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ISOTOPE EDITED NMR STUDIES OF GLYCOSIDASES: DESIGN AND SYNTHESIS OF A NOVEL GLYCOSIDASE INHIBITOR

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Abstract: *N*-^{13}C-methyl-deoxynojirimycin was synthesized and used in isotope-edited NMR studies to probe the binding site of an α-glucosidase. Results from this analysis led to the design and preparation of a novel α-glucosidase inhibitor, *N*-glycyl deoxynojirimycin.

Glycosidase inhibitors continue to be of interest due to their utility for the treatment of HIV, cancer, and diabetes.[1] Rational design of enzyme inhibitors with the necessary specificity requires detailed knowledge of the structure of glycosidases. However, although there are crystal or solution structures of many biologically important glycosidases[2] the structures of only a few mammalian glycosidases have been solved.[3] These molecules are difficult to characterize by NMR or X-ray crystallography because they tend to be large (>100 kDa), are often membrane-bound, and are too numerous to fully characterize rapidly. A method to assay molecular recognition differences between an array of glycosidases would facilitate design of potent selective inhibitors. We report here a study designed to assess the ability of isotopically labeled deoxynojirimycin derivatives and isotope-edited NMR to address molecular recognition features of glycosidases. Results from this analysis led to the design and preparation of a novel α-glucosidase inhibitor.

Observation of correlations between enzyme activity, enzyme–ligand contacts, and enzyme–bound ligand conformation in addition to traditional structure–activity relationship (SAR) studies have led to the design of potent and selective ligands in other systems.[4,5] We are using this approach to discover specificity differences between α-glucosidases that can ultimately be exploited to design enzyme specific inhibitors for use in the treatment of diabetes and HIV. Analysis of protein sequence,[6] folding,[7] and structure[8] indicates that glycosidases with similar sequences, even in the catalytic site, may have differences in sequences close to the catalytic site that determine substrate specificity. We are probing these differences by NMR in order to discover molecular recognition characteristics that would be useful to target.

We investigated yeast α-glucosidase to test our methodology. We used *N*-alkyl deoxynojirimycin analogs (**1**) to probe the catalytic and neighboring sites. Yeast α-glucosidase belongs to a sequence-related family (family 13) that includes α-amylases and oligo-1,6-glucosidases. Although the crystal structure has not yet been determined, the active site nucleophile has been identified as Asp-214.[9]

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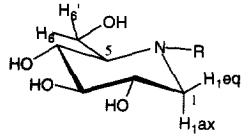
Deoxynojirimycin, **1a**, belongs to the azasugar class of glycosidase inhibitors. The extent and type of N-alkylation of these compounds has been implicated in controlling enzyme specificity and inhibition potency.[10] The hydroxyl groups of the deoxynojirimycin skeleton provide selectivity for glucosidases and the *N*-substituent can be readily varied to achieve specificity for a particular enzyme. Consequently, they are excellent compounds for probing molecular recognition features by NMR.



1a R = H

1b R = CH_3

We investigated the binding of *N*-methyl deoxynojirimycin, **1b**, to brewer's yeast α-glucosidase[11] and effects on enzyme activity. In 0.01M PIPES, 0.2 M NaOAc and 0.01 mM EDTA at pH 6.5, the K_i is reported to be 3.69 x 10^{-4} M.[12] The NMR studies require the use of buffers that are per-deuterated or have no NMR detectable protons. Consequently, we used 0.1 M sodium phosphate buffer at pH 6.55 for the buffer. The enzyme was dialyzed against buffer for 24 h at 4 °C and then either frozen and lyophilized for storage or used immediately in enzymatic or NMR studies. The catalytic activity remained unchanged through these treatments, but decreased over time at room temperature in the absence of EDTA.[13]

Compound **1b** demonstrated a K_i of 5.4 x 10^{-5} M.[14] This differs from the literature value (3.69 x 10^{-4} M)[12] most likely because of the different buffers used.[15] Tris buffer can inhibit glycosidases[16] thus, it is possible that the PIPES N-[2-ethanesulfonic acid] amino moiety affected the kinetics in the other study. Conclusions about the relative effectiveness of inhibitors must take into account buffer conditions as well as pH. Few broad SAR studies on these azasugars have been done in a consistent manner using identical conditions.[12,17]

Isotope-edited NMR studies of ligand–receptor complexes have been done with relatively small proteins (20–30 kDa).[5] We were initially concerned that the bound inhibitor signals might be too difficult to detect and resolve due to the large molecular weight of the enzyme (63 kDa).[18] However, the simplicity of the inhibitor structure allowed us to observe the bound inhibitor effectively.

Labeled *N*-^{13}C Methyl-deoxynojirimycin was synthesized via alkylation of deoxynojirimycin[19] with ^{13}C methyl iodide. The purity and identity of the isolated product was confirmed by spectroscopy.[20] A solution of 1:1 labeled inhibitor/brewer's yeast α-glucosidase[21] was prepared and analyzed by NMR. An isotope-edited NOESY was used where the signals along ω2 correspond to protons bound to both ^{12}C and ^{13}C and the signals along ω1 correspond to protons bound to ^{13}C.[22,23]

Positive NOEs were observed at (ω2,ω1) of (3.99,2.79) and (3.35,2.79) ppm corresponding to free inhibitor NOEs of the methyl protons (2.79) along ω1 with H6/6' (3.99) and $H1_{eq}$ (3.35) respectively, along ω2.[24] Negative NOEs, arising from the methyl protons in the enzyme-bound inhibitor, were observed at (ω2,ω1), (1.73,0.45), and (1.49,0.45) ppm (Figure 1a). These signals were not observed in an identical experiment with an enzyme sample containing 1:1 enzyme/U-^{13}C-glucose and no inhibitor (Figure 1b).[25]

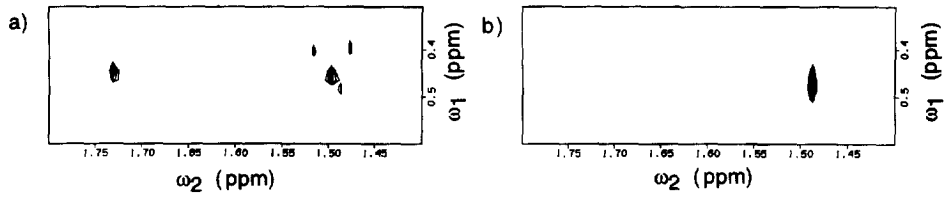


Figure 1.(a) 3.6 x 10^{-4} M *N*-^{13}C methyl deoxynojirimycin, **1b**, 3.5 x 10^{-4} M brewer's yeast α-glucosidase and 1.8 x 10^{-5} M DSS in D_2O. (b) 3.5 x 10^{-4} M U ^{13}C-C6 glucose 3.5 x 10^{-4} M brewer's yeast α-glucosidase and 1.8 x 10^{-5} M DSS in D_2O. Spectra were acquired and processed under identical conditions.[23]

We observed a 2.34 ppm upfield shift of the N-methyl protons of **1b** in the bound versus free inhibitor. Protonation of the nitrogen in the enzyme active site does not account for this upfield shift since protonation of **1b** in solution leads to a downfield shift of the methyl protons.[20] Rather, the large upfield shift is possibly due to the methyl group of **1b** being situated such that it is above or below the plane of an aromatic ring and shielded by aromatic ring current induced diamagnetic anisotropy. The observed unique negative NOEs are most likely between the protons of the ^{13}C labeled methyl group and the H6/6' and $H1_{eq}$ protons, as is the case with the free ligand, all affected to a similar extent by local enzyme-induced anisotropy.

The crystal structure of a sequence-related mammalian α-amylase (porcine pancreatic amylase, family 13) complexed with acarbose (a pseudotetrasaccharide α-amylase inhibitor) reveals several key aromatic rings near the enzyme's active site (Trp58, Trp59, Tyr62, and Tyr151).[26] Comparison of the peptide sequences of porcine pancreatic amylase and yeast α-glucosidase points out certain aromatic residues in the yeast enzyme that are likely situated near the active site. The most notable are residues Tyr71 and Phe165, analogous to Tyr62 and Tyr151 in porcine pancreatic α-amylase, which could be creating the large upfield shift we observed.

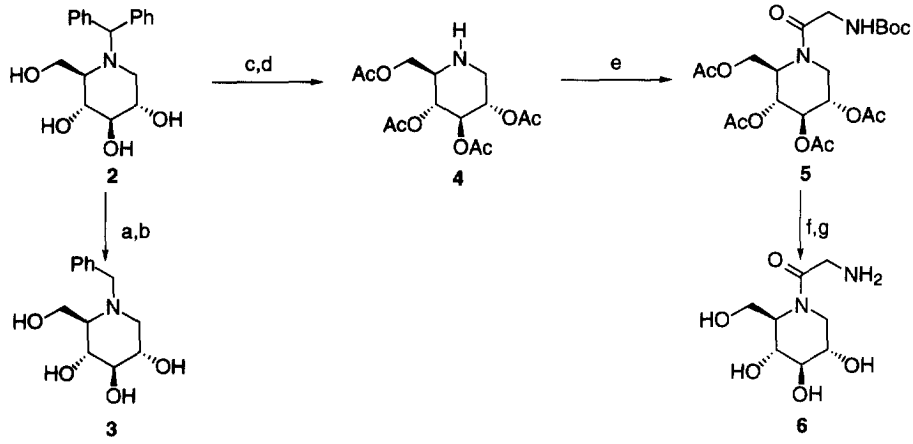
Because the NMR analysis implicated aromatic rings as critical components near the binding site of **1b**, we proceeded to investigate deoxynojirimycin analogs that could take advantage of the local aromatic environment through a mechanism such as π–π stacking or cation–π interactions.[27] We initially prepared N-benzyl deoxynojirimycin[28] **3** as shown in scheme 1. The readily available **2** was hydrogenolyzed to provide deoxynojirimycin.[19] The yield for this hydrogenolysis is low although the isolated product obtained is pure. Treatment with benzyl bromide gave the target compound **3**.

We saw no inhibition with N-benzyl deoxynojirimycin at concentrations up to 3.0 mM using the conditions discussed previously. The lack of inhibition might be due to steric factors. We then investigated a less bulky derivative. For carbohydrate analogs, such as the azasugars, inhibition of α-glucosidases depends on planarity at the anomeric carbon, basicity/hydrogen bonding capabilities and appropriate hydroxyl group orientation.[12,29] The optimal position of the basic heteroatom is in the ring (replacing O_6 or C_1) or at the anomeric position. Abolishing the basicity of this heteroatom, such as with N-oxidation or N-acylation,[30] leads to reduced activity. We had hoped that a carbonyl group would provide the necessary interaction with the possible π system near the binding site and yet be considerably smaller than a benzene ring. In order to maintain a basic (protonatable) amine in or near the ring we chose to prepare analog **6**.[31]

We again started from **2**, peracylated it, then hydrogenolyzed it to generate tetraacetate **4**. We used TBTU to couple N-Boc-glycine to the free amine of **4**. Other coupling agents (e.g., DCC, CDI) were less useful as very

little product was obtained. The Boc group was removed with HCl.[32] Finally, the acetates were removed with Dowex (OH-) to provide our target inhibitor **6**.

Scheme 1



(a) H_2, Pd/C, MeOH, 50%; (b) $PhCH_2Br$, CH_3CN/H_2O, 50%; (c) Ac_2O, pyr, 65%; (d) H_2, Pd/C, MeOH, 51%; (e) Boc-gly-OH, TBTU CH_3CN, iPr_2NEt, 50%; (f) AcCl, EtOAc, MeOH, 20%; (g) Dowex (OH^-), 56%.

As designed, compound **6** did indeed show competitive inhibition with a K_i of 2.3×10^{-5} M, more potent than *N*-methyl deoxynojirimycin. Based on the factors involved in optimal inhibition for azasugars outlined earlier, the N-glycyl derivative is atypical in its activity. Although the amide bond of **6** is planar, the ring itself is not significantly perturbed; therefore, it is not likely that the improvement in inhibition is due to flattening of the ring. It is possible that the carbonyl group is interacting via π–π stacking as designed. Also, the basic nitrogen on the side chain of **6** retains the potential for protonation or hydrogen bonding but is not in one of the optimal positions to take advantage of this interaction. It is possible that the protonated nitrogen is interacting with the enzyme aromatic pocket identified in our NMR studies via cation–π interactions. It is also possible that long-range ionic interactions are involved. Although compound **6** is only moderately more effective than **1b**, the improvement in inhibition indicates new directions for further improvement of deoxynojirimycin derivatives. An additional feature of this new class of inhibitors is the ready availability of analogs via peptide coupling of **4** to alternate amino acids. A combinatorial chemistry approach could be utilized to expand the SAR of analogs of **6**.

Our results indicate that useful information about the binding site of glycosidases can be obtained using isotopically labeled *N*-alkyl deoxynojirimycin derivatives. Our studies led to the design of a novel glucosidase inhibitor. Isotope-edited NMR studies probing molecular recognition features in mammalian glucosidases are currently underway and will be coupled with further studies of N-peptidyl and other deoxynojirimycin derivatives to discover potent, specific inhibitors for the treatment of diabetes and HIV.

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15. The K_i for deoxynojirimycin also differed from the literature value of 8.7×10^{-3} M[12] with a $K_i = 5.1 \times 10^{-3}$ M for synthetic **1a** and 8.0×10^{-3} M for commercial **1a** (Fluka Chemical).
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21. Enzyme was dialyzed against 0.1 M sodium phosphate buffer at pH 6.55 for 24 h at 4 °C. Solvent was exchanged with D_2O via freeze/lyophilize cycles. Final NMR concentrations were 3.5×10^{-4} M enzyme and 3.6×10^{-4} M in inhibitor. Chemical shifts were referenced to 3-(trimethylsilyl-)1-propanesulfonic acid, sodium salt (DSS).
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23. An ω1 1/2X-filtered NOESY[23] with refocusing delay and decoupling (during both t_1 and t_2) was used. 2-D data were recorded on a Bruker DMX 600 in the phase sensitive mode using the TPPI method with irradiation of the residual HDO peak during the relaxation delay and mixing time. Repetition delay was 2 s with 128 scans averaged for each of 256 FIDs of 2K complex data points with a 6009 Hz sweep width. The tuning delay 1/(2J) was set for $J = 140$ Hz (the $^1J_{CH}$ of methyl group on **1b** where the methyl carbon is ^{13}C) with a 200 ms mixing time. Using FELIX (Biosym/Molecular Simulations Inc.) data were zero-filled to 512 points in t_1 and apodized using phase-shifted skewed sinebells (90° shift, 0.7 skew) in both dimensions.
24. Data not shown. These NOEs are identical to those seen with labeled inhibitor alone under the same buffer and NMR conditions. H6/6' are unresolved at pH 6.5.
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