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Enthalpic Nature of the CH/π Interaction Involved in the Recognition of Carbohydrates by Aromatic Compounds, Confirmed by a Novel Interplay of NMR, Calorimetry, and Theoretical Calculations

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Abstract: Specific interactions between molecules, including those produced by a given solute, and the surrounding solvent are essential to drive molecular recognition processes. A simple molecule such as benzene is capable of recognizing and differentiating among very similar entities, such as methyl 2,3,4,6-tetra-*O*-methyl-α-D-galactopyranoside (α-Me_5Gal), methyl 2,3,4,6-tetra-*O*-methyl-β-D-galactopyranoside (β-Me_5Gal), 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose (β-Ac_5Gal), and methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannopyranoside (α-Me_5Man). In order to determine if these complexes are formed, the interaction energy between benzene and the different carbohydrates was determined, using Calvet microcalorimetry, as the enthalpy of solvation. These enthalpy values were -89.0 ± 2.0, -88.7 ± 5.5, -132.5 ± 6.2, and -78.8 ± 3.9 kJ mol^{-1} for the four complexes, respectively. Characterization of the different complexes was completed by establishing the molecular region where the interaction takes place using NMR. It was determined that β-Me_5Gal is stabilized by the CH/π interaction produced by the nonpolar region of the carbohydrate on the α face. In contrast, α-Me_5Man is not specifically solvated by benzene and does not present any stacking interaction. Although α-Me_5Gal has a geometry similar to that of its epimer, the obtained NMR data seem to indicate that the axial methoxy group at the anomeric position increases the distance of the benzene molecules from the pyranose ring. Substitution of the methoxy groups by acetate moieties, as in β-Ac_5Gal, precludes the approach of benzene to produce the CH/π interaction. In fact, the elevated stabilization energy of β-Ac_5Gal is probably due to the interaction between benzene and the methyl groups of the acetyls. Therefore, methoxy and acetyl substituents have different effects on the protons of the pyranose ring.

Introduction

Interactions between carbohydrates and proteins mediate many fundamental and complex phenomena, such as allergic reactions, embryogenesis, tissue development, fertilization, metastasis, bacterial cell wall recognition, and protein hydration and stability.[1−7] It has been demonstrated that these interactions are facilitated by the formation of hydrogen bonds.[8] However, CH/π stacking is very common in the recognition sites of proteins that recognize carbohydrates such as galectins[9] and mediate the interaction between carbohydrates and the aromatic residues of the side chains of Trp, Tyr, Phe, and His present in the receptors. CH/π interactions have their origin in dispersion forces, which have an impact on the enthalpic term of the free energy.[10] Depending on the chemical nature of the interacting groups, the CH/π interaction may contribute more stabilizing energy than a hydrogen bond; therefore, it may become difficult to evaluate the actual energy contribution of each type of interaction when they are competing. Because of the importance of CH/π interactions and the intrinsic difficulty in their proper description, multiple efforts have been made to characterize them. When the C−H group is highly acidic, as is the case for the C−H group of chloroform, the CH/π interaction is intense. However, when the hydrogen group donor is not very acidic, the participation of several C−H bonds in the proper orientation

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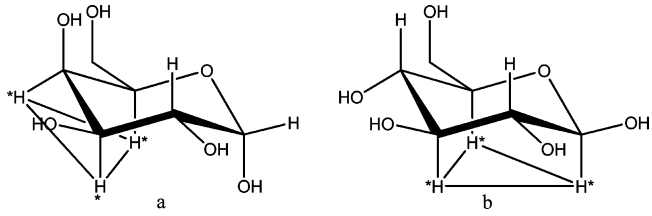

Figure 1. Orientation of the three hydrogen atoms required to interact with the aromatic molecule through the corresponding CH/π interactions. The hydrogen atoms marked with an asterisk establish the molecular region capable of stacking. The disposition indicated in **a**, α-galactose (positions 3, 4, 5) corresponds to a consecutive arrangement; that in **b**, β-glucose, (positions 1, 3, 5) belongs to an alternated arrangement.

is necessary for interaction. This is very relevant when carbohydrates interact with aromatic compounds in the recognition site of a protein. Due to the lack of acidity of the hydrogen atoms of the carbohydrate's pyranose ring, at least three hydrogen atoms must be located in the same molecular region, and there must be enough space to allow their proximity to the aromatic compound (Figure 1). This orientation can be of two types: consecutive, as in α-galactose (**a**, Figure 1), or alternated, as in β-glucose (**b**, Figure 1). Previous studies of the CH/π interaction have been based on the individual use of diverse analytical, biochemical, and biophysical techniques, such as calorimetry,[11] X-ray analysis,[12−14] NMR,[15] IR,[16] and molecular modeling.[17,18] These studies have been published as different attempts to properly describe the interactions.[19−22] However, the method presented herein, with the combination of NMR, calorimety, and theoretical calculations to adequately describe the nature of the interaction, has not been reported before.

Knowledge of all the factors that control this interaction would be of fundamental importance for modifing and modulating the affinity of natural receptors, depending on the process under study, and should be instrumental in improving the design of artificial receptors, as well as for the rational design of carbohydrate- or glycomimetic-based pharmaceuticals.[23]

Undoubtedly, a rigorous way to establish the nature of a weak interaction would be to determine its associated energy, as well as the molecular region where it is produced.[17] Only with this knowledge it is possible to avoid speculation. Thermodynamic data may allow researchers to establish the magnitude of interactions but do not provide direct evidence of their specificity. The nature and structure of the solvent molecules, behaving as individuals or as a bulk, are also of paramount relevance to determine the specificity or lack thereof of the interaction between two different entities, or between any entity and the solvent.

Experimental determination of thermodynamic binding data is essential for understanding the process itself, but also for evaluating the adequacy of the theoretical methods employed to model and quantify molecular interactions. Theoretical data strongly depend on the level of theory as well as on the correction of intrinsic failures in methodology such as the basis set superposition or the scaling of the zero-point energy. Some of the corrections required have their origin in the fact that the energy is usually calculated considering complex molecules as ideal gases.

Simple models are very useful to understand molecular recognition processes. On this basis, we have decided to focus on the investigation of isolated CH/π interactions between sugars and aromatic moieties by eliminating the possibility of hydrogen bonding by using *O*-substituted sugars as well as the proper solvents. In the study presented herein, the sugar hydroxyls have been substituted by two common protecting groups: *O*-methyl and *O*-acetyl moieties. As solvent, we have chosen benzene to mimic the nonpolar, hydrophobic, and aromatic character of the lectin binding sites. Even if these modifications do not reproduce the conditions of the biological systems, it is useful to isolate the interactions to determine if the potential interaction between carbohydrates and aromatic substrates has an enthalpic origin or if it is purely entropic.

A key question arises: Can an aromatic molecule (benzene) differentiate between two isomeric carbohydrates? It is generally accepted that, besides solvation, two factors govern molecular recognition: complementarity and preorganization.[24,25] Our study shows that, in fact, the interacting groups should complement each other but preorganization is not strictly essential for the CH/π interaction to take place. Since weak interactions have their origin in dispersion forces, they also have an impact on the enthalpic term of the free energy; the existence of the CH/π interaction can be demonstrated by measuring the energy involved, which should be stabilizing and dependent on the chemical nature of the interacting molecules, to be specific.[16,26,27] To the best of our knowledge, this is the first time the interplay of calorimetric solvation data, NMR shielding effects, intermolecular NOEs, and theoretical calculations is used to draw conclusions about specific interactions between carbohydrates and proteins, and this could be established as an adequate methodology for this type of study.

Results and Discussion

a. Theoretical Calculations. Benzene was chosen as a good model to perform computational studies, since this molecule maintains the essence of the interaction between an aromatic moiety and a carbohydrate molecule. It is the simplest possible model and could help answer a key question: What is the

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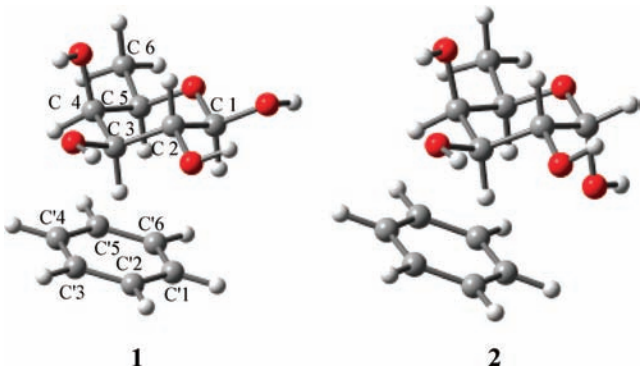

Figure 2. Structures of the benzene/β-fucose (**1**) complex[17] and benzene/α-fucose (**2**) complex at the MP2/6-31G(d,p) level of theory with BSSE correction incorporated during the full geometry optimization procedure.

relationship between molecular complementarities and the specificity of the interaction?

At the beginning of this study, a nonsubstituted regular sugar was employed. Using computer simulations, it is possible to orient the aromatic group (benzene) toward the nonpolar region of the carbohydrate, thus avoiding the participation of hydrogen bonds, which prevails over CH/π interactions *in vacuo*. To simplify the conformational problems posed by the presence of the sugar hydroxymethyl group, it was changed to a simple methyl group, as in 6-deoxy sugars. Therefore, we used fucose as the model for galactose. Moreover, to restrict the degrees of freedom of the hydroxyl groups, intramolecular OH$\cdots$OH hydrogen bonds were forced by orienting the hydroxyls in a cooperative counterclockwise (from the perspective of the carbohydrate's β face) manner.

The supramolecular structures of the β-(**1**) and α-fucose (**2**) associated with benzene obtained at the MP2/6-31G(d,p) level with the basis set superposition error (BSSE) correction incorporated during the full optimization process of the geometry are presented in Figure 2. The interaction energy reported for the benzene/**1** complex was previously described[17] and is -12.5 kJ mol^{-1}, while we calculated that for epimer **2** in this study at the same level of theory to be -11.3 kJ mol^{-1}. Even when in both supramolecules the benzene is oriented laterally, the presence of a hydroxyl group in the anomeric position (C1) of **2** generates a repulsive environment that, on average, places the benzene moiety more separated from **2** than from **1**, especially at this region. Following are the distances for complexes **1** and **2**, respectively: C1$-$C′1, 4.52 and 4.94 Å; C2$-$C′2, 4.82 and 5.04 Å; C3$-$C′3, both 3.96 Å; C4$-$C′4, 4.05 and 3.97 Å; and C5$-$C′5, 3.89 and 4.02 Å.

b. Thermochemistry. Although factors such as the disruption of the packing forces in the presence of a solvent (i.e., benzene), the differences in the solvation of the solutes, and the degree of solvation, among others, may play a role in the enthalpic changes measured in an experiment, it is possible to estimate the energy associated with the interaction generated by van der Waals complexes because the system used as the reference (**6**) lacks the interaction of interest but it can be assumed, through the design of the experiment, that all the other conditions are similar. Thus, by measuring the amount of heat exchanged when benzene binds to the carbohydrate and comparing the systems, it is possible to assume that the difference in energy is related to the CH/π interaction.

Nevertheless, since it is essential that the carbohydrate be soluble in benzene, the direct experiment could not be performed. Therefore, substitutions at the hydroxyl groups were introduced. In principle, the chosen substituent should not interact with benzene to a significant extent; thus, methyl groups were chosen as a first approximation. Moreover, this structural modification has the advantage of removing the possibility of hydrogen bonding, which could compete with the CH/π interaction.

To make the chemical modifications to the compounds of interest, permethylation was done according to the conditions described by Wang et al.[28] using iodomethane as the alkylating agent. Compounds were purified and characterized by different techniques (see Supporting Information). Compound β-Me$_5$Gal (**4**) was crystallized, and its X-ray diffraction-based structure is shown in Figure 3.

One more important issue that could arise is the fact that the methyl groups could generate a congested environment that would interfere with the interaction. Figure 3 shows the two conformers present in the crystalline network of β-Me$_5$Gal. In the solid state, β-Me$_5$Gal has an extended conformation where there is enough space for the approach of a benzene molecule. In fact, there are four C$-$H bonds on the α face of the carbohydrate available for providing CH/π interactions. Mobility of the *O*-methyl groups could challenge the approach of the aromatic ring, and establishment of the interaction could, in addition, constrain the expected motion of these pendant groups in solution with the concomitant conformational restrictions and the corresponding entropic penalty.

Nevertheless, according to the X-ray structure for the putative complex with benzene, in the key sugar regions where three C$-$H vectors exist close in space to generate the patch for benzene interaction, the existing oxygen atoms are located in such a way that they are not able to generate repulsive environment to form the van der Waals complex.

Rigorous analysis of the interaction energy should also consider the solvation energy. In the present context, the term "solvation process" refers to the energetic and structural changes occurring in a system upon transferring solute molecules into the solvent. These changes could result in the disruption of intramolecular interactions within the solvent and solute, as well as the formation of new intermolecular interactions between the molecules of solute and solvent.[29] In our case, when comparing the different carbohydrates, the existing differences in the solvation enthalpies should reflect the magnitude of the interaction of each sugar isomer with the solvent molecules. Scheme 1 shows the terms required to determine this thermodynamic property. The enthalpy of phase change as well as the dissolution enthalpy can be determined by direct measurement in a calorimeter.[11] The dissolution enthalpy values of α-Me$_5$Gal (**3**), β-Me$_5$Gal (**4**), β-Ac$_5$Gal (**5**), and α-Me$_5$Man (**6**) solutes in benzene ($\Delta_{dis}H^{\circ}_{m}$) were then determined.

When considering the obtained $\Delta_{dis}H^{\circ}_{m}$ values, it is necessary to take into account the heat of the broken intramolecular interactions and the heat released when each molecule of solute gets in the bulk of the solution and is surrounded by a shell of solvent molecules.[30] It could be deduced that, for the solid compounds **4** and **5**, a positive value of this quantity was observed, indicating that these compounds are more stable in

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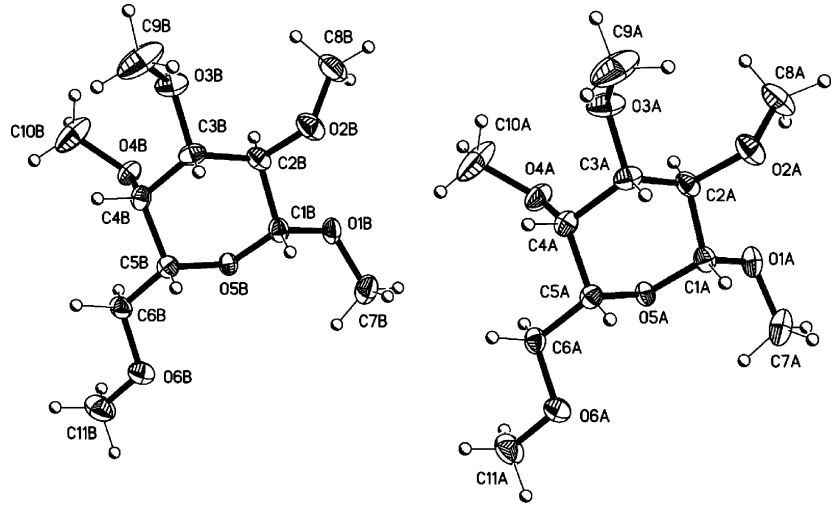

Figure 3. Conformation of β-Me_5Gal (**4**) in the solid state.

Scheme 1. Thermodynamic Determination of the Heat of Solvation

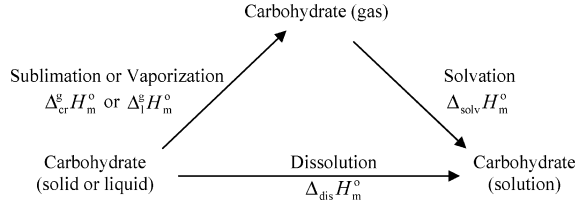

Table 1. Enthalpies of Solvation (in kJ mol^{-1}) of Methyl 2,3,4,6-Tetra-*O*-methyl-α-D-galactopyranoside (α-Me_5Gal, **3**), Methyl 2,3,4,6-Tetra-*O*-methyl-β-D-galactopyranoside (β-Me_5Gal, **4**), 1,2,3,4,6-Penta-*O*-acetyl-β-D-galactopyranose (β-Ac_5Gal, **5**), and Methyl 2,3,4,6-Tetra-*O*-methyl-α-D-mannopyranoside (α-Me_5Man, **6**) in benzene (1:10, mol:mol)[a]

	$\Delta_{dis}H^\circ_m$	$\Delta^g_{cr}H^\circ_m$ or $\Delta^g_lH^\circ_m$[b]	$\Delta_{solv}H^\circ_m$	σ_{tot}, ±[c]
3	−0.67	88.37	−89.04	2.00
4	16.54	105.25	−88.71	5.54
5	12.10	144.64	−132.54	6.19
6	−3.65	75.18	−78.83	3.91

[a] Calculated from the respective enthalpies of dissolution, determined by Calvet microcalorimetry at 303.15 K, and the enthalpies of phase change, determined by scanning calorimetry. [b] For solid compounds **4** and **5** it is reported the heat of sublimation $\Delta^g_{cr}H^\circ_m$, and for liquid compounds **3** and **6** it is reported the heat of vaporization $\Delta^g_lH^\circ_m$. [c] σ_{tot} is the overall uncertainty on the enthalpy of solvation, calculated from the uncertainties on the enthalpies of dissolution and phase change (see Supporting Information).

pure form than in solution. It can be anticipated that breaking the crystalline form of these molecules is energy demanding. In contrast, a negative, exothermic dissolution enthalpy can be observed for the two liquid compounds, **3** and **6**. The results are gathered in Table 1.

The mannose derivative **6** can be used as a reference since, according to our previous studies, the pyranose ring does not have the substitution pattern required for establishing a CH/π interaction[11] (at least three C−H bonds exposed in the same molecular region).[17] Thus, compound **6** cannot interact with benzene as a van der Waals complex. On the other hand, the data for **5** (β-Ac_5Gal) were employed to estimate the effect of the methyl group on the interaction. In this compound, the more acidic hydrogen atoms of the *O*-acetyl group show a distinct chemical nature in comparison to those of the *O*-methyl group (as in **3**, **4**, and **6**).

The phase change enthalpies reflect the cohesive energy of the crystalline lattice in the case of the solid compounds. Strong dipolar intermolecular interactions must be predominant in compound **5** due to the *O*-acetyl groups. In fact, the heat of sublimation of this compound is the highest of the four substances studied. In pure compounds **3**, **4**, and **6**, intermolecular interactions between *O*-methyl groups must be generated by dispersion forces, thus significantly reducing the heat of sublimation of β-Me_5Gal (**4**) with respect to that of β-Ac_5Gal (**5**), and even making compounds **3** and **6** liquids.

It should be noted that, for solid compounds **4** and **5**, the heat of sublimation $\Delta^g_{cr}H^\circ_m$ is reported. On the other hand, for liquid compounds **3** and **6**, the heat of vaporization $\Delta^g_lH^\circ_m$ is reported. The solvation energy $\Delta_{solv}H^\circ_m$ of the mannose derivative, **6** (−78.8 ± 3.9 kJ mol^{-1}), indicates that this is the least stable system in benzene solution. As expected from the stereochemistry of the pyranose ring, the $\Delta_{solv}H^\circ_m$ values are higher for compounds **3** (−89.0 ± 2.0 kJ mol^{-1}) and **4** (−88.7 ± 5.5 kJ mol^{-1}). In principle, we can infer that the energy difference between galactose derivatives **3** and **4**, and the mannose analogue, **6**, is due mainly to the CH/π energy. This can be estimated through eq 1.[11]

$$\text{CH}/\pi \text{ energy} = \Delta_{solv}H^\circ_m(\mathbf{3} \text{ or } \mathbf{4}) - \Delta_{solv}H^\circ_m(\mathbf{6}) \qquad (1)$$

At this point, it is possible to assume that the $\Delta_{solv}H^\circ_m$ term of **6** only includes the interactions between the *O*-methyl groups and the solvent, while the same term $\Delta_{solv}H^\circ_m$ for **3** and **4** also includes the CH/π interaction. According to this approximation, the CH/π interaction between the methyl galactopyranoside epimers and benzene amounts to 10.2 and 9.9 kJ mol^{-1} for **3** and **4**, respectively. Although only an approximation, these values are strikingly in agreement with the values calculated at the MP2/6-31G(d,p) level when the BSSE correction is included during the optimization process, 12.5 kJ mol^{-1} for model complex **1**[17] and 11.3 kJ mol^{-1} for **2**.

Carbohydrate−aromatic interactions have been widely studied using computational methods. The obtained energy values oscillate between 10.5 and 20.9 kJ mol^{-1} when the MP2 method is used. Since the different theoretical methods include different levels of approximation,[31−33] with diverse considerations of the repulsive and attractive, it is of paramount importance to access

the experimental reference values around 10.0 kJ mol^{-1} in order to estimate the quality of the different theoretical treatments.

For the penta-*O*-acetyl compound **5**, the heat of sublimation is 144.6 kJ mol^{-1}, in concordance with its vapor pressure. Interestingly, the interaction of this molecule with benzene amounts to −132.5 kJ mol^{-1}, the most stabilizing one observed in the present study. If there were no stabilization mediated by the CH/π interaction with the hydrogen atoms at the ring, the observed stabilization energy would have its origin in the independent interaction of the acetyl groups with the benzene molecules. If the solvation value of **5** is subtracted from the solvation of compound **6**, there is a difference of −53.7 kJ mol^{-1}. It is possible to contemplate that, on average, each of the *O*-acetyl group interactions contributes with −10.7 kJ mol^{-1}, a value expected for a CH/π interaction.

c. NMR Determinations. As mentioned above, thermodynamic data can be used to establish the existence of the interaction but not the region of the molecule where it happens. NMR can be safely employed for this purpose. This technique has been nicely used for the detection of intermolecular NOEs to study interactions of specific complexes such as cyclodextrins and trifluoroethanol[34] or cyclodextrins and aromatic compounds.[35] In this context, the use of an anisotropic titration procedure has established that β-galactose and benzene[11] or phenol[17] interact in the region of the C3−C6 segment of the carbohydrate, basically as described by the theoretical calculations.

Saccharides are amphipathic substances.[36] Substitution of the carbohydrate hydroxyl with methyl groups increases its hydrophobic properties and, in principle, should facilitate the access of aromatic molecules to the proximity of the pyranose ring. However, a pattern originated by specific and stabilizing CH/π interactions would decrease the translational and rotational degrees of freedom of the molecule. On the other hand, when considering solvation, in the absence of specific interactions, the approach of benzene molecules to the sugar would be random.

Table 2 presents the differences in the ^{1}H NMR chemical shifts of the compounds under study in chloroform-*d* and benzene-d_6. A positive value indicates shielding, thus protection, and the possibility that the corresponding proton interacts with the benzene face. In other words, it is within the anisotropic cone of the aromatic entity and allows the location of one benzene molecule close to a specific region of the carbohydrate ring.

A factor common to all the carbohydrates studied herein is that all the methyl groups are shielded in the presence of benzene. For the *O*-methyl sugars, the observed values oscillate between 0.10 and 0.30 ppm, while for the acetylated systems, the values are much larger, ranging from 0.34 to 0.61 ppm. These observations can be correlated with the different chemical nature of the *O*-acetyl with respect to the *O*-methyl group and its tendency to interact in a much stronger manner with benzene molecules.

Table 2. Chemical Shift Difference, $\Delta\delta = \delta CDCl_3 - \delta C_6D_6$ (in ppm), for Compounds **3**−**6**

	α-Me_5Gal, **3**	β-Me_5Gal, **4**	β-Ac_5Gal, **5**	α-Me_5Man, **6**
H1	0.01	0.01	−0.13	0.15
H2	−0.18	−0.37	−0.38	0.13
H3	−0.08	0.18	−0.09	−0.10
H4	0.17	0.20	0.01	−0.20
H5	−0.05	0.24	0.60	−0.15
H6proS	−0.01	0.04	0.06	0.01
H6proR	−0.04	−0.02	0.06	0.06
Me(1)	0.22	0.13	0.56	0.24
Me(2)	0.25	0.02	0.42	0.24
Me(3)	0.25	0.25	0.41 or 0.29[a]	0.26
Me(4)	0.10	0.10	0.61	0.10
Me(5)	0.27	0.30	0.46 or 0.34[a]	0.19

[a] Chemical shifts of methyl(3) and methyl(5) are interchangeable.

If the ring protons are now considered for β-galactoside **4**, the hydrogen atoms at positions 3, 4, and 5 are strongly shielded, with values that range between 0.18 and 0.24 ppm. This experimental fact contrasts with the observations for the α-mannoside **6**, where there is a lack of protection for the same hydrogen atoms. Indeed, the observed differences are the opposite, with values ranging between −0.10 and −0.20 ppm. This is expected since the presence of an equatorial methoxyl group at position 4 of mannose, just in the region of approach of benzene, generates a repulsive environment, and thus, the CH/π interaction does not take place.

Between these two cases, for **3** and **5** there are intermediate observations. For **3**, the protection of H-4 is high ($\Delta\delta = 0.17$ ppm), while there is lack of protection of H-3 and H-5 (−0.08 and −0.05 ppm, respectively). The presence of one axial methoxy group at the anomeric position should account for these values.

The ^{1}H NMR data of compound **5** are particularly relevant. The annular protons appear downfield from the methylated compounds, although this is a regular behavior for *O*-acetylated sugars. Besides the electron-withdrawing effect of the acetyl groups, there are some structural features that deserve attention. In the solid-state X-ray structures of both β-Ac_5Gal (**5**)[37,38] and α- Ac_5Gal[39] (Figure 3), the OCOC segments (where the initial O is that of the carbonyl group and the final C is part of the carbohydrate ring of the β-Ac_5Gal) have values of 1.61° for C1, 7.19° for C2, 4.99° for C3, 3.13° for C4, and 1.47° for C5. This shows that the different carbonyl groups eclipse the protons that are part of the galactose ring; these protons are paramagnetically shifted because they are located in the deshielding region of the carbonyl group. These conformational preferences have been studied before,[40,41] and it has been reported that the preferred conformation is that where the C−O bond of the carbonyl is parallel to the C−H bond of the pyranose ring. Indeed, our observations, including the chemical shifts, are in accordance with those studies, suggesting that the conformations in solution and in the solid state are similar. At the same time,

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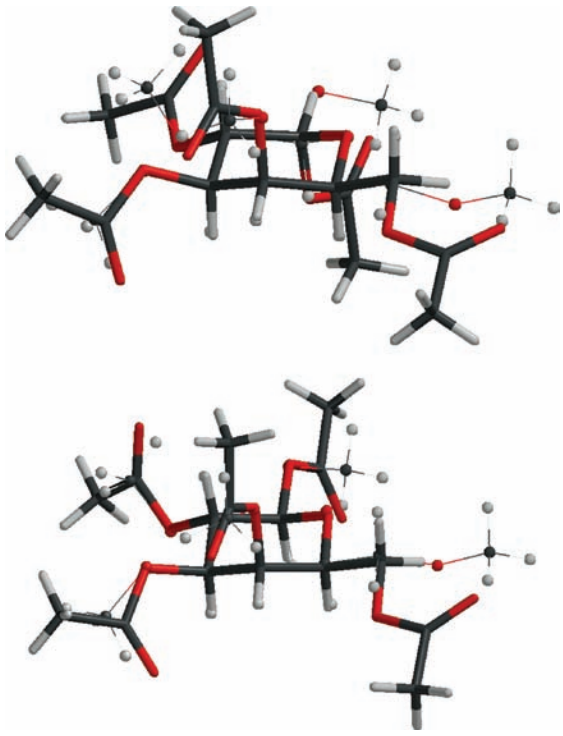

Figure 4. Superposition of β-Me_5Gal (**4**, ball and sticks) with the α-Ac_5Gal (top) and β-Ac_5Gal **5** analogues (bottom) (wire frame).

this conformational arrangement puts the oxygen atom in the region needed for the interaction with benzene. Similar results are observed in the α-Ac_5Gal anomer. Therefore, the interaction of the ring protons with the benzene moieties is geometrically disfavored by the repulsive environment generated by the carbonyl orientation. Figure 4 shows the superposition of one of the β-Me_5Gal conformers, **4** (balls and sticks), with the anomers β-Ac_5Gal, **5** (Figure 3a), and α-Ac_5Gal (Figure 3b) (wire frame), suggesting the difficulty of establishing proper interactions between the ring protons of the acetylated sugars and an approaching aromatic moiety.

Indeed, for **5**, only the region around H-5 on the side of the hydroxymethyl group is available for the interaction with the aromatic moiety. This explains the observed protection and thus shielding of H-5.

The NMR results may also be satisfactorily integrated with the calorimetry data. In fact, although in compound **5** the hydrogen atoms of the ring are not capable of participating in CH/π interactions, the molecule can interact with the aromatic compound through the *O*-acetyl groups. Again, on this basis, it can be assessed that the nature of the *O*-methyl and *O*-acetyl groups is rather different.

NOE experiments were also performed to try to detect the possibility of close proximity between the carbohydrate and benzene. The detection of intermolecular NOEs between solvent and small organic molecules has been used to establish the way in which solutes are solvated.[34,42−45] For the modified carbohydrates **3**−**5** at the same concentration (ca. 0.24 M), similar experimental parameters were employed (Figure 5). The different behavior observed for those molecules is notorious. As expected, for all the molecules, the methyl group showed significant NOEs, indicating its direct interactions with benzene.

In contrast, the NOEs observed for the ring protons of the different molecules are rather different. For β-Me_5Gal (**4**) there are clear intermolecular NOEs on H-3, H-4, and H-5 based upon the difference of the steady NOE spectra, supporting the existence of interactions between the carbohydrate and the aromatic moiety. The observed NOEs for α-Me_5Gal **3** are smaller than those for **4**, and those observed for β-Ac_5Gal (**5**) are just above the noise signal.

Therefore, the experimental NOE data (supported by the chemical shift perturbation analysis) seem to indicate that benzene interacts with the different sugars, but much more strongly with the bottom ring face of β-Me_5Gal (**4**), as also indicated by the theoretical calculations. It is noticeable that the ring protons of mannopyranoside **6** have very little interaction with benzene, although it has been observed that this interaction depends on the sample concentration (the study of this effect is in progress, see Supporting Information). Finally, for the peracetylated compound **5**, very weak NOEs are observed. This observation may be safely explained by the lack of direct interaction between benzene and the sugar ring protons. The tiny effects observed could be due to indirect reorientation effects provoked by the interaction of benzene molecules with the methyl groups of the *O*-acetyl substituents. The different intensities of the NOEs observed in Figure 5 for each carbohydrate can be associated with its proximity to the benzene ring. In all cases, the methyl groups that are necessarily solvated and close to the solvent show intense signals. The intensity of the signals follows the order of approximation established by the theoretical calculations: higher intensities and thus closer proximity of the β-galactose derivative, less for the α isomer, and very small for the acetylated compound (Figure 6). For the peracetylated compound β-Ac_5Gal (**5**), the preferred interaction of the benzene molecules with the acetyl groups could be observed through NOESY cross peaks (see Supporting Information).

A scheme representing the observed interactions is shown in Figure 6. Very few experimental observations on the existence of aromatic−sugar interactions when exploring acetylated or methylated carbohydrate molecules have been reported and systematically compared. Recently, Waters et al.[46−48] studied the attractive interaction between one tryptophan residue and a diagonally cross-stand tetra-O-acetyl-Glc-Ser, fundamental to stabilize the folding of a β-hairpin.[48] NMR data were used to demonstrate the existence of the interaction between the α face of the carbohydrate and the tryptophan residue. The authors stated that, when the acetyl groups are eliminated, the interaction with tryptophan is lost. This result is not expected since CH/π interactions do, indeed, take place for natural carbohydrates (unsubstituted) in aqueous solutions.[17] Thus, the absence of the acetyl groups should maintain the CH/π interaction if it exists. Given the particular geometrical arrangement of the studied glycopeptides, it could be possible that instead of (or besides)

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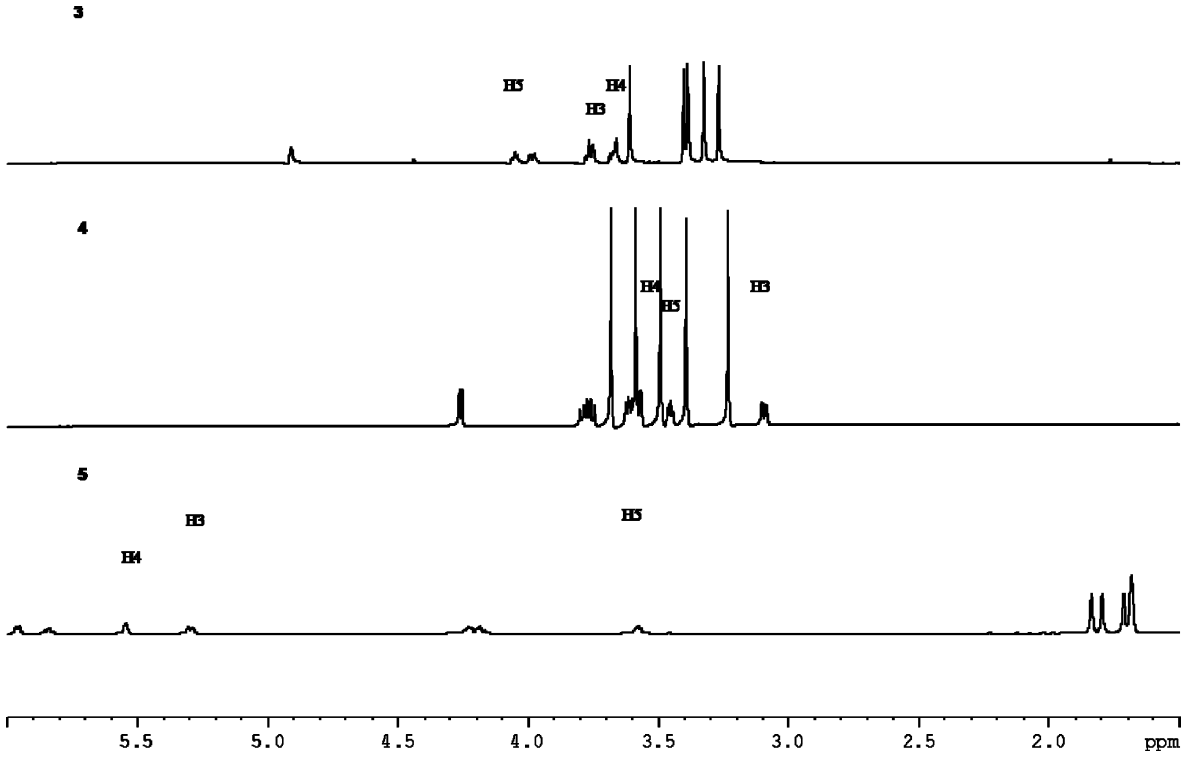

Figure 5. Comparative NOEs on compounds **3**, **4**, and **5** at 298 K upon inversion of the solvent signal (benzene). The intensity scale is the same for all cases.

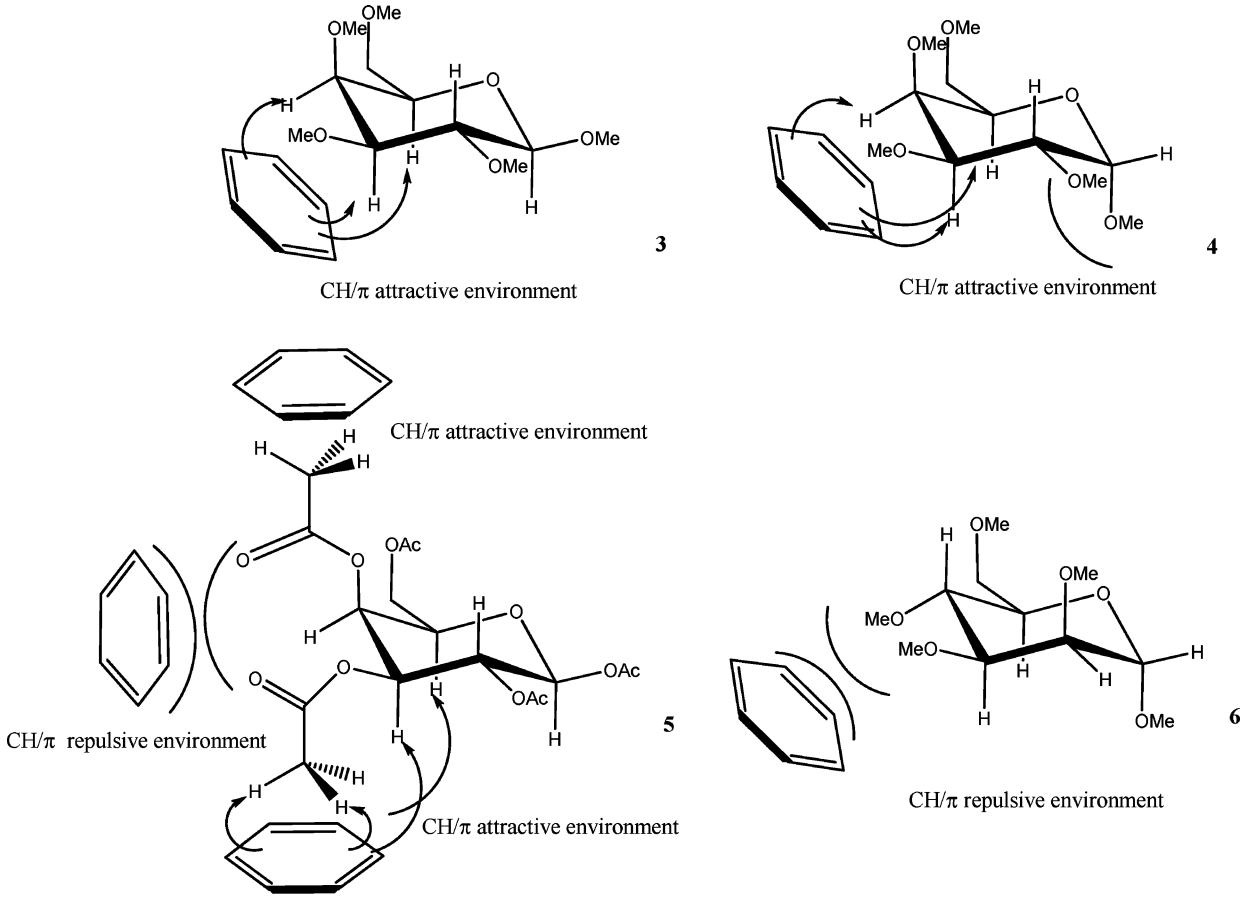

Figure 6. Origin of the observed NOE for compounds **3** and **4** (top), **5** and **6** (bottom).

an increase in the cost of solvation of the unsubstituted glucose suggested by the authors, additional dominant interactions between the methyl group of one of the acetyl groups and tryptophan take place. The energy associated with the phenomenon would certainly point toward this interaction and would be in line with our results. A study of the interaction between the carbohydrate and the aromatic in systems free of other interactions would be needed to better identify its energy and dynamics.

Obviously, in complex molecules, such as those glycopeptides mentioned above, the interaction phenomenon would be a consequence of different processes, including the solvation/desolvation process. However, it has also been shown that nonsubstituted sugars may also effectively interact with aromatic moieties, in a nonambiguous manner.[17] Thus, for each particular case, many factors should be considered, including sugar ring-to-aromatic interactions, methyl-to-aromatic interactions, and the role of solvation, especially for complex biomolecules. In any case, from our perspective, it is sound to systematically study the interaction between carbohydrates and aromatic moieties in systems free of additional interactions, which may substantially modify and modulate the structural observations and energy measurements.

Today, structures of various complexes formed by carbohydrates bound to the recognition sites of proteins determined by X-ray are available in different databases. Many lectin/sugar complexes relevant to this study have been described.[49] For example, the complex formed by evulin I (a nontoxic protein that inactivates ribosomes) and β-galactose, where the relevant interaction occurs with a tryptophan residue of the protein, is in perfect agreement with the results described here.[21] The same is true for the interaction between the nonreducing residue of the disaccharide αGal1,3βGalOMe and a tryptophan residue of isolectin 1-B4 from *Griffonia simplicifolia*.[50] A good example where the aromatic residue is tyrosine is fucosyllactose (αFuc1,2βGal1,4Glc), when this amino acid interacts with lectin II of *Ulex europaeus*.[51] In light of the study presented, it is possible to establish that the interactions between the carbohydrate and the aromatic residues of the proteins mentioned above have a stabilizing nature and an effect on the enthalpic term of the Gibbs free energy. It is noticeable that the CH/π interaction is a key contribution, in addition to rest of the typical interactions of the biological media that include hydrogen bonds, among others.

Conclusions

The interaction energies of α-Me$_5$Gal (**3**), β-Me$_5$Gal (**4**), β-Ac$_5$Gal (**5**), and α-Me$_5$Man (**6**) with benzene, computed as the enthalpy of solvation determined using Calvet microcalorimetry and differential scanning calorimetry, are -89.0 ± 2.0, -88.7 ± 5.5, -132.5 ± 6.2, and -78.8 ± 3.9 kJ mol^{-1}, respectively. This confirms that the nature of these interactions is enthalpic.

From the structural viewpoint, the interacting regions have been determined by using NMR experiments. While for β-Me$_5$Gal, the interaction takes primarily place using the (*R*-) α-face of the carbohydrate for establishing CH/π interactions with benzene, for α-Me$_5$Man no interaction of this type is detected. For acetylated sugars, chemical shifts and NOE data demonstrate that the methyl groups of the *O*-acetyl substituents effectively interact with benzene. These methyl groups generate a repulsive environment around the sugar ring that precludes efficient CH/π interaction with the *R*-face of the sugar. Calorimetry measurements indicate that every methyl group contributes to the stability of the system with an energy of ca. -10.7 kJ mol^{-1}. Moreover, methyl groups in *O*-acetyl substituents behave differently from those in *O*-Me moieties when interacting with benzene. Although not completely substantiated, this fact may have its origin in the polarization of the methyl hydrogens in the *O*-acetyl group, which contributes to its well-known acidity. Benzene can recognize and differentiate the α- and β-anomers of permethylated galactose from the mannose analogue and from the corresponding per-*O*-acetylated galactose derivatives. For the simple per-*O*-acetylated monosaccharide models, such as those studied herein, the acetyl groups do interact intensely with benzene and, in addition, block its access to the (*R*-) α-face of the carbohydrate. Previous reports suggesting that a methyl from an acetyl group is similar to a methyl group of a methoxy group were reinterpreted in terms of our findings. The observed effect could be a consequence of the methyl of the acetyl group−aromatic interaction instead of a carbohydrate−aromatic one.

Our novel use of a combination of NMR measurements with theoretical calculations and calorimetric data has satisfactorily supported an interaction model for substituted carbohydrate−aromatic interactions in simple models and could be adopted for other similar studies. The role of solvent and osmolites for modulating the observed interaction is now under study in our laboratories.

Experimental Section

General Procedure To Carry Out the Permethylation of Carbohydrates. [28] Methyl-D-pyranoside (1 g, 5.15 mmol, 1.0 equiv) was dissolved in dimethyl sulfoxide (7 mL). NaOH aqueous solution (50%, w/w) (10 mL, 50 mmol, 9.70 equiv) was added slowly. The mixture was stirred to form a gel suspension, and CH_3I (3 mL, 48.18 mmol, 9.35 equiv) was added dropwise. The reaction mixture was stirred for 24 h. Water (100 mL) was added, and the aqueous phase was extracted with CH_2Cl_2 (3 $\times$ 100 mL). The combined organic phases were dried (Na_2SO_4), filtered, and concentrated *in vacuo*. All compounds were purified by flash column chromatography (silica gel, hexane/ethyl acetate 9:1). The product, methyl 2,3,4,6-tetra-*O*-methyl-β-D-galactopyranoside (**3**), was dissolved in EtOAc (30 mL), activated charcoal was added (20%, w/w), and the black suspension was stirred for 20 min. The final product was recrystallized from hexane/CH_2Cl_2 ($\geq$99.9%).

Methyl 2,3,4,6-tetra-*O*-methyl-α-D-galactopyranoside (**4**) and methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannopyranoside (**5**) were purified by flash column chromatography (silica gel, hexane/ethyl acetate 9:1) and by activated charcoal column (CH_2Cl_2) to get $\geq$99.9% purity for each compound. NMR signals assignments for compound **4** were done using HSQC, HMBC, NOESY, and COSY. 2D experiments are included in the Supporting Information.

Methyl 2,3,4,6-Tetra-*O*-methyl-β-D-galactopyranoside (3).[52−54] Yield: 875 mg (68%). White solid, mp 45−47 °C. IR (KBr): 2979, 2921, 2836, 1448, 1373, 1334, 1305, 1183, 1073, 1106, 957, 997, 957, 908, 876, 740, 660. ^{1}H NMR (500 MHz, C_6D_6): 2.97 (dd, J = 9.5, 3.0, 1H$_3$); 3.11 (s, 3H, Me$_5$); 3.27 (s, 3H, Me$_3$); 3.33 (ddd, J = 1.5, 5.5, 7.5, 1H$_5$); 3.37 (s, H, Me$_1$); 3.46 (dd, J = 1.0, 3.0,

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$1H_4$); 3.50 (dd, $J = 5.5, 9.0$, $1H_{6a}$); 3.57 (s, 3H, Me_2); 3.64 (dd, $J = 8.0, 9.5$, $1H_{6b}$); 3.68 (dd, $J = 7.5, 10.0$, $1H_2$); 4.15 (d, $J = 7.5$, $1H_1$). ^{13}C NMR (125 MHz, C_6D_6): 56.3 (Me_1); 58.3 (Me_3); 58.8 (Me_5); 60.7 (Me_2); 60.9 (Me_4); 71.4 (C_6); 73.4 (C_5); 75.6 (C_4); 81.3 (C_2); 84.9 (C_3); 105.3 (C_1). CI-MS: 251 ($[M+H]^+$; $[C_{11}H_{22}O_6]^{H+}$).

Methyl 2,3,4,6-Tetra-*O*-methyl-α-D-galactopyranoside (4).[52−54] Yield: 759 mg (59%). Yellow liquid. IR (film): 2979, 2910, 2829, 1450, 1359, 1252, 1199, 1098, 1053, 986, 954, 883, 764, 665. ^1H NMR (500 MHz, C_6D_6): 3.14 (s, 3H, Me_5); 3.20 (s, 3H, Me_1); 3.26 (s, 3H, Me_2); 3.27 (s, 3H, Me_3); 3.52 (m, $1H_{6a}$); 3.53 (m, $1H_4$); 3.60 (dd, $J = 2.5, 9.0$, $1H_{6b}$); 3.62 (m, $1H_3$); 3.82 (dd, $J = 4.0, 10.5$, $1H_{6b}$); 3.90 (m, $1H_5$); 4.77 (d, $J = 3.0$, $1H_1$). ^{13}C NMR (125 MHz, C_6D_6): 55.0 (Me_1); 58.2 (Me_3); 58.4 (Me_2); 58.8 (Me_5); 61.0 (Me_4); 70.0 (C_5); 71.8 (C_6); 77.0 (C_4); 78.8 (C_2); 81.0 (C_3); 98.8 (C_1). CI-MS: 251 ($[M+H]^+$; $[C_{11}H_{22}O_6]^{H+}$).

Methyl 2,3,4,6-Tetra-*O*-methyl-α-D-mannopyranoside (6).[55] Yield: 797 mg (62%). Yellow liquid. IR (film): 2980, 2909, 2829, 1451, 1377, 1291, 1191, 1114, 1065, 997, 971, 871, 795, 662. ^1H NMR (500 MHz, C_6D_6): 3.13 (s, 3H, Me_1); 3.12 (s, 3H, Me_5); 3.22 (s, 3H, Me_3); 3.23 (s, 3H, Me_4); 3.42 (s, H_4); 3.43 (dd, $J = 2.0, 3.0$, $1H_2$); 3.53 (dd, $J = 2.0, 11.0$, $1H_{6a}$); 3.58 (m, $1H_{6b}$); 3.60 (m, $1H_3$); 3.62 (m, $1H_4$); 3.70 (m, $1H_5$); 3.68 (dd, $J = 7.5, 10.0$, $1H_2$); 4.64 (d, $J = 2.0$, $1H_1$). ^{13}C NMR (125 MHz, C_6D_6): 54.4 (Me_1); 57.2 (Me_3); 58.9 (Me_2); 59.0 (Me_5); 60.4 (Me_4); 72.4 (C_6); 72.5 (C_5); 77.0 (C_4); 77.4 (C_2); 82.6 (C_3); 99.0 (C_1). CI-MS: 251 ($[M+H]^+$; $[C_{11}H_{22}O_6]^{H+}$).

Compound 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose (**5**, ≥99.0%) was purchased from Aldrich and used without further purification.

NMR Experiments. Modified methylpyranoside (15 mg, 0.06 mmol) was dissolved in a mixture of $C_6D_6-C_6H_6$ (0.25 mL−0.25 mL) to measure the NOE effect using a Bruker 500 MHz spectrometer. 1D NOE difference spectra were obtained upon irradiation of the benzene signal and internal subtraction of data acquired by on-resonance and off-resonance selective excitation on alternate scans.

X-ray Determination. Solid-state structure was resolved in a Bruker Smart Apex CCD X-ray diffractometer. See Supporting Information for details.

General Procedure To Measure the Enthalpies of Dissolution by Heat Flux Calorimetry. Dissolution experiments were performed by heat flux calorimetry, using a differential Setaram C80 Calvet calorimeter working in isothermal mode at 303.15 K. Sensitivity and temperature control of the calorimetric device has been described elsewhere.[10] For dissolution experiments, stainless steel mixing with membrane vessels were employed, and the mass of each pyranoside and aromatic solvent was calculated in order to generate the maximal possible thermal signal but with a resulting molar relation carbohydrate−solvent, after the dissolution process, as near as possible to 1:10. The masses of the substances involved in each dissolution experiment were measured in an MC210 P Sartorius balance sensitive to 10 μg. After loading of the mixing vessels into the fluxmeters of the Calvet calorimeter, temperature and heat flux were stabilized by 60 min, and then data acquisition was started. Five minutes was enough to get a good initial baseline, and then the aluminum membrane that separates carbohydrate from the solvent inside of the mixing vessel was broken, and reversing of the C80 calorimeter was performed during 15 min to promote a total dissolution process. Analysis of the amplified dissolution curves, generated by the data treatment software of the C80 calorimeter, showed that a lapse of 115 min was enough for a total heat transfer from the dissolution cell to the fluxmeters. The C80 calorimeter works at constant pressure; consequently, integration of the curve of heat flux as a function of time releases directly the enthalpy of dissolution of each carbohydrate in the respective aromatic solvent. Tables including all data of mass and heat involved in each dissolution experiment are provided in the Supporting Information.

General Procedure To Measure the Enthalpies of Sublimation and Vaporization by Differential Scanning Calorimetry. The calorimetric measurements of the enthalpy associated with the change from condensed to gas phase were performed using the isothermal or scanning operation of a modified Perkin -Elmer DSC7 differential scanning calorimeter.[56,57] The sensitive element of this device is a DSC7 calorimetric holder assembly, located inside of a vacuum chamber and connected to the DSC7 analyzer by an electrical feed. The vacuum chamber is evacuated with a rotary vacuum pump, and residual pressure is monitored by a pressure gauge relayed to a Pirani gauge control. Perkin-Elmer 0219-0041 open standard aluminum pans were utilized as vaporization or sublimation cells.

For isothermal vaporization experiments on the methyl 2,3,4,6-tetra-*O*-methyl-β-D-galactopyranoside, a temperature of 323.15 K was established as the most suitable from preliminary tests. For the measurement experiments, samples of around 10 mg of the liquid substance were placed inside the vaporization pan and weighed on a Sartorius 4503 microbalance sensitive to 1.0 μg. Once the prepared sample pans were loaded in the calorimetric sensor, temperature and heat flux were stabilized and data acquisition began. Three minutes was enough to get a good initial baseline, and then a valve relaying the vacuum chamber to the vacuum pump was opened, the pressure inside the chamber was downloaded quickly to promote the vaporization process, and the complete calorimetric curve was registered in a lapse of 30 min.

For the phase change experiments with the solid 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose, from preliminary tests, scanning operation and a range of temperature from 403.15 to 473.15 were established as the most appropriate. For the measurement experiments, samples of around 11 mg of the solid carbohydrates were placed inside the sublimation pan, and the set was weighed to 1.0 μg of sensitivity. The prepared sample pans were loaded in the calorimetric sensor, and then the pressure inside the vacuum chamber was fixed in the range of 100−150 Pa, while the temperature of the sample was held at 403.15 K. After 3 min for stabilizing the calorimeter's heat flux signal, scanning the temperature at a rate of 10 K/min started. In the range of 428−443 K, each calorimetric curve showed a sharp peak due to the melting of the sample, immediately followed by a wide rounded signal in the interval of 443−455 K, due to the vaporization of the melted substance.

In isothermal as well as in scanning methodology, throughout loading and thermal stabilization of the calorimetric system, a small fraction of the substance vaporizes or sublimes; therefore, an accurate quantification of the mass lost in this part of the experimental procedure is necessary and was performed by independent experiments as previously described.[46,47]

Data acquisition and integration of the area under each calorimetric curve were performed using the Pyris software of the DSC-7 calorimeter. Prior to the measurements, the calorimetric system was calibrated for energy and temperature using high-purity samples of indium and zinc. Tables providing detailed experimental data and the procedure to calculate the enthalpy of the phase change are supplied in the Supporting Information.

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Supporting Information Available: Full optimized geometries of the supramolecular complexes; complete ref 26; ^{1}H 500 MHz NMR spectra of compounds **3**−**6** (Figures S13−S16, pp S12−S15); enthalpies of dissolution and vaporization of compound **3** (Tables S4 and S6, pp S8, S9); enthalpies of dissolution and sublimation of compound **5** (Tables S5 and S7, pp S8, S10); NOESY 500 MHz NMR spectrum of compound **5** (Figure S24, p S34); and CIF file for **4**. This material is available free of charge via the Internet at http://pubs.acs.org. CCDC-746171 β-Me_5Gal (**4**) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from the Cambridge Crystallographic Data Center via www.ccdc.cam.ac-uk/data_request/cif.

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