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Structural Investigation of the Binding of 5-Substituted Swainsonine Analogues to Golgi α-Mannosidase II

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Dedicated to the memory of Warren Delano (1972–2009).

Golgi α-mannosidase II (GMII) is a key enzyme in the N-glycosylation pathway and is a potential target for cancer chemotherapy. The natural product swainsonine is a potent inhibitor of GMII. In this paper we characterize the binding of 5α-substituted swainsonine analogues to the soluble catalytic domain of *Drosophila* GMII by X-ray crystallography. These inhibitors enjoy an advantage over previously reported GMII inhibitors in that they did not significantly decrease the inhibitory potential of the swainsonine head-group. The phenyl groups of these analogues occupy a portion of the binding site not previously seen to be populated with either substrate analogues or other inhibitors and they form novel hydrophobic interactions. They displace a well-organized water cluster, but the presence of a C(10) carbonyl allows the reestablishment of important hydrogen bonds. Already approximately tenfold more active against the Golgi enzyme than the lysosomal enzyme, these inhibitors offer the potential of being extended into the N-acetylglucosamine binding site of GMII for the creation of even more potent and selective GMII inhibitors.

Introduction

The glycosylation profile on the surface of a tumor cell correlates with its metastatic potential,[1] and control of aberrant glycosylation offers potential for chemotherapy.[2] The enzyme Golgi α-mannosidase II (GMII, E.C.3.2.1.114) is a promising target for intervention in the glycosylation process. Swainsonine, one of the most potent inhibitors of GMII yet discovered, and related pyrrolidines offer promising antitumor activity;[3] however, unwanted swainsonine co-inhibition of the lysosomal α-mannosidase (LM, E.C.3.2.1.24) has resulted in a search for highly potent inhibitors that are more selective for GMII.

The α(1–3)/α(1–6)-mannosidases, including GMII and LM are members of the family 38 glycoside hydrolases in the CAZy classification system (http://www.cazy.org). These enzymes cleave the bond between two mannose residues, by sequential cleavage in a single active site.[4] The cleavage mechanism involves formation of a covalent glycosyl-enzyme intermediate and results in net retention of configuration.[5]

GMII is involved in the creation of glycoproteins that contain complex carbohydrates. It is responsible for the formation of the "core" trimannose structure to which all complex carbohydrates are appended. It catalyses the hydrolysis of an α(1–6)- and an α(1–3)-linked mannose from GlcNAc-Man5-GlcNAc2 (which is N-linked to an asparagine residue) to form GlcNAc-Man3-GlcNAc2-Asn-X.[6]

Lysosomal α-mannosidases, in contrast, are involved in the degradation of complex sugars derived from glycoproteins. If LM activity is impaired, either chemically or through mutation, there is buildup of partially processed oligosaccharides in large vacuolar structures within cells; this can have severe neurological consequences.[7]

Branched complex-type carbohydrates have been observed to increase following malignant transformation and appear to play a role in metastasis. Treating murine tumor cells with the plant derived indolizidine alkaloid swainsonine (Table 1) results in cells that are less metastatic.[8] At the same time swainsonine has positive effects on cellular immunity, and alleviates tumor-associated immune suppression and increases bone marrow cell proliferation.[8] Despite these positive aspects, the potential for neurological damage resulting from lysosomal mannosidase inhibition has spurred the search for new, more GMII-specific inhibitors.

Placing substituent groups on swainsonine at all positions save the C(3) and C(5) positions (numbering as indicated in

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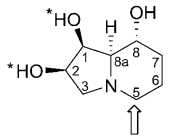
Table 1. IC_{50} and K_i values for swainsonine derivatives in both lysosomal and Golgi mannosidase II. Numbering of the swainsonine compound is shown below. The arrow indicates the position where the substituents are attached. Compounds **2**–**4** and **6** are referred to as α-configured, while **5** and **7** are β-configured. The carbonyl group in **2**–**5** is at C(10).



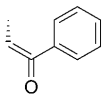
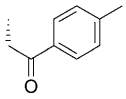
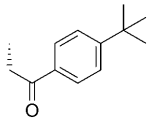
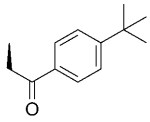
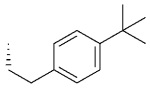
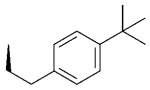
		Lysosomal		Golgi	
		(Jack Bean)	(*Drosophila*)	(*Drosophila*)	(*Drosophila*)
	Substituent at C(5)	IC_{50} [nM]	IC_{50} [nM]	IC_{50} [nM]	K_i [nM]
swainsonine	H	250	220	37	3.0
2		510	n.d.	30	2.8
3		250	310	29	2.7
4		220	340	29	2.7
5		2500	2550	250	n.d.
6		420	930	44	n.d.
7		3700	n.d.	250	n.d.

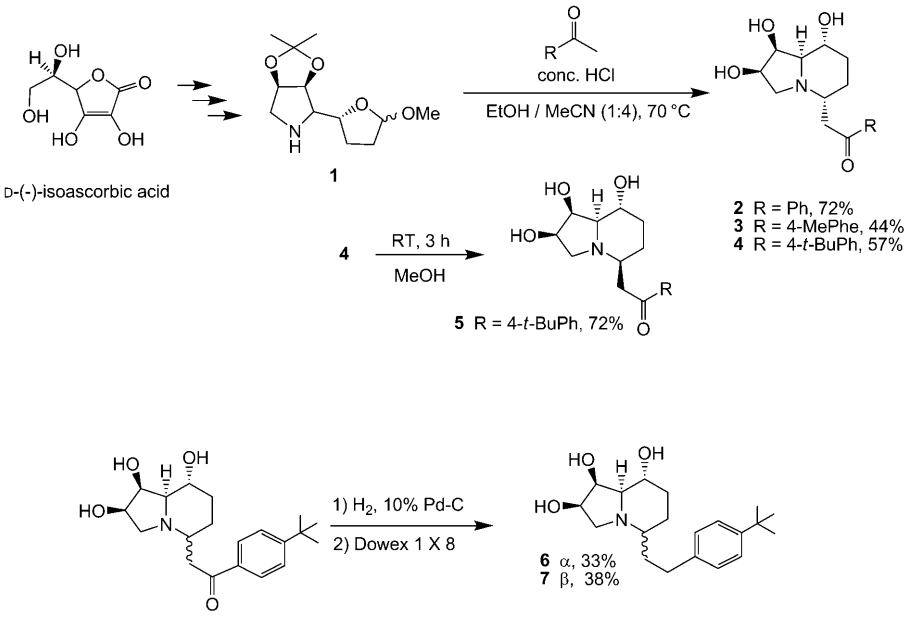
Table 1) results in compounds with a dramatic loss of inhibitory activity. The C(3)-substituted swainsonine analogues[9] were reported to be more active than the parent compound against jack-bean mannosidase (an enzyme with lysosomal-like properties). However, their potency was seen to be considerably worse than that of swainsonine towards GMII from *Drosophila melanogaster* (dGMII) (D.A.K., L. Guo, W. Pearson, D.R.R., unpublished observation). We have previously reported on C(5)-substituted analogues of swainsonine and showed that the inhibitory activity of the (5α)-substituted swainsonine analogues was similar to the activity of swainsonine towards jack-bean mannosidase.[10] As reported here, the (5α)-substituted swainsonine analogues are almost indistinguishable from swainsonine in their ability to inhibit dGMII, and so provide a promising starting point from which to extend swainsonine to exploit other binding groups within the GMII active site. Knowledge of the spatial positioning of and interactions formed with the 5-substituted swainsonine analogues is invaluable in the design of future analogues. We have utilized X-ray crystallography to determine the high-resolution structures of dGMII with both (5α)- and (5β)-substituted swainsonine analogues. The aromatic substituents are found in a region of the active site pocket not occupied in previous dGMII structures. Both the (5α) and (5β) analogues are found in a similar position. A well-organized cluster of water molecules is displaced by the substituents and a water-free region close to the backbone around Arg876 is created.

Results and Discussion

C(5α)-substituted analogues are as potent as swainsonine

A series of C(5)-substituted swainsonine analogues was synthesized from amine acetal **1**, which was obtained from D-(−)-isoascorbic acid through the Mannich reaction[10] as shown in Scheme 1. Their inhibitory potency (IC_{50}) was tested on jack-bean α-mannosidase, *Drosophila* lysosomal mannosidase, and the recombinant catalytic domain of Golgi α-mannosidase II from *Drosophila* (dGMII) (Table 1). For swainsonine and the (5α)-carbonyl-containing analogues **2**–**4**, the IC_{50} values measured under standard conditions against dGMII were virtually identical, ranging from 30–40 nM. These compounds are all poorer inhibitors of the lysosomal enzyme of *Drosophila* or jack bean with IC_{50} values ranging from 220–~500 nM. Under these conditions the selectivity for the Golgi enzyme is thus ~9–17-fold; this selectivity is encouraging but inadequate for the prevention of long-term side effects. The C(5β) analogues were six- to eight-fold worse than the corresponding C(5α) analogues but had similar selectivity (10–15×) in comparison to the jack-bean enzyme.

The standard conditions of the assay to assess IC_{50} were identical to those previously used and utilize commercially available *para*-nitrophenyl α-D-mannopyranoside (*p*NP-mannose) as the substrate with an enzyme concentration of 40 nM for 45 min. At these concentrations the IC_{50} of the swainsonine analogues is similar to the enzyme concentration and it is likely that a considerable proportion of the inhibitor will be bound to the enzyme. Thus, to determine K_i values, reactions were carried out at 25 °C with 80-fold less enzyme (0.5 nM) in the presence of the stabilizing agents, 50 μg mL^{-1} bovine serum albumin, 0.1% Triton X-100 and 10 μM $ZnSO_4$. However, the stabilizing agents were insufficient in preventing loss of activity during the long time course of the reaction that was necessitated by the slow turnover rate with PNP-mannose. 2,4-dinitrophenyl α-D-mannopyranoside (DNP-mannose) proved to be a much better substrate and allowed us to measure the reaction directly within an hour. We used DNP-mannose to de-



Scheme 1. Synthesis of 5-substituted swainsonine analogues.

termine the K_i of the dGMII with the three best inhibitors (**2**–**4**) and with swainsonine. Again, for all these compounds, almost identical inhibition constants were obtained and were in the range of 2.5 to 3 nM (Table 1), the lowest values so far reported with GMII.

The aromatic C(5) substituents occupy similar positions

The binding of the C(5)-substituted compounds to the active site of dGMII was studied by using X-ray diffraction data collected at the CHESS synchrotron. High-resolution data from cocrystals of **2**–**7** with dGMII were obtained, which allowed the visualization of the electron density for the bound compounds. Statistics for data collection and refinement are shown in Table S1 in the Supporting Information. The electron density for the bound compounds is shown in Figures 1 and S1. High-quality, unambiguous density can be seen for compounds **2**–**4**, which are C(5α) substituted and contain a carbonyl group at C(10). For these compounds the F_o–F_c omit map can be visualized clearly when the contour level is set to 6σ. For compounds **5**–**7** the electron density for the complete molecules only becomes clear when the F_o–F_c omit maps are plotted at 3σ. The electron density for the phenyl ring of **6**, which lacks the C(10) carbonyl, is much more poorly resolved than that of **4**, and this indicates the importance of the C(10) carbonyl in anchoring the aromatic moiety. The average temperature factors (B-factors) are also shown for both the swainsonine moiety and the C(5) substituent. The B-factors are an indication of molecular movement, with lower B-factors denoting more rigid atoms. It can be seen in all cases that the B-factors of the multiply hydrogen-bonded swainsonine moiety are lower than those of the aromatic ring, and the aromatic rings of **6** and the C5β analogues have the highest B factors. In the following discussion we will focus on the dGMII:**4** because it had the lowest K_i and the best-defined electron density. It is therefore the best starting point for the design of future compounds.

The dGMII:complex structures were superimposed with the high resolution (1.3 Å) structure of dGMII bound to swainsonine [PDB ID: 3BLB]. The active site amino acids overlap very closely (not shown) as do the bound inhibitors (especially the C(10) carbonyl-containing inhibitors **2**–**4**; Figure 2A). The compounds lacking the C(10)-carbonyl substituent (**6** and **7**) have the largest difference (Figure 2B and C). The presence of the C(10) carbonyl in the β-oriented inhibitor **5** results in an orientation quite similar to that of **4** (Figure 2C). Distances for the interactions made by the compounds are listed in Table S2 and they are all very comparable with the interactions made by swainsonine. The swainsonine moieties of the C(5β)-substituted compounds are displaced slightly from the parent

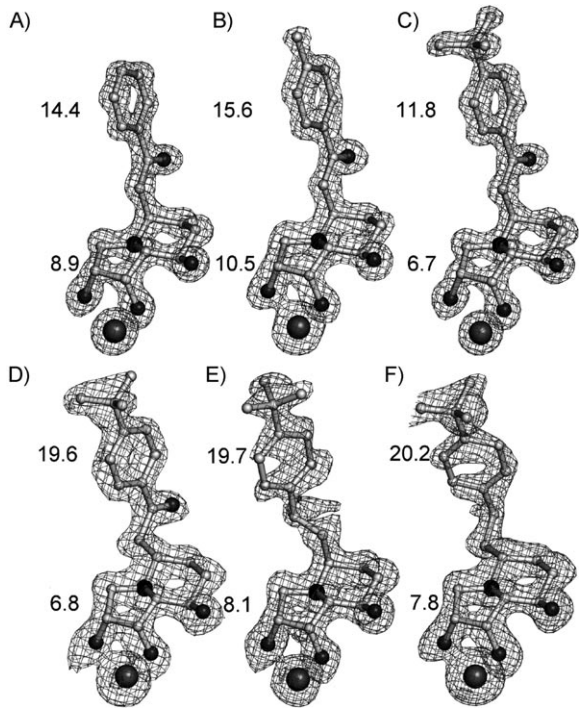


Figure 1. Electron density in the active site of the dGMII complexes. Simulated annealing (to 3000 K) F_o-F_c omit maps were calculated after removal of the compound and zinc from the final model. Electron density is shown contoured at 6σ for A–C, or at 3σ for the poorly bound compounds (D–F). Carbons are colored white, oxygens grey, and nitrogens black. Zinc is shown as a large sphere. Average temperature factors for the swainsonine moiety and the phenyl moiety are indicated to the left of each compound. A) **2**; B) **3**; C) **4**; D) **5**; E) **6**; and F) **7**.

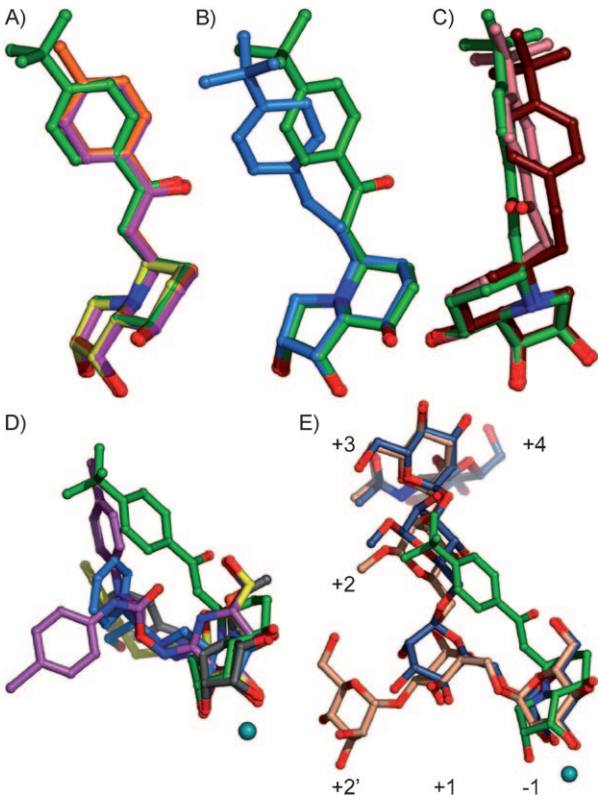


Figure 2. Comparison of bound **4** with new and previously solved bound inhibitors and substrates. Coordinates of the dGMII complexes were superimposed on the dGMII:swainsonine structure. The carbon atoms for **4** are colored green, and the active site zinc is shown as a cyan sphere. A) The binding of **4** is compared with swainsonine (yellow), **2** (magenta), and **3** (orange). B) Comparison of the binding of C(5α) compounds with (**4**) or without (**6**, light blue) the C(10) carbonyl. C) Comparison of C(5α)- (**4**) and C(5β)-containing compounds (**5**, salmon) and (**7**, brown). D) Comparison with benzyl-substituted inhibitors, benzyl-mannostatin (**SK2**, grey), **GHR** (plum, both conformers of the aromatic substituent are shown), **LKS** (yellow), and **GB6** (sky-blue). E) Comparison of **4** with substrate analogues; $GlcNAcMan_5$ (tan), $GlcNAcMan_4$ (dark blue). The sugars are labeled in terms of the various sites to which they bind on GMII; −1 is the cleavage site; the +2′ site or "holding site" contains the α(1–3) linked mannose, which will be cleaved in the second reaction after rotation of the +1 "swivel" sugar; the +4 "anchor" site binds GlcNAc and is absent in lysosomal mannosidases.

compound; this might contribute to the lowered inhibitory potential of these compounds.

The C(5) substituents occupy a position not previously observed to be occupied in other complexes

We next compared the position of the bound 5-substituted swainsonine analogues with other aromatic-substituted inhibitors of dGMII whose binding was characterized by X-ray crystallography.[11–14] Structures and inhibitory activities of these inhibitors are shown in Figure 3.

These previously investigated compounds include benzyl-mannostatin (**SK2**, from PDB ID: 2F7P),[11] an N-aryl carbamate derivative of gluco-hydroxyiminolactam (**GHR**, from PDB ID: 3D52),[12] a benzyl 5-thio-D-mannopyranosylamidinium derivative (**LKS**, from PDB ID: 1R34),[13] and a phenyl-(ethyl)aminomethyl pyrrolidone derivative (**GB6**, from PDB ID: 3DDF).[14] In the case of **SK2**, **GHR**, and **LKS** the addition of the phenyl moiety was markedly detrimental and reduced the inhibitory potency 7, 7.4, and 13-fold respectively in comparison to the parental compounds.[11–13] (There is no unsubstituted "parent" for **GB6**). This was in contrast with the C(5α)-substituted swainsonine analogues studied here, which had similar inhibitory potencies as the parent compound.

The overlay of the four previously solved structures with one of the substituted swainsonine analogue complexes, (dGMII:**4**) is shown in Figure 2D and Figure S5. It can be seen that the phenyl groups of the other compounds are found in a position completely different from what is observed in dGMII:**4**. The aromatic moiety of **GHR** was found in two different locations, one of which more closely resembles that of **4**, but its very weak electron density was in marked contrast to the well-defined position of **4**.

The dGMII:**4** structure was also superposed with solved structures containing bound sugars (Figures 2E and S6). These included synthetic GlcNAc-Man_4 (from PDB ID: 3BVX),[15] and natural derived GlcNAc-Man_5-GlcNAc (from PDB ID: 3CZN).[4] It is clear that the sugars in the +1 position do not match the position of the phenyl group attached to C(5) of swainsonine. In contrast, the other previously investigated phenyl-containing inhibitors reside in a region of the active site that is much closer to that where the +1 sugar is found, as can be seen from Figure 2D.

In the study of other glycosyl hydrolase inhibitors addition of an aromatic group often leads to dramatically improved affinity, as stacking interactions are important at the +1 (aglycone) site (some examples are given in ref. [16]). In contrast, from the results presented here and previously, it seems that in the case of GMII the +1 site is not a good target. We have previously shown that the +1 sugar is not tightly bound.[15] This is a reflection of the mechanism of GMII cleavage in which the α(1–3) linked mannose has to swing around the +1 sugar (the "swivel" position) in order to reposition itself in the cleav-

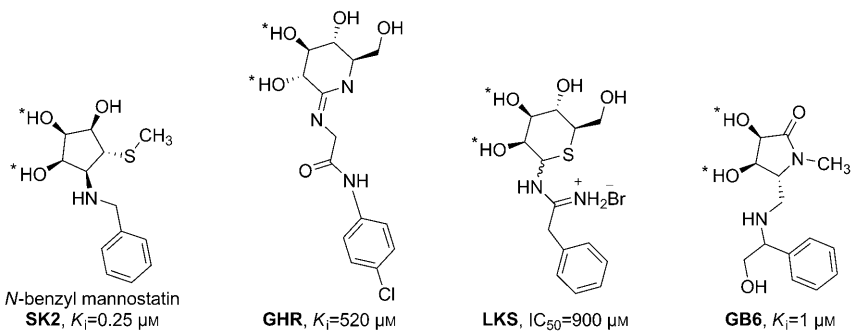


Figure 3. Previously studied compounds discussed in this paper. Compounds are identified by their PDB ligand code, and their inhibitory activity against dGMII is indicated.

age pocket following the initial cleavage of the α(1–6)-linked mannose.[4,15,17] Thus, lack of interacting residues at the +1 position supports the observation that aromatic groups that target this region do not demonstrate improved inhibitory properties. The synthetic C(5) analogues produced here target a unique region of GMII that would not be readily obvious from modeling studies based on previous inhibitor or substrate structures and appear to derive binding energy from previously unobserved interactions.

Both polar and hydrophobic interactions mediate binding

Figure 4A and B illustrate the principal polar and hydrophobic interactions, respectively, made between dGMII and **4**. Stereoviews of the interactions are given in Figures S2 and S3. Of particular importance for binding of the swainsonine moiety are the polar interaction between the positively charged nitrogen (N4) and the OD2 oxygen of the catalytic nucleophile Asp204, interactions of the O1 and O2 to the active site zinc and aspartates (Asp92, Asp204, Asp472), and the hydrogen-bonding network at O8 that involves Trp95 NE1, Asp472 OD1, and Try727 OH. A new interaction not found in swainsonine is between the C(10) carbonyl oxygen and Arg228, both directly and through an intermediate water. Arg228 in turn interacts with the catalytic nucleophile Asp204. The OD1 of Asp204 is 2.9 Å from both the NH2 and NE atoms of Arg228. Table S2 compares the polar interaction distances observed in the complexes of the six compounds presented here with those seen in the dGMII:swainsonine structure. No significant differences are seen except in the O10:Arg228 interaction.

Hydrophobic interactions seem to play an important role in the binding of the swainsonine analogues, as there are numerous close contacts (<4 Å) between aromatic groups in the active site of dGMII and the rings of the inhibitor (Figures 4B and S3). The close contacts (3.5–3.6 Å) with Phe206 should be particularly strong. Of particular note is a new stacking interaction between the phenyl ring of **4** with the backbone of the protein at Arg876 with distances of 3.3–4 Å (see below).

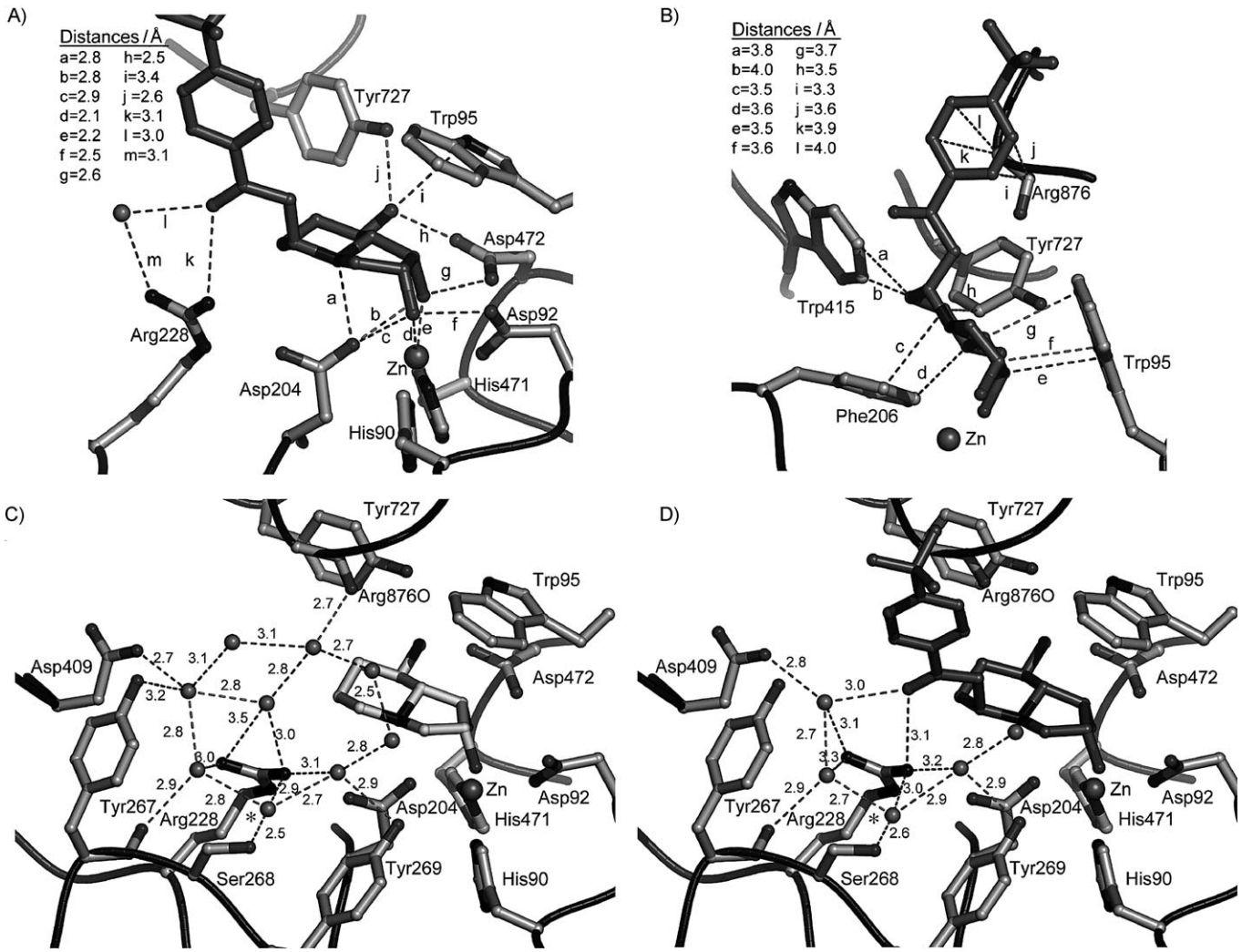


Figure 4. Binding of **4** in the active site of dGMII. A) Polar interactions. Distances [Å] are indicated in the top left corner. B) Hydrophobic interactions. C) and D). A water cluster that occurs in the dGMII:swainsonine structure (PDB ID: 3BLB) is disrupted on binding of **4** . Waters are shown as small spheres. The asterisk denotes the position of a water molecule missing in the complex with **5**–**7** (see text).

The C(5) substituents displace a well defined water cluster

Figure 4C and D provide a side-by-side comparison of swainsonine and the C(5α) analogue **4** bound in the active site of dGMII. A well defined water structure that occurs in the dGMII:swainsonine structure (PDB ID: 3BLB), bridging the backbone carbonyl of Arg876 (Arg876O) and Arg228 (Figures 4C and S4A), is displaced by the binding of the phenyl ring. Five water molecules are displaced, including a central low B factor water at the center of this cluster. Importantly, in the complexes formed with the tight inhibitors (**2**, **3** and **4**) this key central water is replaced by the C(10)-carbonyl group, which re-establishes hydrogen bonds with Arg228 and results in virtually unchanged geometry in this region (shown with dGMII:**4** in Figures 4D and S4B).

We have previously shown[18] that removal of another conserved water molecule in the active site (indicated by an asterisk in Figure 4C and D) caused by the displacement of Arg228 results in considerable loss of inhibitor potency (PDB ID: 2OW6). In that case addition of a single hydroxyl group to 8-*epi*-thiolentiginosine changed the IC_{50} from 14 μM to 2000 μM.[18] We had not previously observed this Arg228-mediated loss of water in other dGMII:inhibitor complexes, and this water is present in the complexes with **2**–**4**. Interestingly, in the complexes of dGMII with the three least potent inhibitors (**5**–**7**) Arg228 is displaced to a significant extent and the critical water is missing in the electron density (results not shown). In the case of the β-substituted noncarbonyl-containing **7** the movement of Arg228 is even greater (by 0.5 Å) than was observed in PDB: 2OW6.[18]

The C(5)α substituents form new hydrophobic interactions with Arg876

The backbone carbonyl of Arg876 (Arg876O) has been shown to form hydrogen bonds with the C(6)OH of the natural substrate as well as many inhibitors including **LKS**, **GB6**, and **GHR**.[4,13–15,18] Further, in a study of mannostatin analogues, interaction with the Arg876O appeared to be a key component.[19] In that study, removal of the thiomethyl group of mannostatin A (or a hydroxyl group in an analogue) to which Arg876O formed hydrogen bonds, resulted in a greater than 1000-fold reduction in inhibitor potency even though other interactions remained the same.[19] In contrast, Arg876O does not seem to contribute greatly to the potency of swainsonine, the binding of which appears to be facilitated by the considerable number of hydrophobic interactions with the region of the cleavage pocket formed by Trp95, Phe206, Trp415, and Tyr727. In the binding of **4**, the displacement of the water cluster by the C(5) substituent results in the formation of a new hydrophobic region, and the phenyl ring stacks against the backbone of the protein chain around Arg876O, creating new, additional, hydrophobic interactions (Figures 4B, D and S3).

Implications for inhibitor design: Bridging the gap between the C5 substituents and the GlcNAc binding site

An important consideration in the creation of new Golgi α-mannosidase inhibitors is the selectivity that they show for the Golgi enzyme in comparison to their ability to inhibit the lysosomal enzyme. The effective small molecule inhibitors such as swainsonine and mannostatin, as well as the C(5)-substituted swainsonine analogues studied here, bind to residues in the −1 site that are highly conserved between the lysosomal and Golgi enzymes, and thus the limited amount of selectivity (ca. tenfold) exhibited by these compounds is not surprising. GMII is dependent on the prior action of *N*-acetylglucosaminyltransferase I (GnT1), which adds GlcNAc to $Man_5GlcNAc_2$ to create $GlcNAcMan_5GlcNAc_2$ the substrate of GMII.[6] Lysosomal mannosidase, in contrast, cleaves the Man5 substrate but not the GlcNAc-modified one.[15,20] Our previous structural work[4,15] has shown the position of the GlcNAc binding site in GMII (the +4 site) is located within a loop of the protein that is absent in the structure of the lysosomal enzyme.[21] In GMII GlcNAc forms an important stacking interaction with Tyr267 and makes other polar interactions. GlcNAc bound to the +4 site is quite well-ordered in the electron density, and its presence on the mannose-containing substrates completely alters their binding pattern in the active site.[4,15] The GlcNAc binding site thus presents an attractive region to target for developing highly selective inhibitors.

Ideally the distance between the −1 site and GlcNAc site could be spanned without compromising the binding affinity to the −1 site. The C(5α)-substituted analogues presented here are a big step towards this goal as they extend the inhibitor from the −1 site without significant loss of activity. In the superposed structures, the terminal methyl group of **4** is only 0.7 Å away from C2-OH of the mannose bound in the +2 site of native-sugar complex (PDB:3CZN). A superposition of **4** with the terminal residues GlcNAc-Man_4 (from PDB:3BVX) is shown in Figures 5 and S7. In this case the branching carbon (C17) is 0.9 Å away from the O5 of the +2 sugar, while the terminal methyl (C20) is 1.5 Å from C1. It is easy to imagine extension of the C5 analogues by addition of a short linker, a mannose and GlcNAc to create an inhibitor with the desired properties of potency and selectivity. We have noted previously that the +2 sugar-binding site is quite weak and does not seem to contribute significantly to the substrate binding. The replacement of the +2 mannose by a very short linker to the +3 mannose should have no detrimental effect on the binding. The +3 mannose is slightly better defined in the electron density but does not appear to make many contacts with the enzyme. It should also be possible to replace this with an extended acyl chain in a novel multisite inhibitor.

Conclusions

We have determined the mode of binding of a new class of substituted swainsonine analogues, the phenyl substituents of which displace a well-defined water cluster and form new stacking interactions with the backbone of GMII. This previous-

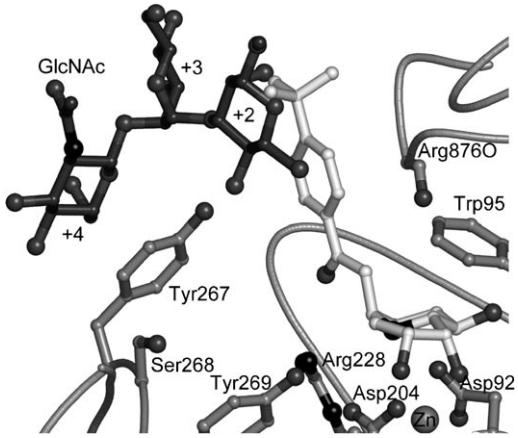


Figure 5. Extension of **4** should allow spanning of the catalytic site and the specificity determining GlcNAc-binding site. The dGMII:**4** (light grey) structure was superposed with the dGMII:$GlcNAcMan_4$ structure from PDB: 3BVX. Only the terminal sugars of $GlcNAcMan_4$ are shown (dark grey). Tyr267 forms an important stacking interaction with GlcNAc.

ly unobserved binding mode provides a direct pathway between the cleavage site and specificity determining GlcNAc-binding site, and could lead to the synthesis of selective GMII inhibitors.

Experimental Section

Substrates and inhibitors: PNP-mannose was purchased from Sigma, and DNP-mannose was a kind gift of Steve Withers (University of British Columbia). Swainsonine was purchased from Toronto Research Chemicals (Toronto, ON). Stock concentrations of inhibitors were made up in DMSO and stored at −70 °C.

Chemistry: All reactions were conducted under an atmosphere of dry nitrogen. Thin-layer chromatography (TLC) was carried out on Merck silica gel 60 F_{254} plates and detected by UV light, iodine vapor, and phosphomolybdic acid solution. Flash column chromatography separations were carried out by using Merck Kieselgel 60 silica gel (0.063–0.200 mm) or Kanto Chemical silica gel 60 N (40–50 μm, spherical, neutral). Reagents and solvents were purchased from common suppliers. ^{1}H NMR and ^{13}C NMR spectra were recorded on a JNM-EX-400 (400 MHz) spectrometer. Chemical shifts (δ, ppm) were determined by using TMS as internal standard. MS spectra were recorded on JEOL JMS-AX-500 and JEOL JMS-DX-303 mass spectrometers. Elemental analysis for C, H, and N were performed on a Yanaco CHN CORDER MT-5 analyzer (Kyoto, Japan). High-performance liquid chromatography (HPLC) was performed on Shiseido fine chemicals C_{18} column (Tokyo, Japan) by using a JASCO PU-1586 pump equipped with a UV/Vis detector JASCO UV-1570.

General synthetic procedure for swainsonine analogues via the Mannich reaction: The 5-substituted swainsonine analogues **2**–**4** are synthesized by the Mannich reaction of amine acetal **1** with the corresponding ketone according to the method reported previously.[10] Briefly, a solution of the amine acetal **1** (10 mg, 0.04 mmol) in EtOH/acetonitrile (1:4; 500 μL) was added to a solution of EtOH/acetonitrile (1:4, 500 μL) containing excess ketone (250–500 μL) and a few drops of conc. HCl. The mixture was stirred for 3 h under reflux. After the reaction, the mixture was evaporated and dissolved in water. The aqueous phase was washed with Et_2O and lyophilized. The residue was purified by preparative reversed-phase HPLC (C18, acetonitrile/water gradient elution system). The solution of **4** (29.7 mg, 0.086 mmol) in MeOH (6 mL) was stirred at room temperature for 3 h and then evaporated. The residue was purified by reversed-phase HPLC (C18, acetonitrile/water gradient elution system) to afford **5** (21.4 mg, 72%).

(1*S*,2*R*,5*S*,8*R*,8a*R*)-5-(2-(4-*tert*-Butylphenyl)ethyl]octahydroindolizine-1,2,8-triol [(5*S*)-5-(4-*tert*-butylphenethyl)-swainsonine] (6): 10% Pd-C (10 mg, 26 mol%) was added to a solution of **4** (24.2 mg, 0.070 mmol) in ethanolic HCl (2.6 mL of 50 mM). The flask was evacuated by aspirator and purged with hydrogen three times. The reaction mixture was heated at 50 °C. After 22 h, the hydrogen was evacuated and the mixture was filtered through Celite and rinsed with MeOH. The solution was then concentrated. The residue was applied to an ion exchange column (Dowex 1X8 200 OH^-), which was eluted with water. The solution was freeze-dried. The residue was purified by using reversed-phase HPLC (C18, acetonitrile/water gradient elution system) to afford **6** (8.1 mg, 33%) as a white solid.: ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.93$ (m, 2H), 7.56 (m, 2H), 4.17 (m, 2H), 3.80 (m, 1H), 3.60 (m, 1H), 3.04 (dd, $J = 8.2$, 15.5 Hz, 1H), 2.87 (m, 1H), 2.75 (dd, $J = 3.5$, 10.4 Hz, 1H), 2.45 (m, 1H), 1.79 (m, 2H), 1.52 (m, 2H), 1.34 (s, 9H), 1.34 (m, 1H); IR (KBr): 3415, 2950, 2864, 1513, 1459, 1130, 1087, 1032, 838 cm^{-1}; EI-HRMS *m/z* calcd for $C_{20}H_{31}NO_3$ $[M]^+$ 333.2304, found 333.2307.

(1*S*,2*R*,5*R*,8*R*,8a*R*)-5-(2-(4-*tert*-Butylphenyl)ethyl]octahydroindolizine-1,2,8-triol [(5*R*)-5-(4-*tert*-butylphenethyl)-swainsonine] (7)): 10% Pd-C (9.0 mg, 26 mol%) was added to a solution of **5** (21.4 mg, 0.062 mmol) in ethanolic HCl (50 mM, 3.8 mL). Similarly, in a hydrogen atmosphere, the reaction mixture was heated at 50 °C for 9 h. After the reaction, the reaction solution was treated in the same way as mentioned above to afford **7** (7.1 mg, 38%) as a white solid. ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.29$ (d, $J = 8.3$, 2H), 7.09 (d, $J = 8.3$ Hz, 2H), 4.18 (m, 2H), 3.79 (m, 1H), 3.05 (dd, $J = 1.7$, 10.2 Hz, 1H), 2.69 (m, 1H), 248 (m, 1H), 2.38 (m, 1H), 2.06 (m, 1H), 1.89 (m, 3H), 1.80 (m, 1H), 1.57 (m, 1H), 1.29 (s, 9H), 1.27 (m, 2H); IR (KBr): 3369, 2939, 2864, 2818, 2776, 1518, 1457, 1368, 1131, 1082, 1040, 823 cm^{-1} ; EI-HRMS *m/z* calcd for $C_{20}H_{31}NO_3$ $[M]^+$ 333.2304, found 333.2308.

Enzymes: Jack-bean α-mannosidase was purchased from Sigma. Recombinant *Drosophila* lysosomal α-mannosidase (CG6206 gene product) was purified as described previously.[12] dGMII purification was performed essentially as described previously[17] with an added anion-exchange step to further purify the enzyme. Briefly, the Golgi lumenal located catalytic domain of *Drosophila* GMII (residues 76–1108) containing an N-terminal His_6 tag was expressed in a secreted form in *Drosophila* S2 cells. The medium was batch bound to Blue F3GA agarose (Sigma). dGMII was eluted with NaCl (0.35 M) and directly batch bound to Ni-NTA agarose (Qiagen) from which it was eluted with imidazole (30 mM). After dialysis the protein was further purified by salt elution from a MonoQ anion-exchange column (GE Biosciences), prior to buffer exchange, concentration, and freezing in liquid N_2.

Inhibition assays: Inhibition assays with jack-bean mannosidase were performed at pH 5 in acetate buffer essentially as outlined by Dennis et al.,[8] while assays on *Drosophila* lysosomal mannosidase were carried out at pH 4.5. Inhibition of dGMII was measured at pH 5.75.[13] Determination of the IC_{50} values (concentrations of inhibitor at which 50% of activity remains) was carried out with *p*NP-mannose (4 mM) and enzyme (40 nM) as described previously.[22]

The K_i values for the tight binding inhibitors (swainsonine and **2**–**4**) were assessed in microtiter plates with dGMII (0.5 nM) in a buffer

containing MES (40 mM, pH 5.75), bovine serum albumin (50 $\mu g\,mL^{-1}$), zinc (10 μM), Triton X100 (0.1%), phosphate (1 mM), NaCl (10 mM), and DMSO (10%). Final reaction volume was 80 μL and the reaction was carried out at 25°C. Due to the low enzyme concentration, DNP-mannose was used as a substrate because it has a much faster turnover rate than PNP-mannose. The reaction was followed directly by monitoring the change in absorption at 405 nm in a microplate reader. DNP-mannose concentrations ranged from 2–30 mM, and inhibitor concentrations ranged from 1–15 nM. The program GraFit 4.0.21 (Erithacus Software, Horley, UK) was used to calculate apparent K_i and standard deviations. This program employs nonlinear fitting of the data for each inhibitor concentration to the Michaelis–Menten equation.

Structure determination: Crystallization, data collection, and refinement of the complexes were essentially performed as outlined in detail previously.[14,15] All crystals were prepared as cocrystals and all data was collected on beamlines A1 and F1 at the Cornell High Energy Synchrotron Source (CHESS, Ithaca, NY, USA). Model building was carried out in Coot.[23] Final refinement was with Refmac5[24] as implemented in CCP4.[25] Simulated annealing omit maps were generated in CNS.[26] Both zinc and the inhibitor were removed from the final model, which was subject to simulated annealing to 3000 K. Graphics were generated with Molscript[27] and Conscript[28] and rendered with Pymol.[29]

For the comparisons of substrate-binding positions, each of the independently refined structures was superposed to the 1.3 Å structure of swainsonine bound to dGMII (PDB ID: 3BLB; the reference structure). To prevent bias, an overlay of the Cα backbone of the entire protein was used as the fitting criteria. The superposition was carried out by using the SSM subroutine[30] of Coot and the coordinates of the inhibitors extracted.

Structures and structure factors were deposited in the Protein Data Bank with the accession numbers 3EJP, 3EJQ, 3EJR, 3EJT, 3EJU, 3EJS for the complexes of dGMII with **2**–**7**, respectively.

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[1] a) R. J. Bernacki, M. J. Niedbala, W. Korytnyk, *Cancer Metastasis Rev.* **1985**, *4*, 81–101; b) J. W. Dennis, M. Granovsky, C. E. Warren, *Biochim. Biophys. Acta Gen. Subj.* **1999**, *1473*, 21–34; c) S. Hakomori, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 10231–10233.

[2] D. H. Dube, C. R. Bertozzi, *Nat. Rev. Drug Discovery* **2005**, *4*, 477–488.

[3] a) P. E. Goss, C. L. Reid, D. Bailey, J. W. Dennis, *Clin. Cancer Res.* **1997**, *3*, 1077–1086; b) H. Fiaux, F. Popowycz, S. Favre, C. Schuetz, P. Vogel, S. Gerber-Lemaire, L. Juillerat-Jeanneret, *J. Med. Chem.* **2005**, *48*, 4237–4246; c) S. Favre, H. Fiaux, C. Schuetz, P. Vogel, L. Juillerat-Jeanneret, S. Gerber-Lemaire, *Heterocycles* **2006**, *69*, 179–192.

[4] N. Shah, D. A. Kuntz, D. R. Rose, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 9570–9575.

[5] S. Numao, D. A. Kuntz, S. G. Withers, D. R. Rose, *J. Biol. Chem.* **2003**, *278*, 48074–48083.

[6] N. Harpaz, H. Schachter, *J. Biol. Chem.* **1980**, *255*, 4894–4902.

[7] a) H. P. Broquist, *Annu. Rev. Nutr.* **1985**, *5*, 391–409; b) D. R. Tulsiani, H. P. Broquist, L. F. James, O. Touster, *Arch. Biochem. Biophys.* **1988**, *264*, 607–617; c) A. C. Crawley, S. U. Walkley, *J. Neuropathol. Exp. Neurol.* **2007**, *66*, 687–697.

[8] J. W. Dennis, S. L. White, A. M. Freer, D. Dime, *Biochem. Pharmacol.* **1993**, *46*, 1459–1466.

[9] W. H. Pearson, L. Guo, *Tetrahedron Lett.* **2001**, *42*, 8267–8271.

[10] T. Fujita, H. Nagaswa, Y. Uto, T. Hashimoto, Y. Asakawa, H. Hori, *Org. Lett.* **2004**, *6*, 827–830.

[11] S. P. Kawatkar, D. A. Kuntz, R. J. Woods, D. R. Rose, G. J. Boons, *J. Am. Chem. Soc.* **2006**, *128*, 8310–8319.

[12] D. A. Kuntz, C. A. Tarling, S. G. Withers, D. R. Rose, *Biochemistry* **2008**, *47*, 10058–10068.

[13] L. M. Kavlekar, D. A. Kuntz, X. Wen, B. D. Johnston, B. Svensson, D. R. Rose, B. M. Pinto, *Tetrahedron: Asymmetry* **2005**, *16*, 1035–1046.

[14] H. Fiaux, D. A. Kuntz, R. C. Janzer, S. Gerber-Lemaire, D. R. Rose, L. Juillerat-Jeannere, *Bioorg. Med. Chem.* **2008**, *16*, 7337–7346.

[15] W. Zhong, D. A. Kuntz, B. Ember, H. Singh, K. W. Moremen, D. R. Rose, G.-J. Boons, *J. Am. Chem. Soc.* **2008**, *130*, 8975–8983.

[16] a) T. Granier, N. Panday, A. Vasella, *Helv. Chim. Acta* **1997**, *80*, 979–987; b) L. Gartenmann Dickson, L. Emmanuel, J.-L. Reymond, *Org. Biomol. Chem.* **2004**, *2*, 1217–1226; c) T. M. Gloster, J. M. Macdonald, C. A. Tarling, R. V. Stick, S. G. Withers, G. J. Davies, *J. Biol. Chem.* **2004**, *279*, 49236–49242; d) H. C. Dorfmueller, V. S. Borodkin, M. Schimpl, S. M. Shepherd, N. A. Shpiro, D. M. van Aalten, *J. Am. Chem. Soc.* **2006**, *128*, 16484–16485.

[17] J. M. H. van den Elsen, D. A. Kuntz, D. R. Rose, *EMBO J.* **2001**, *20*, 3008-3017,.

[18] N. S. Kumar, D. A. Kuntz, X. Wen, B. M. Pinto, D. R. Rose, *Proteins Struct. Funct. Bioinf.* **2008**, *71*, 1484–1496.

[19] D. A. Kuntz, W. Zhong, J. Guo, D. R. Rose, G.-J. Boons, *ChemBioChem* **2009**, *10*, 268–277.

[20] C. Park, L. Meng, L. H. Stanton, R. E. Collins, S. W. Mast, Yi, H. Strachan, K. W. Moremen, *J. Biol. Chem.* **2005**, *280*, 37204–37216.

[21] P. Heikinheimo, R. Helland, H.-K. S. Leiros, I. Leiros, S. Karlsen, G. Evjen, R. Ravelli, G. Schoehn, R. Ruigrok, O. K. Tollersrud, S. McSweeney, E. Hough, *J. Mol. Biol.* **2003**, *327*, 631–644.

[22] D. A. Kuntz, A. Ghavami, B. D. Johnston, B. M. Pinto, D. R. Rose, *Tetrahedron: Asymmetry* **2005**, *16*, 25–32.

[23] P. Emsley, K. Cowtan, *Acta Crystallogr. Sect. D Biol. Crystallogr.* **2004**, *60*, 2126–2132.

[24] G. N. Murshudov, A. A. Vagin, E. J. Dodson, *Acta Crystallogr. Sect. D Biol. Crystallogr.* **1997**, *53*, 240–255.

[25] Collaborative Computational Project, Number 4, *Acta Crystallogr. Sect. D Biol. Crystallogr.* **1994**, *50*, 760–763.

[26] A. T. Brünger, P. D. Adams, G. M. Clore, W. L. DeLano, P. Gros, R. W. Grosse-Kunstleve, J.-S. Jiang, J. Kuszewski, M. Nilges, N. S. Pannu, R. J. Read, L. M. Rice, T. Simonson, G. L. Warren, *Acta Crystallogr. Sect. D Biol. Crystallogr.* **1998**, *54*, 905–921.

[27] P. J. Kraulis, *J. Appl. Crystallogr.* **1991**, *24*, 946–960.

[28] M. C. Lawrence, P. Bouke, *J. Appl. Crystallogr.* **2000**, *33*, 990–991.

[29] W. L. DeLano, *The PyMol Molecular Graphics System*, DeLano Scientific, Palo Alto, CA, **2008**.

[30] E. Krissinel, K. Henrick, *Acta Crystallogr. Sect. D Biol. Crystallogr.* **2004**, *60*, 2256–2268.

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