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Conformational behaviour of mannanuronic acid based azasugars

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Dedicated to the memory of Professor Dr Werner Reutter

Abstract: A set of mannanuronic acid based iminosugars, comprising the C-5-carboxylic acid, -methyl ester and -amide analogues of 1-deoxymannojirimycin (DMJ), was synthesized and their pH dependent conformational behavior studied. Under acidic conditions the methyl ester and the carboxylic acid took up an “inverted” ¹C₄ chair conformation as opposed to the “normal” ⁴C₁ chair at basic pH. This conformational change is explained by the stereoelectronic effects of the ring substituents and it parallels the behavior of the mannanuronic acid ester oxocarbenium ion. Because of this solution phase behavior, the mannanuronic acid ester azasugar was probed as an inhibitor for a *Caulobacter* GH47 mannosidase that hydrolyzes its substrates following a reaction itinerary that proceeds through a ³H₄ transition state. No binding was observed for the mannanuronic acid ester azasugar, but sub-atomic resolution data were obtained for the DMJ-CkGH47 complex, showing two conformations, ³S₁ and ¹C₄, for the DMJ inhibitor.

Introduction

Stereoelectronic substituent effects have a profound effect on the three-dimensional structure of molecules. Where substituents on a cyclic compound generally have a preference for an (pseudo)-equatorial position for steric reasons, the electronic spatial preferences depend on different forces such as charge-charge and dipole-dipole interactions.^[1] The conformation and reactivity of carbohydrates is determined to a large extent by the nature and orientation of the substituents. This influence becomes apparent in glycosylation reactions, where the amount, nature and orientation of the hydroxyl groups, protected with electron withdrawing esters or more electron neutral ether groups, determine the overall reactivity.^[2] It has long been known that in glycosylations, axial substituents are less deactivating or “disarming” than their equatorially positioned equivalents.^[3] Similarly, the basicity of iminosugars (or “azasugars”), carbohydrates of which the endocyclic oxygen is replaced by an amine, is influenced by the orientation of the ring substituents and azasugars bearing more axially positioned hydroxyl groups are more basic than their stereoisomers bearing

equatorially positioned substituents.^[4] These effects can be explained by the more favorable interaction of the axially positioned electronegative oxygen substituents with the positive charge present on the azasugar ring in a protonated state and the (partial) positive charge of oxocarbenium ion (-like) intermediates in glycosylation reactions.^[4-7]

In mannanuronic acids, mannosides of which the C6-OH is oxidized to a carboxylic acid functionality, the carboxylic acid has a profound effect on the conformation and reactivity of the pyranoside.^[8] In the context of the construction of bacterial oligosaccharides we have studied the glycosylation behaviour of a variety of mannanuronic acid donors in detail and we have found these to be unexpectedly reactive.^[9] In addition, glycosylations involving these donors proceed with an extraordinary selectivity to provide 1,2-*cis* glycosidic linkages. These findings have been rationalized through the conformational preferences of (partially) positively charged mannanuronic acid oxocarbenium ion (-like) intermediates that are governed by the ring substituent effects. These species prefer to adopt a “flipped” ring structure and in the ³H₄ (-like) oxocarbenium ion all substituents take up the most stabilizing (or least destabilizing) orientation: the C2-OR *pseudo*-equatorial and the C3-, C4-OR and C5-COOR groups *pseudo*-axial. Indeed DFT calculations indicate that the ³H₄ oxocarbenium ion is significantly more stable than the alternative (“non ring-flipped”) ⁴H₃ ion (See Scheme 1A).^[10]

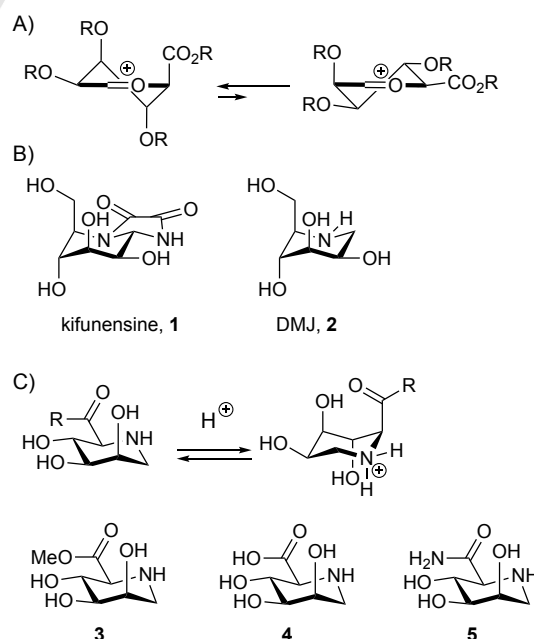


Figure 1. A) Conformational equilibrium for the mannanuronic acid oxocarbenium ion. B) Structures of the mannosidase inhibitors kifunensine (1) and 1-deoxymannojirimycin (DMJ, 2) in their ¹C₄ conformation. C) Mannuronic acid azasugars studied here.

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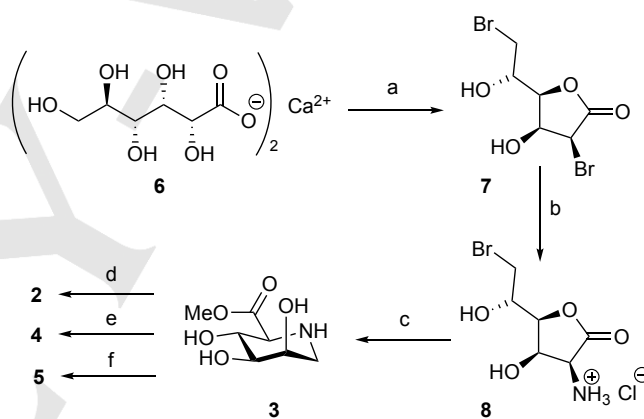
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Carbohydrate processing enzymes, such as glycoside hydrolases, may induce a chemical transformation by forcing the carbohydrate substrate into an unusual conformation.^[11] α -Mannosidases that belong to the CAZY family GH47, are inverting glycoside hydrolases that cleave α -1,2-mannosidic linkages. The mammalian GH47 mannosidases can be found in the Golgi and endoplasmic reticulum (ER) where they cleave mannose residues from *N*-glycans, thereby playing an important role in protein biosynthesis and quality control. The mechanism by which these hydrolases cleave the 1,2-mannosidic bonds is notable as they employ an unusual catalytic itinerary. The substrate that is to be cleaved binds in a ${}^3\text{O}B / {}^3\text{S}_1$ conformation and is hydrolyzed in a reaction that proceeds through a transition state in which the mannose ring adopts a ${}^3\text{H}_4$ conformation.^[12] Kifunesine (**1**, Scheme 1B), a potent inhibitor of the mannosidase I enzyme, has been shown to adopt a ring flipped ${}^1\text{C}_4$ conformation and a similar conformation was found for 1-deoxymannojirimycin (DMJ, **2**, Scheme 1B) bound in the active site of the yeast homologue *Saccharomyces cerevisiae*.^[12] We were inspired by the conformation of the inhibitors of the GH47 enzymes to explore the behaviour of mannanuronic acid based azasugars. We here report on the synthesis of mannanuronic acid based azasugars **3**, **4**, and **5** (Scheme 1C) and a study on their conformational behaviour. We show that the stereoelectronic effects that determine the structure of the mannanuronic acid oxocarbenium ions also impact the three-dimensional structure of these azasugars and that protonation of the ring nitrogen can induce a ring flip leading to an axial rich ${}^1\text{C}_4$ conformation in solution. We build on this to show how deoxymannojirimycin, the "parent" compound, binds to a bacterial GH47 enzyme from *Caulobacter* sp. K31^[12] but also that, unfortunately despite improved solution behaviour, the mannanuronic acid derivatives do not bind to the GH47 enzyme, likely by virtue of their altered C5-substituent.

Results and Discussion

The synthesis of DMJ **2** and its C5 analogs was achieved according to the route devised by Wrodnigg and co-workers.^[13] As depicted in Scheme 2, methyl mannanuronic acid ester azasugar **3** was obtained in four steps from the commercially available calcium D-gluconate monohydrate (**6**).^[14] The gluconate **6** was treated with HBr in acetic acid to form 3,5-di-O-acetyl-2,6-dideoxy-2,6-dibromo-D-manno-1,4-lactone after a series of acid catalyzed transformations (i.e. substitution of the C2 and C6 hydroxyl groups, intramolecular ring closure and acetylation of the remaining hydroxyl groups). Next the acetyl groups at O3 and O5 were removed in an acid catalyzed transesterification with methanol to provide the pure dibromolactone **7** after crystallization from chloroform/water in 26% yield over the two steps. Regioselective displacement of the C2-bromide with an azide occurred with retention of configuration, explained by Bock *et al.*^[15] with epimerization of the C2-bromide to the more reactive glucose configured dibromide and subsequent regioselective substitution by the azide. Thereafter palladium catalyzed reduction of the

intermediate azide and subsequent crystallization from ethanol gave 2-amino-6-bromo-lactone (**8**) as its hydrochloric acid salt in 55%. Treatment of this salt with triethylamine in methanol led to ring opening and intramolecular bromide displacement by the C2 amine to give crude azasugar methyl ester **3**. Purification of this compound from the triethylammonium and sodium salts formed in the reaction proved difficult, because of the high polarity of the compound as well as the lability of the methyl ester towards hydrolysis. Attempts to crystallize the compound were to no avail. Therefore, all hydroxyl groups in **3** were capped with trimethylsilyl groups^[16] to allow for the purification of the compound by chromatography. After desilylation, the pure methyl ester **3** was obtained as its hydrochloric acid salt. DMJ (**2**) was synthesized from **3** by a sodium borohydride mediated reduction and was obtained in 29% yield after column chromatography. D-Mannuronic acid azasugar **4** and amide **5** were obtained from **3** through saponification with sodium hydroxide or aminolysis with methanolic ammonia, respectively.



Scheme 2. Synthesis of DMJ and its C5 analogs for this study. Reaction conditions: a) (i) HBr, AcOH; (ii) MeOH, 26% over 2 steps; b) (i) NaN₃, acetone; (ii) H₂, Pd/C, HCl (aq.), MeOH, 55% over 2 steps; c) (i) Et₃N, MeOH, quant.; (ii) HMDS, CuSO₄·H₂O (cat.), CAN; (iii) MeOH, AcCl (cat), 70% over three steps. d) NaBH₄, EtOH, 29%; e) NaOH, H₂O, quant.; f) NH₃, MeOH, quant..

With the set of azasugars in hand we established their pK_a values by titration and investigated their conformational behaviour at different pH* (the pH measured in D₂O) values by NMR spectroscopy. Table 1 summarizes the results of these studies. For DMJ a pK_a value of 7.4 was measured, which is in line with the pK_a previously established for this compound (7.5).^[4c] The pK_a values of methyl ester **3**, amino acid **4** and amide **5** were determined to be 5.3, 7.5 and 5.8, respectively. The drop in pK_a value for the ester and the amide is a clear manifestation of the electron withdrawing effect of the carboxylic acid ester and amide functionalities. At higher pH*, where acid **4** is deprotonated, the electron withdrawing effect of the carboxylate is lowered because of its negative charge.

Table 1. pK_a values for compounds **2-5** and observed and calculated coupling constants and determined ${}^4\text{C}_1$: ${}^1\text{C}_4$ conformer ratio

Compound	pH*	$J_{3,4}$ (obs.)	$J_{3,4}$ (calc.) 4C_1 1C_4	NMR Ratio 4C_1 : 1C_4	pK_a
2	9	9.5	9.0 3.3	100:0	7.4
2	2	9.5	9.4 4.3	100:0	
3	8	9.4	9.4 3.9	99:1	5.3
3	2	7.5	9.5 4.9	56:44	
4	11	9.8	9.3 3.1	100:0	7.5
4	5	8.8	9.1 3.8	94:6	
4	1	8.3	9.5 4.7	75:25	
5	9	9.7	9.0 2.8	100:0	5.8
5	2	9.3	9.1 4.7	100:0	
3	MeOD	9.2	9.3 4.2	99:1	
3	MeOD (+TFA)	4.8	9.3 4.7	1:99	

Figures 2-5 show the 1H NMR spectra of azasugars **2-5** recorded at varying pH*. In Figure 2, the 1H NMR spectra of DMJ (**2**) in D_2O at pH* 1-12 are collected. From pH* 1 to pH* 6.5 no changes are observed in either chemical shifts or coupling constants. The coupling constants are indicative of a “normal” 4C_1 chair conformation for the azasugar ring. Going from pH* 6.5 to pH* 12 a significant shift in chemical shift is observed for all ring protons, with the direct neighbours of the amino group experiencing the largest shift. No changes occur in the coupling constants of the ring protons, indicating that no major conformation change takes place.

In Figure 3, the 1H NMR spectra of methyl ester **3** at different pH* values are displayed. Because hydrolysis of the methyl ester was observed above pH* 8, no spectra were recorded above this pH*. Large chemical shift changes are seen with increasing pH*. Especially H5 undergoes a large chemical shift change and shifts from δ 4.04 at pH* 2 to 3.22 at pH* 8. Also a change in coupling constants is observed for the ring protons. For example, the $J_{3,4}$ changes from 9.4 Hz at basic pH* to 7.5 Hz at acidic pH*, indicative of a change in conformation of the azasugar ring. At high pH* the azasugar adopts a single conformation, while both the 1C_4 and 4C_1 conformers are present at low pH* (*vide infra*).

Mannuronic acid **4** can occur in three different charged states: the fully protonated state, the neutral zwitterionic state and the negatively charged state. In Figure 4, the 1H NMR spectra of **4** are shown from pH* 1 to pH* 12. Again large chemical shift changes are observed upon changing pH* (especially for H5 shifting from δ = 3.9 to δ = 2.9 ppm). Also a small change in coupling constants is apparent. The $J_{3,4}$ changes from 9.8 Hz at high pH* to 8.8 Hz at neutral pH* to 8.3 Hz at acidic pH*. Thus, in line with the conformational behaviour of methyl ester **3**, mannonic acid **4** can change its conformation in a pH-dependent manner.

Figure 5 displays the collection of 1H NMR spectra for amide **5** at different pH* values. Smaller changes are observed for the chemical shift change of H5 and there is no significant change of the coupling constants, indicating minimal conformation changes going from high to low pH* for this azasugar.

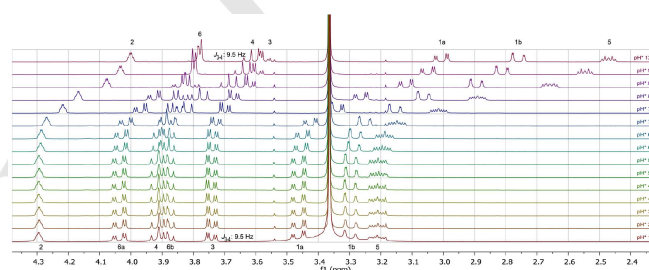


Figure 2. 400 MHz 1H NMR spectra for DMJ (**2**) at different pH*; spectra are referenced to residual methanol.

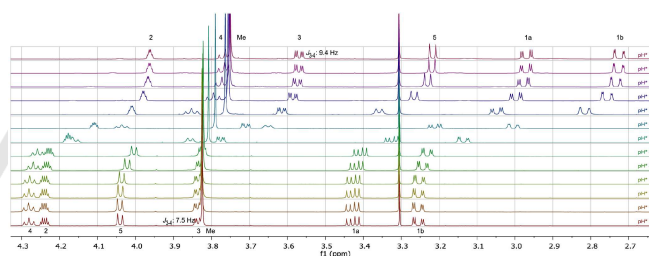


Figure 3. 1H NMR spectra for 2,6-dideoxy-2,6-imino-mannuronic acid methyl ester (**3**) at different pH*; spectra are referenced to residual methanol.

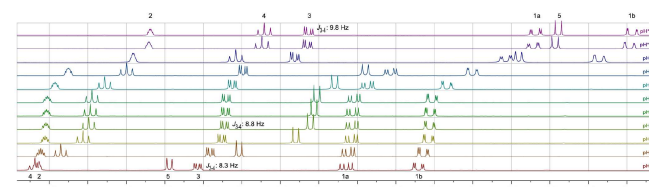


Figure 4. 1H NMR spectra for 2,6-dideoxy-2,6-imino-mannonic acid (**4**) under different pH*; spectra are referenced to water.

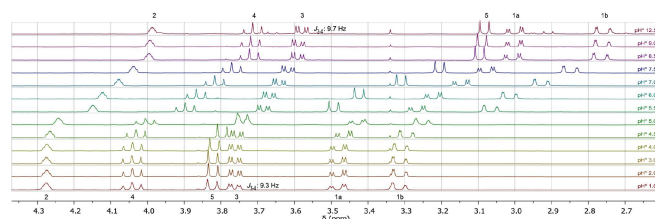


Figure 5. ^1H NMR spectra for 2,6-dideoxy-2,6-imino-mannuronic amide (**5**) at different pH*; spectra are referenced to water.

To establish the ratio of $^1\text{C}_4$ and $^4\text{C}_1$ conformers for the different azasugars we used DFT calculations to determine the coupling constants of the two conformers of both the protonated and deprotonated azasugars (See SI for details).^[17] Table 1 shows the measured coupling constants ($J_{3,4}$) for the four azasugars at low and high pH*, the calculated $J_{3,4}$ values for the $^4\text{C}_1$ and $^1\text{C}_4$ conformers and the ratio of the two conformers, established from the measured average coupling constants. As can be seen from Table 1, there is good agreement between the calculated and measured coupling constants at high pH*. With the two values for $J_{3,4}$ the ratio of the $^1\text{C}_4$ and $^4\text{C}_1$ conformers was established and it is clear that DMJ **2** takes up a single $^4\text{C}_1$ conformation at both low and high pH values. For the methyl ester **3** the situation is different. With the calculated values for the coupling constants of both conformers ($J_{3,4} = 9.5$ Hz and 4.9 Hz, for the $^1\text{C}_4$ and $^4\text{C}_1$ azasugars, respectively) and the measured average coupling constant ($J_{3,4} = 7.5$ Hz) the ratio of the two conformers was established to be 56:44, indicating that the two chair conformers are equally stable. In a similar vein the ratio of the two chair conformers of the acid (**4**) was determined at three different pH values. As can be seen in Table 1, at high pH, the anionic azasugar **4** is present as a single conformer while the measured average coupling constant at pH = 5 indicates a 94:6 mixture of conformers. At low pH two conformers are observed in a 75:25 $^4\text{C}_1 : ^1\text{C}_4$ ratio. For the amide **5**, at both high and low pH the $^4\text{C}_1$ chair is almost exclusively present.

To investigate the conformational behaviour in a less polar environment the azasugar showing the largest conformational change, methyl ester **3**, was investigated in MeOD. In Figure 6 the spectra of the non-protonated and protonated azasugar are depicted. In this medium the $J_{3,4}$ coupling constant changes from 9.2 Hz to 4.8 Hz upon protonation, indicating that the non-protonated azasugar resides in the $^4\text{C}_1$ conformation where the protonated species is found in the opposite $^1\text{C}_4$ conformation.

The NMR results show that DMJ analogues having a methyl ester or carboxylic acid at C5 (as in **3** and **4**, respectively) can change their conformation from the $^4\text{C}_1$ chair to the opposite $^1\text{C}_4$ chair upon protonation. This conformational change is seen even in a highly polar medium such as water and is significantly enhanced in a more apolar solvent (MeOD). The nature of the substituent at the C5 of the DMJ analogues is of major importance, as DMJ (**2**) and the C5 amide DMJ (**5**) do not display a conformational change upon changing pH. The

difference between the ester and amide is notable, because both functional groups, the C5 carboxylic acid ester and C5 carboxamide respectively, have a similar effect on the basicity of the azasugars. The electron withdrawing effect of both groups leads to a significant drop in the pK_a values for **3** and **5**, with the strongest electron withdrawing functionality -the ester- having the strongest inductive effect. The conformational flip of ester **3** and acid **4** can be accounted for by taking into consideration that electron withdrawing groups prefer to occupy an axial position on a positively charged pyranose ring to minimize their destabilizing effect.^[4-7] The fact that amide **5** does not change its conformation to accommodate this intrinsic preference may be due to internal hydrogen bonds that can be formed between the amide $-\text{NH}_2$ and the C4-OH which provides an extra stabilizing factor in the $^4\text{C}_1$ amide.^[18]

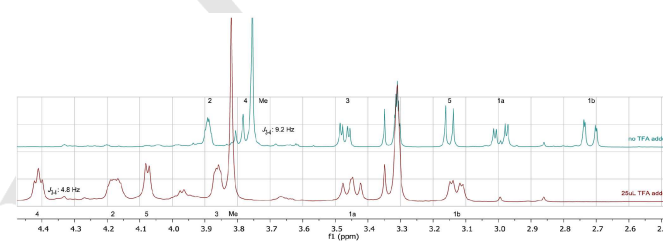


Figure 6. DMJ Methyl ester (**3**) in MeOD (± 0.7 mL). The non-protonated azasugar is shown on top (no TFA added), the protonated azasugar on the bottom (25 μl TFA added). Spectra are referenced to CD_2HOD .

The NMR results show that DMJ analogues having a methyl ester or carboxylic acid at C5 (as in **3** and **4**, respectively) can change their conformation from the $^4\text{C}_1$ chair to the opposite $^1\text{C}_4$ chair upon protonation. This conformational change is seen even in a highly polar medium such as water and is significantly enhanced in a more apolar solvent (MeOD). The nature of the substituent at the C5 of the DMJ analogues is of major importance, as DMJ (**2**) and the C5 amide DMJ (**5**) do not display a conformational change upon changing pH. The difference between the ester and amide is notable, because both functional groups, the C5 carboxylic acid ester and C5 carboxamide respectively, have a similar effect on the basicity of the azasugars. The electron withdrawing effect of both groups leads to a significant drop in the pK_a values for **3** and **5**, with the strongest electron withdrawing functionality -the ester- having the strongest inductive effect. The conformational flip of ester **3** and acid **4** can be accounted for by taking into consideration that electron withdrawing groups prefer to occupy an axial position on a positively charged pyranose ring to minimize their destabilizing effect.^[4-7] The fact that amide **5** does not change its conformation to accommodate this intrinsic preference may be due to internal hydrogen bonds that can be formed between the amide $-\text{NH}_2$ and the C4-OH which provides an extra stabilizing factor in the $^4\text{C}_1$ amide.^[18]

Having established that the mannuronic acid azasugars readily undergo a ring flip upon protonation we probed the binding of the azasugars in the binding pocket of an α -1,2-mannosidase, GH47, from *Caulobacter* K31 strain. All four compounds were

tested for binding using X-ray crystallography and isothermal titration calorimetry. Initially we analysed the binding of the parent compound DMJ **2**.

DMJ **2** binds to CkGH47 with a K_D of 481 nM (determined by isothermal titration calorimetry, Figure 7A). Although DMJ binding is essentially as observed previously for the mammalian GH47 structures,^[19] the sub-atomic resolution data (See SI), in this case allows us to observe DMJ bound in the active site of CkGH47 in two different ring conformations (Figure 7B). In the -1 subsite, the conformation of DMJ is in both 3S_1 and 1C_4 , each with a modelled occupancy of 0.5. Both conformations are consistent with the conformational itinerary of GH47 in which the substrate is moulded in a 3S_1 conformation in the Michaelis complex to react via a 3H_4 transition state forming the product in a 1C_4 conformation.^[12,19,20] This dual-conformation observation could be perhaps explained by the proximity of the pH of the crystallisation conditions, 6.5, to the pK_a of DMJ, 7.5 and the protonation of the species; although one cannot deconvolute which conformer relates to which protonation state).

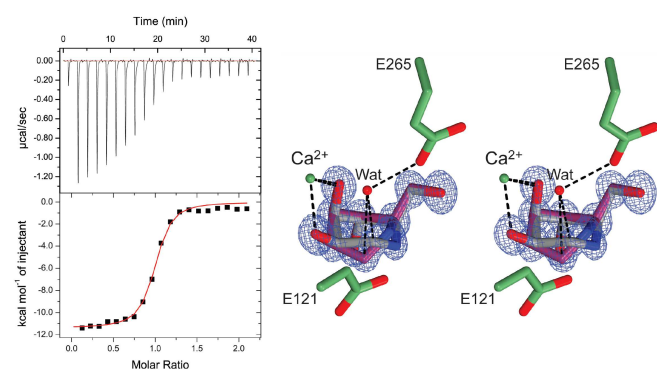


Figure 7. Binding of DMJ **2** to CkGH47. (a) ITC-derived thermodynamics of binding. The stoichiometry, n , was 0.96 ± 0.01 sites. The association constant, K_A , was $2.1 \times 10^6 \pm 3.3 \times 10^5 \text{ M}^{-1}$. The enthalpy change, ΔH , was $1140 \pm 150 \text{ kcal mol}^{-1}$. (b) Divergent wall-eyed stereo electron density for the structure of CkGH47 in complex with DMJ **2** (with the two conformations shown with grey/purple bonds). The map shown is a maximum-likelihood / σ_A weighted $2F_{\text{obs}} - F_{\text{calc}}$, contoured at 0.87 electrons / \AA^3 . The active centre calcium is shown as a green sphere and a water - likely equating to the nucleophilic water in catalysis, is shown as a red sphere. Key active centre residues discussed in the text are labelled.

The structure confirms proposals made by others, and us, concerning the catalytic apparatus.^[12,19,20] Briefly, catalytic base, E365, is hydrogen bonded to the O6 of DMJ, 2.6 Å. It is held in place by the nucleophilic water, which in turn is coordinated by calcium. The indirect route to protonic assistance is by the Oε2 of E121, facilitated by a water molecule, which is hydrogen bonded to O4 of DMJ. The riding hydrogens of this bond are visible, matching the level of detail of structure by Thompson *et al.*^[12a] With the assistance of a metal ion, the O2-C2-C3-O3 torsion angle of a 4C_1 conformation is tightened from $\sim 60^\circ$ to $0-15^\circ$, consistent with the known conformational pathway of GH47 via a 3H_4 transition state.^[12,19,20]

Unfortunately, despite promising solution characteristics, Table 1, we were not able to detect binding of mannanuronic acid derivatives **3**, **4** or **5**. Simple modelling of these compounds in the active centre, using the DMJ complex as a template,

suggested that the likely reason would be steric clashes with residues in the active site, in particular E427. To test this hypothesis, a mutant was produced containing an E427A mutation to increase the size of the active site; however attempts at complexes with this variant still did not allow observation of **3-5** in the -1 subsite of the enzyme (data not shown) suggesting that further steric clashes might also be contributing to lack of inhibition.

Conclusions

Mannuronic acid based azasugars can change their conformation upon protonation of the endocyclic amine from a "normal" 4C_1 chair to the inverted 1C_4 chair conformation. The molecules thereby position their substituents such that they are optimally positioned to accommodate the positive charge. Although the conformational behavior of any other glycuronic acid based azasugars, having different substituent configurations has not yet been studied in detail, it is likely that the spatial preferences of the substituents in the mannanuronic acid azasugar work in concert to affect the ring flip. This behavior is in line with the conformational effects observed for fully protected mannanuronic acid glycosyl donors and therefore the results described here provide an extra indication that the positive charge at the anomeric center of a mannanuronic acid oxocarbenium ion is responsible for the observed unusual ring flip. This intriguing ring-flipping behavior pointed to the potential use of the mannanuronic acid azasugars as inhibitors for mannosidases that hydrolyze their substrates through a ring flipped conformational itinerary. Unfortunately, the mannanuronic acid azasugars did not bind to the studied GH47 mannosidase. Although the concept of chemically-flipped inhibitors worked in solution, especially in low polarity buffers, they sadly highlight the challenges of conformationally-specific enzyme inhibition. For whilst the introduction of favourable chemistry including, in some cases, locking groups frequently introduces substituents that prevent binding for steric reasons - in enzyme active centres that have evolved to harness the interactions of and thus distort unsubstituted sugars. For example, the elegant locking of a mannoside mimic into $B_{2,5}$ conformation with a three-carbon bridge^[21] - in order to target $B_{2,5}$ transition-state mannosidases specifically, simply resulted in steric clashes with the target β -mannosidase and no inhibition of the wild type enzyme.^[22] Indeed, whilst the concept of conformation-specific targeted inhibition is one of the most exciting in glycochemistry, it is only rarely achieved - the use of ring-flipped kifunensine **1** to inhibit "Southern Hemisphere" mannosidases is one of the very few where a conformationally-restrained inhibitor works and has indeed found considerable application in cell biology.^[23] The challenge therefore remains to provide the specific tools and therapeutic compounds required for cellular or patient use, whilst also maintaining binding to the target enzyme.

Experimental Section

General methods for organic synthesis: All reagents were of commercial grade and used as received unless stated otherwise. Reactions were performed at room temperature unless stated otherwise. Molecular sieves (4Å) were flame dried before use. Flash column chromatography was performed on silica gel (40-63 μm). ^1H and ^{13}C NMR spectra were recorded on a Bruker AV 600, Bruker AV 400 or a Bruker DPX 400 spectrometer in D_2O or CD_3OD . Chemical shifts (δ) are given in ppm relative to the solvent residual signals. Coupling constants (J) are given in Hz. All given ^{13}C spectra are proton decoupled. Compound names are given using the standard iminosugar nomenclature numbering.

2,6-dibromo-2,6-dideoxy-D-mannono-1,4-lactone (7): Calcium D-gluconate monohydrate **5** (126 g, 280 mmol) was put under an argon atmosphere before being dissolved in 33% HBr in acetic acid (500 ml, 3.0 mol). The reaction mixture was stirred for 18 hours to form acetylated **6**. MeOH (1 l) was added and the mixture was refluxed for 2 hours. After refluxing the mixture was concentrated to half the volume under reduced pressure before adding another 500 ml of MeOH. The reaction was left to stir overnight after which the mixture was concentrated resulting in a slightly oily residue. This was co-evaporated with 100 ml of MeOH and three times with H_2O . The residue was extracted with diethyl ether (4 x 100 ml), the organic layers were combined, dried with MgSO_4 , filtered and concentrated under vacuum yielding a yellow oily residue. This was crystallized from $\text{CHCl}_3 / \text{H}_2\text{O}$ to yield a white crystalline solid (44 g, 146 mmol, 26% yield). ^1H NMR (400 MHz, D_2O): δ 5.20 (1H, d, $J = 4.5$ Hz, C-2), 4.64 (2H, m, C-4, C-3), 4.19 (1H, m, C-5), 3.77 (1H, dd, $J = 11.4$, 2.4 Hz, C-6a), 3.65 (1H, dd, $J = 11.4$, 4.9 Hz, C-6b). ^{13}C NMR (101 MHz, D_2O): δ 174.0 (C-1), 81.6 (C-4), 69.1 (C-3), 66.2 (C-5), 47.6 (C-2), 36.6 (C-6). Melting point: 130 °C. $[\alpha]_{\text{D}}^{20}$: +58.6° ($c = 1$, MeOH).

2-amino-6-bromo-2,6-dideoxy-D-mannono-1,4-lactone hydrochloride (8): 2,6-Dibromo-2,6-dideoxy-D-mannono-1,4-lactone (**6**, 5.0 g, 16.5 mmol) was put under argon and dissolved in dry acetone (MgSO_4 , 100 ml). Sodium azide (15.0 g, 231 mmol) was added and the suspension was refluxed for 20 hours. The mixture was filtrated and the filtrate concentrated under reduced pressure. The residue was dissolved in H_2O (50 ml) and extracted with diethyl ether (5 x 100 ml), the organic layers were combined, dried over MgSO_4 , filtered and concentrated under reduced pressure to give a brown oil which was identified as the 2-azido compound but included some of its diastereoisomer. ^1H NMR (400 MHz, D_2O): δ 4.68 (1H, dd, $J = 4.5$, 3.3 Hz, C-3), 4.56 (1H, d, $J = 4.6$ Hz, C-2), 4.46 (1H, dd, $J = 9.2$, 2.7 Hz, C-4), 4.09 (1H, m, C-5), 3.69 (1H, dd, $J = 11.4$, 2.7 Hz, C-6a), 3.56 (1H, dd, $J = 11.5$, 4.9 Hz, C-6b). ^{13}C NMR (101 MHz, D_2O): δ 174.1 (C-1), 81.0 (C-4), 69.6, 65.7 (C-3, C-5), 62.3 (C-2), 36.6 (C-6). The crude compound (16.5 mmol) was put under argon and dissolved in MeOH (100 ml). Palladium on activated carbon (10%, 300 mg, 0.3 mmol) and HCl (37% in H_2O , 10 ml, 121 mmol) were added and the suspension charged with hydrogen atmosphere. The reaction mixture was stirred for 22 hours after which the catalyst was filtered off over a Whatman microfilter. The filtrate was concentrated under reduced pressure and co-evaporated once with HCl (37% in H_2O , 60 ml), thrice with toluene (60 ml) and once with CHCl_3 (50 ml). Crystallization from EtOH yielded 2-amino-6-bromo-2,6-dideoxy-D-mannono-1,4-lactone hydrochloride (**8**) as white crystals (2.6 g, 9.2 mmol, 55% over two steps). ^1H NMR (400 MHz, D_2O): δ 4.83 (1H, dd, $J = 4.8$, 2.8 Hz, C-3), 4.63 (1H, dd, $J = 9.2$, 2.7 Hz, C-4), 4.59 (1H, d, $J = 4.9$ Hz, C-2), 4.20 (1H, m, C-5), 3.77 (1H, dd, $J = 11.5$, 2.6 Hz, C-6a), 3.65 (1H, dd, $J = 11.4$, 5.0 Hz, C-6b). ^{13}C NMR (101 MHz, D_2O): δ 172.1 (C-1), 78.9 (C-4), 64.1 (C-3), 62.9 (C-5), 50.2 (C-2), 33.7 (C-6). Melting point: 207 °C (decomposed). $[\alpha]_{\text{D}}^{20}$: +41.6° ($c = 1$, MeOH).

1-Deoxymannojirimycin (2): 2-amino-6-bromo-2,6-dideoxy-D-mannono-1,4-lactone hydrochloride (**7**, 501 mg, 1.8 mmol), was three times co-

evaporated with dry toluene, put under argon and suspended in dry MeOH (10 ml). The suspension was cooled to 0 °C before addition of distilled triethylamine (1.0 ml, 7.2 mmol) and the resulting clear solution was stirred overnight. The reaction mixture was concentrated under reduced pressure yielding the methyl ester as a white semi-crystalline solid. The residue was put under argon, dissolved in dry EtOH (molsieves, 10 ml) and cooled to 0°C. Sodium borohydride (709 mg, 19 mmol) was added and the suspension was stirred overnight. Dry MeOH (20 ml) was added before the mixture was filtered, concentrated under reduced pressure and co-evaporated with 1M HCl in MeOH (3x, 10 ml). The residue was purified by column chromatography (1:1 EtOAc/EtOH " 100% EtOH) yielding a pure sample of DMJ (**2**) in 29% yield (105 mg, 0.50 mmol). ^1H NMR (399 MHz, D_2O): δ 3.99 (1H, dt, $J = 2.9$, 1.6 Hz, C-3), 3.76 (1H, dd, $J = 12.5$, 3.9 Hz, C-7), 3.71 (1H, dd, $J = 12.5$, 5.5 Hz, C-7a), 3.60 (1H, t, $J = 9.7$ Hz, C-5), 3.53 (1H, dd, $J = 9.6$, 3.1 Hz, C-4), 3.03 (1H, dd, $J = 14.2$, 2.8 Hz, C-2a), 2.80 (1H, dd, $J = 14.2$, 1.5 Hz, C-2b), 2.57 (1H, ddd, $J = 9.7$, 4.9, 3.4 Hz, C-6). ^{13}C NMR (101 MHz, MeOD): δ 74.4 (C-4), 67.6 (C-5), 67.5 (C-3), 62.4 (C-6), 59.6 (C-7), 48.9 (C-2). $[\alpha]_{\text{D}}^{20}$: -14.0° ($c = 0.5$, MeOH).

Methyl 2,6-dideoxy-2,6-imino-D-mannonate hydrochloride (3): 2-amino-6-bromo-2,6-dideoxy-D-mannono-1,4-lactone hydrochloride (**7**, 0.60 g, 2.2 mmol), was co-evaporated thrice with dry toluene, put under argon and suspended in dry MeOH (12 ml). The suspension was cooled to 0 °C before addition of distilled triethylamine (1.2 ml, 8.7 mmol) and the resulting clear solution was stirred overnight. The reaction mixture was concentrated under reduced pressure before being taken up in acetonitrile (15 ml) charged with 1,1,1,3,3,3-hexamethylidisilazane (2.5 ml, 12 mmol) and copper sulphate pentahydrate (cat.). After 1 hour, the reaction mixture was concentrated and a fraction of 234 mg (0.57 mmol) was purified by column chromatography (1-2.5% 1,4-dioxane/DCM) to give 162 mg (0.40 mmol) of the per-TMSylated compound. The protected product was put under argon, dissolved in MeOH (8 ml) and acetyl chloride (1 eq) added to generate HCl in situ. The mixture was stirred for 0.5 hour, after which the compound was concentrated and coevaporated with MeOH yielding the title compound (98 mg, 0.40 mmol, 70% over 2 steps). ^1H NMR (400 MHz, MeOD) δ 4.40 (dd, $J = 5.3$, 4.8 Hz, 1H, C-5), 4.17 (ddd, $J = 9.5$, 4.1, 2.8 Hz, 1H, C-3), 4.09 (d, $J = 4.4$ Hz, 1H, C-6), 3.86 (dd, $J = 5.6$, 2.7 Hz, 1H, C-4), 3.82 (s, 3H, OCH₃), 3.44 (dd, $J = 12.2$, 9.6 Hz, 1H, C-2a), 3.13 (dd, $J = 12.2$, 4.2 Hz, 1H, C-2b). ^{13}C NMR (101 MHz, MeOD) δ 168.1 (C-7), 71.1 (C-4), 69.9 (C-5), 64.2 (C-3), 58.8 (C-6), 53.4 (OCH₃), 43.5 (C-2). $[\alpha]_{\text{D}}^{20}$: +31.8 ($c = 1$, MeOH).

Sodium 2,6-dideoxy-2,6-imino-D-mannonate (4): Methyl 2,6-dideoxy-2,6-imino-D-mannonate hydrochloride (**3**, 24 mg, 0.10 mmol), was dissolved in H_2O (0.5 ml). A sodium hydroxide solution (1M aq., 170 μl , 0.17 mmol) was added and the mixture stirred for 2 hours. The mixture was concentrated under reduced pressure yielding the title compound, pure but with added sodium hydroxide. ^1H NMR (400 MHz, D_2O): δ 4.01 (1H, m, C-3), 3.71 (1H, t, $J = 9.7$ Hz, C-5), 3.60 (1H, dd, $J = 9.6$, 3.2 Hz, C-4), 3.01 (1H, dd, $J = 14.6$, 2.7 Hz, C-2a), 2.95 (1H, d, $J = 9.8$ Hz, C-6), 2.75 (1H, dd, $J = 14.6$, 1.6 Hz, C-2b). ^{13}C NMR (101 MHz, D_2O): δ 178.4 (C-7), 74.1 (C-4), 70.6 (C-5), 69.1 (C-3), 65.2 (C-6), 47.9 (C-2). $[\alpha]_{\text{D}}^{20}$: -7.2 ($c = 1$, MeOH).

2,6-dideoxy-2,6-imino-D-mannonic amide (5): 2-amino-6-bromo-2,6-dideoxy-D-mannono-1,4-lactone hydrochloride (**7**, 500 mg, 1.8 mmol), was co-evaporated thrice with dry toluene, put under argon and suspended in dry MeOH (10 ml). The suspension was cooled to 0 °C before addition of distilled triethylamine (1.0 ml, 7.2 mmol) and the resulting clear solution was stirred overnight. The reaction mixture was concentrated under reduced pressure yielding the methyl ester as a white semi-crystalline solid. The residue was dissolved in 6M ammonia in MeOH (10 ml, 60 mmol) and was stirred overnight. The reaction mixture

was concentrated under reduced pressure yielding 2,6-dideoxy-2,6-imino-D-mannonic amide (**5**) in quantitative yield. An analytical sample was made by crystallisation from pure MeOH (133 mg, 0.76 mmol, 42%). ¹H NMR (399 MHz, D₂O): δ 3.97 (1H, m, C-3), 3.70 (1H, t, J = 9.7 Hz, C-5), 3.57 (1H, dd, J = 9.6, 3.1 Hz, C-4), 3.07 (1H, d, J = 9.8 Hz, C-6), 2.99 (1H, dd, J = 14.6, 2.7 Hz, C-2a), 2.75 (1H, dd, J = 14.6, 1.6 Hz, C-2b). ¹³C NMR (101 MHz, DMSO): δ 173.5 (C-7), 74.9 (C-4), 70.0 (C-5), 68.7 (C-3), 63.3 (C-6), 49.1 (C-2). [α]_D²⁰: -31.6° (c = 0.5, H₂O). HR-MS: [M+H]⁺ calculated for C₆H₁₂O₄N₂: 177.08698; found: 177.08683

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Keywords: Azasugar • Conformation • Stereoelectronic effects • Inhibitor design • Mannosidase

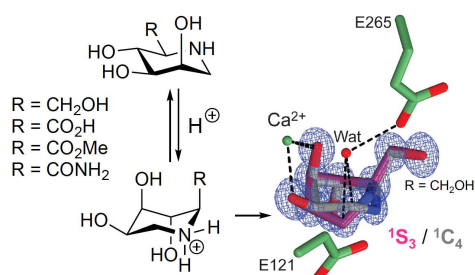
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Entry for the Table of Contents (Please choose one layout)

Layout 1:

FULL PAPER

Mannuronic acid azasugars can undergo a conformational change from the conventional 4C_1 to the 1C_4 chair upon protonation. This behaviour was shown to be dependent on the functionality at C5. Binding is studied with a GH47 mannosidase that employs a hydrolysis itinerary proceeding through a 3H_4 transition state.



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Conformational behaviour of mannuronic acid based azasugars