen bonding) between the amino acids and the carbohydrates.

Experimental Section

All the sugars are from the D series, except the L-fucose. All *O*-methylated sugars, except for the ribose derivatives, as well as the L-amino acids were purchased from Aldrich, with purities higher than 98%. Deuterated water 99.9% (D_2O) was purchased from Cambridge Isotope Laboratories (CIL). The ribose derivatives were synthesized by the protocol described in the Supporting Information.

NMR—general aspects

In Brussels: Stock solutions of the different carbohydrates, phenol, and the amino acids were prepared in D_2O. The pH of each solution was adjusted by addition of small amounts of concentrated DCl or NaOD and measured with a thin electrode (Wilmad) fitted directly in the NMR tube or in the preparation test-tube. No corrections were made for isotopic effects. Typical experimental conditions used were sugar concentrations between 2.5 and 10 mM, 20 molar excesses of aromatic moiety, pH between 5 and 6 (far enough from the pK_a of phenol (9.95), the pK_a values of the carbohydrate hydroxyl groups (16), and the pK_a values of the different functions of the amino acids). Experiments with the amino acids at pH between 11 and 12 were also performed for purposes of comparison. For titration experiments, a separate NMR tube was prepared for each titration point with use of the stock solutions. The carbohydrate concentration was kept constant throughout each titration (1 or 10 mM, depending on the sugar). Phenol concentration was varied between 0 and 650 mM (and verified by comparison of the integrals of the phenol protons with those of the carbohydrate protons).

For the experiments with phenol, the ^{1}H NMR experiments were acquired at 298 K on a 600 MHz Varian spectrometer with a digital resolution of 0.35 Hz per point before zero-filling, with 5 mm high-resolution tubes. In each set of experiments, the 90° pulse was adjusted, and the relaxation delay was set so as to ensure a minimum of 95% relaxation between two acquisitions. 16 to 512 scans were recorded, depending on the sample concentration. Two levels of zero-filling were used for the data processing. DSS was used as an internal reference, but its chemical shift was adjusted according to an external reference through a calibration experiment: 1D ^{1}H NMR spectra were recorded at 298 K on a 400 MHz Varian spectrometer with a digital resolution of 1.25 Hz per point before zero-filling, with 10 mm tubes and an external reference (methanol) in a Wilmad spherical bulb (5 mm), which was maintained in the center of the detection area. The chemical shift of DSS present in the outer area was recorded as a function of phenol concentration.

For the experiments with the aromatic amino acids, the same method was used, except that the 1D ^{1}H NMR spectra were recorded on a 400 MHz Varian spectrometer with a digital resolution of 1.25 Hz per point before zero-filling, in 10 mm tubes and with an external reference (DSS) in a Wilmad spherical bulb (5 mm), which was maintained in the center of the detection area.

In Madrid: The NMR spectra were recorded at 500 MHz on a Bruker Avance spectrometer at 298 K. The spectra were processed by use of Topspin software (Bruker, Inc.). For all the experiments, the high-field resonance of [D_4](trimethylsilyl)propionic acid sodium salt (TSP, 10 μM) was used as an internal chemical shift reference. All the samples were prepared as mixtures of deuterated and normal water (10:90) as the overall solvent composition. Sample pH values were kept around 8 and tested with a thin electrode (Wilmad) fitted directly in a 5 mm NMR tube. The sample concentrations were 4–20 mM in carbohydrate, and the aromatic/sugar molar ratio varied from 1:1 to 20:1. The solutions were not degassed. One-dimensional high-resolution experiments were recorded with 32k complex data points, and 16–32 scans were collected at a spectral width of 4500 Hz. Water suppression was accomplished by use of the WATERGATE pulse sequence.[43] The original FID was zero-filled to 64 k, and Fourier transformation with use of an exponential window function was applied (exponential multiplication, lb = 1 Hz).

The DPFGSE-NOE method was used for the 1D intermolecular NOE measurements with subsequent solvent suppression as described elsewhere.[34] The measurements were recorded at different mixing times from 50 ms to 2 s with a relaxation delay of 30 s and typically 1 K transients.

Theoretical calculations: The calculations were performed on a "HP Cluster Superdome" computer, at the Supercomputing Center of Galicia, Spain (CESGA).

Full geometry optimizations were done with Gaussian98[44] for Fuc/Trp, FucMe4/$C_6H_4F_2$, and FucMe4/C_6F_6 complexes with use of Density Functional Theory (DFT), at the B3LYP/6-31G(d,p) level. Vibrational frequency calculations were done in order to characterize the nature of the stationary points. To determine the interaction energy precisely, basis set superposition error correction (BSSE)[45] was calculated. The counterpoise method proposed by Boys and Bernardi was used,[46] so the proper correction for changes in geometry of the components of the complex was considered.

Moreover, molecular mechanics minimizations were performed with the AMBER* force field (as implemented in the Maestro Program[41]).

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