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A Water Soluble Pd$_2$L$_4$ Cage for Selective Binding of Neu5Ac

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Abstract: The sialic acid N-acetylmuramidic acid (Neu5Ac) and its derivatives are involved in many biological processes including cell-cell recognition and infection by influenza. Molecules that can recognize Neu5Ac might thus be exploited to intervene in or monitor such events. A key obstacle in this development is the sparse availability of easily prepared molecules that bind to this carbohydrate in its natural solvent; water. Here, we report that the carbohydrate binding pocket of an organic soluble [Pd$_2$L$_4$]$_n$ cage could be equipped with guanidinium-terminating dendrons to give the water soluble [Pd$_2$L$_4$][NO$_3$]$_4$ cage 7. It was shown by means of NMR spectroscopy that 7 binds selectively to anionic monosaccharides and strongest to Neu5Ac with $K_a = 24 \text{ M}^{-1}$. The cage had low to no affinity for the thirteen neutral saccharides studied. Aided by molecular modeling, the selectivity for anionic carbohydrates such as Neu5Ac could be rationalized by the presence of charge assisted hydrogen bonds and/or the presence of a salt bridge with a guanidinium solubilizing arm of 7. Establishing that a simple coordination cage such as 7 can already selectively bind to Neu5Ac in water paves the way to improve the stability, affinity and/or selectivity properties of M$_2$L$_4$ cages for carbohydrates and other small molecules.

Introduction

Sialic acids, a class of $\alpha$-keto acid sugars, have been found on the distal ends of cell surface glycoconjugates and play a wide variety of biological roles, especially in cell-to-cell recognition.[1] The most common member is N-acetylmuramidic acid (Neu5-

Figure 1. a) Sialyl Lewis$^x$ 1 (sLe$^x$, Figure 1a) and clinical studies showed the importance of sLe$^x$ in leukocyte adhesion deficiency,[3] inflammatory response,[4] (in vitro) fertilization,[5] coronavirus binding,[6] and cancer metastasis.[7] An example is the study of molecular sensors for sLe$^x$, which facilitates extravasation of cancer cells out of the blood stream (metastasis), displaying leukocyte mimicry.[8] While Neu5Ac is typically O-linked to other molecules as $\alpha$-anomer (axial carboxylate),[9] Neu5Ac, which is utilized by influenza or other viruses to enter mammalian cells,[2] Neu5Ac is also found at the end of tetrasaccharide Sialyl Lewis$^x$ 1 (sLe$^x$, Figure 1a) and clinical studies showed the importance of sLe$^x$ in leukocyte adhesion deficiency,[3] inflammatory response,[4] (in vitro) fertilization,[5] coronavirus binding,[6] and cancer metastasis.[7] An example is the study of molecular sensors for sLe$^x$, which facilitates extravasation of cancer cells out of the blood stream (metastasis), displaying leukocyte mimicry.[8] While Neu5Ac is typically O-linked to other molecules as $\alpha$-anomer (axial carboxylate),[9]

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the chemical antecedent to such linkages is cytidine-5′-monophospho-β Neu5Ac.\textsuperscript{[5,9]} Unlinked Neu5Ac is predominantly present as the β-anomer in solution and has a rich in vivo chemistry.\textsuperscript{[1,10]} Molecules that can bind selectively to Neu5Ac and its derivatives might thus be exploited to understand, monitor or intervene in a range of biological processes.

Inspiration for the development of Neu5Ac binders can be drawn from selectins, a subclass of lectins (carbohydrate binding proteins). As is illustrated in Figure 1a for crystal structure 1G1T (human E-selectin), there is a high degree of interaction complementarity in the binding mode with sLeX.\textsuperscript{[11]} Notably, the anionic Neu5Ac fragment of sLeX forms a salt-bridge with an arginine residue and the galactose fragment has strong charge assisted hydrogen bonds (HBs) with the same arginine residue (highlighted in magenta).

The beneficial effect of employing charge assisted HBs to bind sialic acid derivatives has been mimicked by the artificial receptors 2\textsuperscript{[12]a} and 3\textsuperscript{[12b]} shown in Figure 1b. The benzoboroxole-based receptor 2 can bind covalently to Neu5Ac with its borane part and binding is further enhanced by the presence of the nearby guanidinium group.\textsuperscript{[12b]} Pyrenyl platform 3 was intended to show carbohydrates in water using CH⋯π interactions and showed enhanced affinity for Neu5Ac when equipped with guanidinium-terminating dendrons (R\textsuperscript{π}).\textsuperscript{[12b]} Another binding strategy is the use of covalent macrocyclic compounds that can encapsulate a carbohydrate in aqueous media.\textsuperscript{[13,16]}

For example, macrocycle 4 (Figure 1c, left)\textsuperscript{[13]} is highly selective for GlcNAC-β-OMe (K\textsubscript{d} \approx 18 000 M\textsuperscript{−1} in water) by encapsulating the carbohydrate by regular HBs and CH⋯π interactions.\textsuperscript{[15] However, such covalent macrocycles are not selective for Neu5Ac or related anionic carbohydrates.\textsuperscript{[16a–e]} This can be rationalized by the presence of anionic dendrimers (R\textsuperscript{π}) used to solubilize the hydrophobic binding pockets.

Contrarily, coordination cages based on a dipyridyl ligand (L) an a square planar d\textsuperscript{2} metal (M, for example Pd\textsuperscript{4+} or Pt\textsuperscript{4+}) are positively charged and are known to have affinity for anionic guests.\textsuperscript{[18]} Another advantage of such coordination cages is the reversibility of the pyridine-metal bond. This allows for non-productive oligomerization products to become intermediates towards the desired macrocycle, thus evading low-yielding macrocyclization reactions needed in the synthesis of covalent cages. Recently, two examples of coordination cages with the structure [M\textsubscript{6}L\textsubscript{4}]\textsuperscript{4+} have been reported with affinity for carbohydrates.\textsuperscript{[14–15]} As is exemplified in Figure 1c (right), [Pt\textsubscript{4}L\textsubscript{4}]\textsuperscript{4+} cage 5 is based on dipyridyl ligands separated by anthracene moieties. This cage bound selectively to D-sucrose (K\textsubscript{d} \approx 1 000 M\textsuperscript{−1}) by virtue of shape-complementarity and multiple CH⋯π interactions between the carbohydrate and the polyaromatic cavity of 5.\textsuperscript{[14]} A similar [M\textsubscript{6}L\textsubscript{4}]\textsuperscript{4+} cage was reported (6 in Figure 1d) where the dipyridyl ligands are separated by isophtalamides, similar to macrocycle 4.\textsuperscript{[15]} Cage 6 had an organic solubility handle and could be studied in CD\textsubscript{2}Cl\textsubscript{2} containing 10% DMSO-d\textsubscript{6}, where selectivity towards n-octyl-β-glucoside (K\textsubscript{d} \approx 51 M\textsuperscript{−1}) versus n-octyl-β-galactoside (K\textsubscript{d} \approx 29 M\textsuperscript{−1}) was observed.

We thus wondered what the binding properties of a cage such as 6 would be in aqueous solution, in particular for anionic carbohydrates like Neu5Ac. To this end, the solubility handles of the ligands in 6 were replaced by guanidinium-terminating dendrons to make the [Pd\textsubscript{4}L\textsubscript{4}]\textsuperscript{4+} cage 7 (Figure 1d). Herein, we report that 7 has selective affinity for anionic sugars, particularly for Neu5Ac, and that 7 has very low to no affinity for common neutral mono- and disaccharides.

Results and Discussion

The synthesis of the ligand precursor to cage 7 (penta nitric acid salt 13) is shown in Scheme 1. The starting trimesic pentfluorophenyl (PFP) ester 8 and amine 9 were synthesized according to literature procedures\textsuperscript{[19]} and then coupled to each other to form bis-PFP ester 10 in 62% yield by using a previously reported protocol.\textsuperscript{[15,19]} Subsequently, the remaining PFP esters of 10 were substituted by 3-aminopyridine to afford 11.\textsuperscript{[20]} Deprotection of the Boc groups of 11 followed by basification and treatment with bis-boc-pyrazolocarbaximidine afforded hexa-boc guanidine 12 in 69% yield.\textsuperscript{[20]} The desired guanidinium ligand 13 could be obtained in 74% yield after treatment of 12 with 1 M nitric acid in a water/1,4-dioxane solvent mixture. The pyridyl rings in 13 could be selectively deprotonated by the addition of two equivalents of sodium hydride. The subsequent addition of a Pd(NO\textsubscript{3})\textsubscript{4} solution (0.55 eq.) gave cage 7 in a quantitative yield based on \textsuperscript{1}H NMR. (Note: As is detailed in Section S2 of the Supporting Information).

![Scheme 1. Synthesis of cage 7 from ligand 13, prepared from previously reported building blocks following (adjusted) literature protocol.\textsuperscript{[15,19–20]}](Image)

PFP = pentfluorophenyl, Boc = tert-Butyloxycarbonyl, Sol = guanidinium solubilizing group. Conditions: i) N,N-diisopropylethylamine, 42 h at room temperature (RT) in tetrahydrofuran; ii) 6 eq. 3-aminopyridine, 41 h at 100 °C in pyridine; iii) 4 h at RT in 4 M HCl in dioxane/water; iv) neutralization with NaOH and basification with NET\textsubscript{3}; v) 6 eq. bis-Boc-pyrazolocarbaximidine, 20 h at RT (with dichloromethane); vi) 1 M HNO\textsubscript{3}, 22 h at 50 °C in dioxane/water; vii) 2 eq. NaOH in D\textsubscript{2}O, 0.55 eq. Pd(NO\textsubscript{3})\textsubscript{4} in D\textsubscript{2}O (see also Figure 2). See Section S2 for experimental details and full characterizations.
In the synthesis of 7 from 13, the reaction was unproductive due to reaction compatibility issues between PFP-esters and the Boc-linked NH of diBoc-protected guanidines.

Furthermore, the isotope distribution of a species with the largest monoisotopic mass [M+H] is in agreement with a simulated distribution of monoisotopic mass m/z 476.1823. The modelled molecular formula of [7(NO3)3Cl]2+ includes some deuterium and Cl− because the solution was measured from a D2O sample in undeuterated solvent containing trace amounts of salts (see Supporting Information and Figures S74–S90 for details).

With the water-soluble [Pd2L4]16− cage 7 in hand, the binding affinity for the carbohydrate ligands listed in Table 1 was investigated by 1H NMR titration experiments in D2O. Titration curves for charge neutral carbohydrates 14–26 to about 140 mM only resulted in minor near-linear peaks shifts of some resonances of 7 with Δδmax ≈ 0.02 p.p.m. on average (see Figures S92–S104). These shifts could not result from the dilution of 7, as a dilution study in the concentration range used during titrations revealed that all resonances remained stationary (see Figure S91). Attempts to fit these shifts to a binding model was not feasible and could only be roughly modelled (not fitted) to binding with an affinity around or below the detection limit of ~ 3 M−1. We thus interpret these shifts as resulting from very weak binding of ≤ 3 M−1 (entry 1, Table 1), spanning only the very start of possible binding curves.

The emerging Pd-complex and its parent ligand thus appear to be in slow exchange relative to the NMR time scale. All proton resonances of the resulting well-defined spectrum could be identified and are consistent with cage formation (see bottom of Figure 2a).

The 2D DOSY NMR of this sample reveals that the diffusion constant (D) of 7 (log(D) = −9.83) is substantially larger than that of the neutralized ligand (log(D) = −9.57) which is also consistent with cage formation (see bottom of Figure 2a). Furthermore, the isotope distribution of a species with largest monoisotopic mass m/z = 476.1798 (Figure 2b) measured with CSI HRMS is in agreement with a simulated distribution of [7(NO3)3Cl]2+ with largest monoisotopic mass of m/z = 476.1823. The modelled molecular formula of [7(NO3)3Cl]2+ is consistent with cage formation (see bottom of Figure 2a).

Upon addition of 2 equivalents of NaOH the resonances belonging to the pyridyl ring in 13 were found significantly upfield, which is in line with deprotonation of the pyridyl nitrogens.

Subsequent stepwise addition of Pd(NO3)2 resulted in the disappearance of dipyridyl ligand signals with the proportional appearance of a new set of resonances.

The emerging Pd-complex and its parent ligand thus appear to be in slow exchange relative to the NMR time scale. All proton resonances of the resulting well-defined spectrum could be identified and are consistent with cage formation (see bottom of Figure 2a). Furthermore, the isotope distribution of a species with largest monoisotopic mass m/z = 476.1798 (Figure 2b) measured with CSI HRMS is in agreement with a simulated distribution of [7(NO3)3Cl]2+ with largest monoisotopic mass of m/z = 476.1823. The modelled molecular formula of [7(NO3)3Cl]2+ includes some deuterium and Cl− because the solution was measured from a D2O sample in undeuterated solvent containing trace amounts of salts (see Supporting Information and Figures S74–S90 for details).

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Addition of neutralized solutions of 27–29, did result in significant non-linear shifting of the resonances of 7, with clear signs of saturation (Figures S105–S107). These shifts could be fitted accurately to a 1:1 binding model resulting in the binding constants listed in entries 2–4 of Table 1. Selected spectra of the titration with 29 are shown in Figure 3a. With increasing concentration of Neu5Ac 29, aro-
matic signals a, b, d, f and g shifted downfield and broadened slightly. In the presence of a large excess of 29, a minor species with lower symmetry arose, marked with blue asterisks in Figure 3a. This species disappeared after heating at 60 °C (‘A.H.’), while the major species of [7-29] persisted and another minor symmetrical species appeared (marked with diamonds). This new set of resonances was nearly identical to a solution of deprotonated ligand 13 and neutralized 29 at the same concentration as present in the titration (shown at the top of Figure 3a and assigned with subscript ‘L’). The minor symmetrical species (diamonds) present after heating is thus probably ligand bound to the major symmetrical species (a-g), and a small impurity present in 29 (I and II) that does not bind (see Figure S108); b) HypNMR fit (black lines) using a 1:1 binding model to the shifting of indicated resonances (grey symbols) of the symmetrical signals of 7 to give \(K_a = 24.0 \pm 0.2\) M\(^{-1}\) with indicated goodness of fit of \(r^2\). The speciation is also shown as unbound 7 (host, green line) and the [7-29] adduct (blue line).

Figure 4. a) structures of 7 and β-Neu5Ac 29 with reference \(^1\)H NMR spectrum. Assignment of 29 in main text is with a ‘c’ for ‘carbohydrate’ in front of the number to distinguish it from other assignments. b) 1D selective NOESY spectrum of [7-29] \(\text{II}^+\) with \(t_m = 500\) ms after heating at 60 °C. See section S3 for full structural elucidation of β-Neu5Ac 29.

The titration data (Table 1) clearly show that 7 is selective for anionic monosaccharides, in particular for Neu5Ac 29, and that 7 binds about equally well to glucoronate 27 and galacturonate 28. To rationalize these observations, some molecular models were generated of cage 7 bound to the β-anomers of anionic carbohydrates 27, 28 and 29 using density functional theory (DFT, see Section S4 for details).

Shown in the top of Figure 5a is the space filling representation of a model of 7 that fully encapsulates glucoronate 27, with OH-3 protruding from one of the portals. Interestingly, as is shown in the bottom of Figure 5a, OH-3 is not involved in a HB to an amide, while all the other hydroxyl groups are. Rather, OH-3 is involved in the only (and weak) charge assisted HB involving the pyridyl C–H (blue arrow). The carbonylate is furthermore held in place by four amidic HBs with H–O distances in the range of 2.0–2.3 Å. This four-pronged charge assisted HB interaction might rationalize the selectivity of 7 for the anionic glucuronate 27 over the neutral glucose 14. The model of 7 with galacturonate 28 is very similar

Figure 3. a) Partial \(^1\)H NMR spectra of 7 titrated with Neu5Ac 29 in D\(_2\)O, a spectrum at the end of the titration after heating (‘A.H.’) at 60 °C, and a spectrum of 13 + 29 at the same concentration as at the end of the titration. Assignment of the proton signals: [7-29] major symmetrical species (a-g), asymmetric species (‘), [13-29] minor symmetrical species (a–g), and a small impurity present in 29 (I and II) that does not bind (see Figure S108); b) HypNMR fit (black lines) using a 1:1 binding model to the shifting of indicated resonances (grey symbols) of the symmetrical signals of 7 to give \(K_a = 24.0 \pm 0.2\) M\(^{-1}\) with indicated goodness of fit of \(r^2\). The speciation is also shown as unbound 7 (host, green line) and the [7-29] adduct (blue line).
to the [7-27] model, which is evident from the overlaid HB patternning at the bottom of Figure 5a (see also Figure S116). Here too the carboxylate is held in place by four amidic HBs and OH-3 is the only hydroxyl that has no HB to an amide and only weakly to a pyridyl C–H (blue arrow). This absence of an amidic HB with OH-3 in both 27 and 28 might explain the lack in selectivity observed between these two carbohydrates. As can be seen in the top of Figure 5b, modelling the binding of 7 to Neu5Ac 29 resulted in a complex where the anomeric center (C2) protrudes through one of the four portals of 7. The glyceryl (C7–C9) and acetyl (C10, C11) fragments point out of two other portals (see Figure S117 for details). Presumably, the carboxylate and hydroxyl group on C2 of 29 are too large to be accommodated in the interior of 7 in the same manner as modelled for the carboxylates in 27 and 28 (Figure 5a). Moreover, the guanidinium arm that was incorporated in this model of 7 formed a strong salt-bridge with the carboxylate of 29. This is particularly evident from the HB pattern in the [7-29] model shown in the bottom of Figure 5b. Highlighted in magenta are the three strong charge assisted HBs with H···O distances in the range of 1.8–2.1 Å, which resemble the charge assisted HBs found in PDB entry 1G1T (Figure 1a).\(^{[1]}\) Additionally, there are three amidic HBs and four relatively strong HBs involving pyridyl C–H’s with H···O = 2.2 Å (blue arrows). The salt-bridge formation, together with the encapsulation of 29 via three of the four portals of 7, the three amidic HBs and the four HBs with pyridyl C–H’s observed in the model can offer a rationale for the ~3–4 fold selectivity observed for 29 over 27/28 (see Table 1).

**Conclusions**

A previous reported \([\text{Pd}_{2}\text{L}_{3}]^{16+}\) cage was rendered water soluble by introducing guanidinium solubility groups on the ligand to form the \([\text{Pd}_{2}\text{L}_{3}]^{16+}\) cage 7. Titrations of 7 with common carbohydrates revealed selectivity for anionic carbohydrates. While the binding affinities towards neutral mono- and disaccharides was near or under the detection limit of \(K_a \approx 3\ M^{-1}\), the binding affinities for anionic carbohydrates 27, 28 and 29 were found to be 6.6, 8.3 and 24 M\(^{-1}\), respectively. Cage 7 is thus at least 8 times more selective for 29 than for neutral saccharides. This selectivity could be rationalized based on DFT modelling and likely originates from complementary ion-pair formation between the anionic sugars and cationic 7 by various (charge assisted) HBs, much like those found in nature.

The selectivity for anionic carbohydrates in aqueous solution is rare\(^{[12a]}\) and has not yet been reported with similar isophthalimide macrocyclic structures held together by either covalent\(^{[13,16a–k]}\) or coordination\(^{[15]}\) bonds. While the affinities found are on the lower end, this study establishes the principle that a coordination cage such as 7 can selectively bind to anionic carbohydrates in water. This finding thus paves the way for further improvements of coordination cages to enhance their stability, affinity and/or selectivity properties. Ultimately, such developments could lead to selective synthetic lectins for Neu5Ac and its α/β-derivatives that can be used in biological studies such as Western blotting, targeted drug delivery.

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**Conflict of Interest**

There are no conflicts to declare.

**Keywords:** carbohydrate recognition • host-guest systems • molecular recognition • saccharides • self-assembly

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