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Conformational behaviour of mannuronic acid based azasugars


Dedicated to the memory of Professor Dr Werner Reutter

Abstract: A set of mannuronic acid based iminosugars, comprising the C5-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” 1C4 chair at basic pH. This conformational change is explained by the stereoelectronic effects of the ring substituents and it parallels the behavior of the mannuronic acid ester oxocarbenium ion. Because of this solution phase behavior, the mannuronic 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 1H4 transition state. No binding was observed for the mannuronic acid ester azasugar, but sub-atomic resolution data were obtained for the DMJ-αCrGH47 complex, showing two conformations, 1S 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 those that carry 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.[3-7]

In mannuronic 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 mannuronic 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 mannuronic 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 1H4 (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 1H4 oxocarbenium ion is significantly more stable than the alternative (“non ring-flipped”) 1H2 ion (See Scheme 1A).[10]

Abstract:

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Figure 1. A) Conformational equilibrium for the mannuronic 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.
Carbohydrate processing enzymes, such as glycoside hydrolases, may induce a chemical transformation by forcing the carbohydrate substrate into an unusual conformation\cite{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 endoplasmatic 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 $^{10}$B $^{3}$S conformation and is hydrolyzed in a reaction that proceeds through a transition state in which the mannose ring adopts a $^{2}$H$_{4}$ conformation.\cite{12} Kifunesine (1, Scheme 1B), a potent inhibitor of the mannosidase I enzyme, has been shown to adopt a ring flipped $^{1}$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*.\cite{13}

We were inspired by the conformation of the inhibitors of the GH47 enzymes to explore the behaviour of mannuronic acid based azasugars. We here report on the synthesis of mannuronic 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 mannuronic 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 $^{1}$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\cite{14} but also that, unfortunately despite improved solution behaviour, the mannuronic 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.\cite{15} As depicted in Scheme 2, methyl mannuronic acid ester azasugar 3 was obtained in four steps from the commercially available calcium d-glucuronic monohydrate (6).\cite{16} The glucuronic 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.*\cite{17} 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\cite{18} 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.

With the set of azasugars in hand we established their pK\textsubscript{a} values by titration and investigated their conformational behaviour at different pH\textsuperscript{+} (the pH measured in D\textsubscript{2}O) values by NMR spectroscopy. Table 1 summarizes the results of these studies. For DMJ a pK\textsubscript{a} value of 7.4 was measured, which is in line with the pK\textsubscript{a} previously established for this compound (7.5).\cite{19} The pK\textsubscript{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\textsubscript{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\textsuperscript{+}, where acid 4 is deprotonated, the electron withdrawing effect of the carboxylate is lowered because of its negative charge.

**Table 1.** pK\textsubscript{a} values for compounds 2-5 and observed and calculated coupling constants and determined $^{1}$C\textsubscript{i} : $^{1}$C\textsubscript{e} conformer ratio.
Figures 2-5 show the $^1$H NMR spectra of azasugars 2-5 recorded at varying pH*. In Figure 2, the $^1$H NMR spectra of DMJ (2) in D$_2$O 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” $^4$C$_1$ 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 $^1$H 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 $\delta$ = 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 $^4$C$_1$ and $^4$C$_4$ 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 $^1$H 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 $\delta$ = 3.9 to $\delta$ = 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, mannotonic acid 4 can change its conformation in a pH-dependent manner.

Figure 5 displays the collection of $^1$H 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.
To establish the ratio of $^1$C and $^3$C 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).[@] Table 1 shows the measured coupling constants ($J_{C,D}$) for the four azasugars at low and high pH*, the calculated $J_{D,A}$ values for the $^4$C$_1$ and $^1$C$_1$ azasugars 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 $J_{C,D}$ the ratio of the $^1$C$_1$ and $^3$C$_1$ conformers was established and it is clear that DMJ 2 takes up a single $^4$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 ($J_{C,D}$ = 9.5 Hz and 4.9 Hz, for the $^1$C$_1$ and $^4$C$_1$ azasugars, respectively) and the measured average coupling constant ($J_{C,D}$ = 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 $^4$C$_1$ : $^1$C$_1$ ratio. For the amide 5, at both high and low pH the $^1$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 MeOD. In Figure 6 the spectra of the non-protonated and protonated azasugar are depicted. In this medium the $J_{C,D}$ coupling constant changes from 9.2 Hz to 4.8 Hz upon protonation, indicating that the non-protonated azasugar resides in the $^4$C$_1$ conformation where the protonated species is found in the $^1$C$_1$ 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 $^1$C$_1$ chair to the opposite $^4$C$_1$ 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.[@] 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 -NH$_2$ and the C4-OH which provides an extra stabilizing factor in the $^4$C$_1$ amide.[@]
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 Kd of 481 nM (determined by isothermal titration calorimetry, Figure 7A). Although DMJ binding is essentially as observed previously for the mammalian GH47 structures, in this case, it is observed that the residue in the active site of CKGH47 in two different ring conformations (Figure 7B). In the -1 subsite, the conformation of DMJ is in both "C" and "C", 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 "S" conformation in the Michaelis complex to react via a "H" transition state forming the product in a "C" conformation. This dual-conformation observation could be perhaps explained by the proximity of the pH of the crystallisation conditions, 6.5, to the pKd of DMJ, 7.5 and the protonation of the species; although one cannot deconvolute which conformer relates to which protonation state.

The structure confirms proposals made by others, and us, concerning the catalytic apparatus. 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 O2 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. With the assistance of a metal ion, the O2-C2-C3-O3 torsion angle of a "C" conformation is tightened from ~60° to 0–15°, consistent with the known conformational pathway of GH47 via a "H" transition state.

Unfortunately, despite promising solution characteristics, Table 1, we were not able to detect binding of mannuronic 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" "C" chair to the inverted "C" 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 gluconic 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 mannuronic acid azasugar work in concert to affect the ring flip. This behavior is in line with the conformational effects observed for fully protected mannuronic acid glycosyl donors and therefore the results described here provide an extra indication that the positive charge at the anomeric center of a mannuronic acid oxocarbenium ion is responsible for the observed unusual ring flip. This intriguing ring-flipping behavior pointed to the potential use of the mannuronic acid azasugars as inhibitors for mannosidases that hydrolyze their substrates through a ring flipped conformational itinerary. Unfortunately, the mannuronic 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 B2,5 conformation with a three-carbon bridge - in order to target B2,5 transition-state mannosidases specifically, simply resulted in steric clashes with the target β-mannosidase and no inhibition of the wild type enzyme. 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. 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

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, Ks, was 2.1±0.3 ± 3.3±0.01 M⁻¹. The enthalpy change, ΔH, was 1140 ± 150 kcal mol⁻¹. (b) Divergent wall-eyed stereo electron density for the structure of CKGH47 in complex with DMJ 2 (with two conformations shown with grey/purple bonds). The map shown is a maximum-likelihood / χ-weighted 2Fobs-Fcalc contour at 0.87 electrons / Å. The active centre calcium is shown as a green sphere and a water - likely equating to the nucleophile in catalysis, is shown as a red sphere. Key active centre residues discussed in the text are labelled.

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was concentrated under reduced pressure yielding 2,6-dideoxy-2,6-
imino-α-mannonic amide (5) in quantitative yield. An analytical sample
was made by crystallisation from pure MeOH (133 mg, 0.76 mmol, 42%).
H NMR (399 MHz, D2O): δ 3.97 (1H, m, C-3), 3.70 (1H, 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]D 20: −31.8° (c = 0.5, H2O). HR-MS: [M+H]+ calculated for C21H23O11N2: 377.1369; found: 377.1368.

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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.