Inhibition of Golgi Mannosidase II with Mannostatin A Analogues: Synthesis, Biological Evaluation, and Structure–Activity Relationship Studies

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Mannostatin and aminocyclopentitetrol analogues with various substitutions at the amino function were synthesized. These compounds were tested as inhibitors of human Golgi and lysosomal α-mannosidases. Modification of the amine of mannostatin had only marginal effects, whereas similar modifications of aminocyclopentitetrol led to significantly improved inhibitors. Ab initio calculations and molecular docking studies were employed to rationalize the results. It was found that mannostatin and aminocyclopentitretrol could bind to Golgi α-mannosidase II in a similar mode to that of the known inhibitor swainsonine. However, due to the flexibility of the five-membered rings of these compounds, additional low-energy binding modes could be adopted. These binding modes may be relevant for the improved activities of the benzyl-substituted compounds. The thiomethyl moiety of mannostatin was predicted to make favorable hydrophobic interactions with Arg228 and Tyr727 that would possibly account for its greater inhibitory activity.

Introduction

Cells that have undergone oncogenic transformation often display abnormal cell-surface oligosaccharides and these changes in glycosylation are important determinants of the stage, direction, and fate of tumor progression.[1] Inhibition of the mannose-trimming enzyme human Golgi α-mannosidase II (HGMII), which acts late in the *N*-glycan processing pathway, is one method of blocking the oncogene-induced changes in cell-surface oligosaccharide structures.[2]

HGMII selectively cleaves α1–3 and α1–6 mannosyl residues present in the natural substrate $GlcNAcMan_5GlcNAc_2$.[3] It is a retaining glycosylhydrolase, which employs a two-stage mechanism involving two carboxylic acids positioned within the active site to act in concert: one as a catalytic nucleophile and the other as a general acid/base catalyst.[4–8] Protonation of the exocyclic glycosyl oxygen atom of the substrate leads to bond breaking and simultaneous attack of the catalytic nucleophile to form a glycosyl enzyme intermediate. Subsequent hydrolysis of the covalent intermediate by a nucleophilic water molecule gives an α-mannose product with overall retention of configuration. Studies on retaining mannosidases with 5-fluoro-substituted pseudosubstrates and deuterium-labeled substrates have shown that the transition states on either side of the covalent intermediate have marked oxocarbenium ion character.[4–8] Potent inhibitors of glycosidases are thought to mimic oxocarbenium-like transition states; for example, the inhibitory activity of the natural product swainsonine has long been attributed to its five-membered ring resembling a flattened six-membered ring that has been forced to attain an oxocarbenium-like structure. In fact, the crystal structure of swainsonine complexed with Golgi α-mannosidase II from *Drosophila* shows the inhibitor to be tilted in such a way as to bring its equivalent of an anomeric carbon atom close to the presumed catalytic nucleophile.[9]

Swainsonine has been much investigated as a consequence of its potent inhibitory properties. Clinical studies have shown[10–13] it to exhibit potent antitumor and antimetastatic activity. An unfortunate side effect resulting from this compound is blockage in oligosaccharide catabolism; this arises from inhibition of a related catabolic α-mannosidase found in lysosomes.[2,13] It is clear that to develop a drug appropriate for antimetastatic therapy, an alternative lead compound is required, one that is amenable to easy modification in a combinatorial manner to give compounds that specifically inhibit HGMII without affecting the function of human lysosomal α-mannosidase (HLM).

Mannostatins A and B, isolated from the soil microorganism *Streptoverticillus*, are the most potent inhibitors of class II α-mannosidases reported[14] thus far and are interesting compounds for combinatorial modification to give more selective derivatives (Scheme 1). They were the first nonazasugar-type

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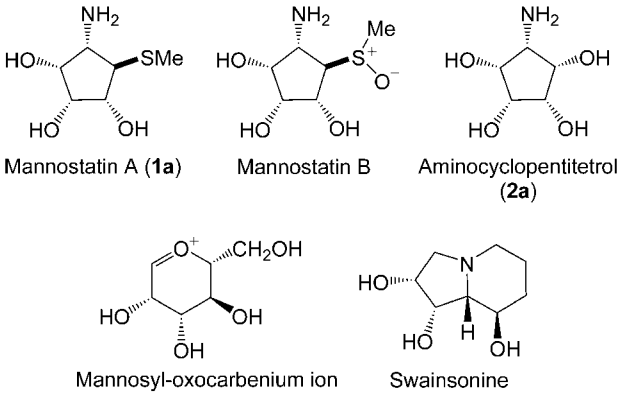


Scheme 1. *Aminocyclopentitol inhibitors of human Golgi mannosidase II.*

inhibitors to be discovered that possess an aminocyclopentitol structure. The inhibitors are of the reversible, competitive type and do not show the slow-binding phenomenon exhibited by swainsonine and its analogues. The synthesis and biological evaluation of a small number of mannostatin analogues has revealed that the basicity of the primary amine and also the neighboring *cis*-diol function are essential for inhibitory activity.[15–21] The thiomethyl moiety could, for example, be replaced by a hydroxymethyl group.[21,22] Most mannostatin derivatives have been tested as inhibitors for Jack-bean and almond mannosidases and structure–activity relationships for the more relevant Golgi and lysosomal enzymes are scarce.

Here we report the synthesis of a range of mannostatin analogues that have various aromatic substituents at the amino function. In parallel, a range of aminocyclopentitetrols, which are structurally simpler than the mannostatins and, as an additional feature, are nonchiral, were modified in a similar fashion. It was hoped that the aromatic substitution on **1a** and **2a** would enable favorable interactions with aromatic residues in the binding site of the mannosidase enzymes. These two families of compounds have been tested for their ability to inhibit HGMII and HLM and the activities of the two series of compounds were compared. Computational studies have been performed to rationalize the data.

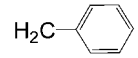
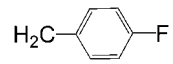
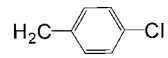
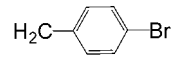
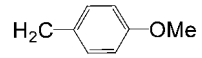
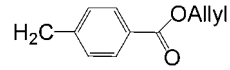
Results and Discussion

Synthesis and biological evaluation of mannosidase inhibitors

Optically pure mannostatin A (**1a**) and the *meso*-aminocyclopentitetrol **2a** were prepared by a modified literature procedure.[23] A key step of this approach involved an aldol condensation of nitromethane with a dialdehyde derived from myo-inositol. The physical and spectroscopic data of the two compounds were in agreement with previously reported data. The amino functionalities of **1a** and **2a** were modified by reductive amination with a range of aromatic aldehydes. Conventional procedures involving $Na(CN)BH_3$ in combination with solvents such as trimethyl *ortho*-formate (TMOF),[24] TMOF/methanol, or dichloromethane/methanol in the presence or absence of benzotriazole[25] resulted in mixtures of products. Alkylation of the amines with benzyl bromide in the presence of CsOH[26] gave, apart from the required product, a substantial amount of disubstituted amine. Fortunately, addition of a methanolic solution of the hydrochloride salts of **1a** and **2a**, pretreated with methanolic sodium hydroxide (0.2 equiv), to a slurry of an aromatic aldehyde (2.5 equiv), $Na(CN)BH_3$ (1.0 equiv), and molecular sieves (3 Å), followed by stirring for 18 hours, gave the secondary amines **1b–g** and **2b–g** in reasonable to good yields.[15] The compounds were purified by Iatro-bead column chromatography and satisfactory compositional and spectroscopic data were obtained in each case.

Recently, we described recombinant forms of human Golgi α-mannosidase II[27] and human lysosomal α-mannosidase.[28] For these enzymes, the rate of hydrolysis of different concentrations of 4-methylumbelliferone alone and in the presence of different concentrations of inhibitor was measured fluorometrically and inhibition constant (K_i) values were determined from Dixon plots. As can be seen in Table 1, mannostatin A (**1a**) is a markedly better inhibitor than the corresponding aminocyclopentitetrol **2a**. Benzylation of the amino functionality of mannostatin A gave compounds **1b–g**, which were slightly less potent inhibitors of the Golgi mannosidase II than the lead compound; *N*-benzyl, *N*-(*p*-fluorobenzyl), *N*-(*p*-chlorobenzyl), *N*-(*p*-bromobenzyl), and *N*-(*p*-methoxybenzyl) substitution caused similar reductions in inhibitory activity (3- to 5-fold). Modification of the amino functionality with allyl 4-hydroxymethylenebenzadehyde (to form **1g**) had a larger impact, with a 15-fold loss of activity being observed. Surprisingly, the chemical modifications to the inhibitors had no significant effect on HLM, a result indicating that they do not lead to either favorable or unfavorable interactions with the binding site.

Table 1. *Inhibition of human Golgi mannosidase II (HGMII) and human lysosomal mannosidase (HLM) by compounds* ***1a–g*** *and* ***2a–g****.*

	R	(**1a–g**) HGMII K_i [μM]	(**1a–g**) HLM K_i [μM]	(**2a–g**) HGMII K_i [μM]	(**2a–g**) HLM K_i [μM]
a	H	0.21	0.09	50	6.6
b		0.88	0.11	10	0.45
c		0.53	0.17	6.0	0.33
d		0.91	0.10	8.1	0.67
e		0.51	0.05	7.6	0.33
f		0.52	0.10	6.6	0.48
g		3.22	0.14	4.4	0.16

The inhibition data for aminocyclopentitetrols **2a**–**g** displayed a different profile than for the mannostatin analogues; for each enzyme, the introduction of a substituted benzyl moiety resulted in an improvement of inhibitory activity. In the case of HGMII, modification of the amino group of **2a** with substituted benzyl moieties led to small improvements in inhibitory activity (5- to 10-fold), whereas large increases in activity (10- to 40-fold) were measured with the lysosomal enzyme. Compound **2g**, with the allyl ester on the *para* position of the benzyl substituent, proved to be the best inhibitor tested; it displayed a 40-fold more favorable K_i value than parent compound **2a**. Surprisingly, this substituent caused the largest loss in inhibitory activity for mannostatin A.

Despite the fact that the introduction of substituted benzyl moieties resulted in less favorable ratios of K_i(HGMII)/K_i(HLM), the compounds described here provide important leads for the development of selective inhibitors of human Golgi mannosidase II. This Golgi enzyme is able to recognize two potential aglycones during cleavage of the substrate GlcNAc-$Man_5GlcNAc_2$ to $GlcNAcMan_3GlcNAc_2$. Since the corresponding alternative substrate, $Man_5GlcNAc_2$, is >1000-fold less effective as a substrate, the Golgi enzyme must have an obligatory binding site for recognition of one branch of the aglycone to enable a high-affinity substrate interaction. There is no data to support the idea that the broad specific lysosomal α-mannosidase is able to recognize an extended aglycone to any significant degree. Thus, by combinatorial extension of the benzyl derivatives with other chemical functionalities, the possibility exists that favorable interactions may be established with the extended binding pocket for the aglycone of HGMII, thereby accentuating the selectivity of this enzyme.

Computational studies

The mode of inhibition by azasugars, such as swainsonine, has been rationalized by their resemblance to the mannosyl oxycarbenium ion, a putative intermediate in the hydrolysis of mannosidases.[29] There has, however, been some debate as to whether this model can be extended to the mode of inhibition of mannostatin (**1a**). It has been contended that in their lowest energy conformations, aminocyclopentitols, such as mannostatin, do not superimpose well onto the hypothetical oxycarbenium ion intermediate.[15,30] An alternative mode of inhibition has been proposed[15] that is based on the resemblance of **1a** to β-mannopyranosylamine.

Recently, crystal structures have been reported for *Drosophila* GMII (DGMII) in the absence and presence of the inhibitor swainsonine.[9] This enzyme has 41% sequence identity and 61% similarity with HGMII and, most importantly, amino acids in catalytic domain are preserved. Furthermore, it has been shown that the two enzymes display similar kinetic and inhibitory properties.[31] Thus, it has been proposed that *Drosophila* GMII is a good model system for the analogous human enzyme.

Molecular docking of mannostatin (**1a**) and aminocyclopentitretrol (**2a**) to the crystal structure of DGMII may provide an opportunity to study the mode of inhibition of these compounds. Furthermore, some of the inhibitory data summarized in Table 1 may be rationalized by comparing the docking modes of the two compounds with those of their benzylated counterparts (**1b** and **2b**).

Five-membered ring systems, such as those in compounds **1a** and **2a**, are inherently flexible due to their ability to assume several twist and envelope conformations, which can interconvert with relative ease through pseudorotational itineraries.[32,33] Therefore, it is necessary to consider all the possible low-energy envelope conformers of **1a** and **2a** for the docking studies.

Conformational properties of isolated five-membered rings such as furanoses have been studied by geometry optimizations of the ten possible envelope conformations through the use of ab initio molecular orbital calculations.[34–36] Thus, the conformational properties of **1a** and **2a** were studied by optimizing the ten possible envelope conformers by constraining a specific endocyclic torsion angle to 0° and allowing all other parameters to be optimized at the B3LYP/6-31G* level. The hydroxy and amino groups of the resulting structures were placed in each of the three staggered orientations, which were then reoptimized without any restraints at the same level of theory. The effects of solvation were approximated by single-point calculations of gas-phase and solution-phase energies with the Poisson–Boltzmann treatment[37,38] in the Jaguar program[39] (Table 2). It was observed that, for both compounds, many hydroxy rotamers converged to a single structure. In the case of **1a**, 30 initial structures led to 20 unique structures, which represented only 6 envelope conformations (E_1, ^1E, E_2, E_3, ^3E, and ^4E). In the case of **2a**, 15 unique structures were obtained, which represented 9 envelope conformations (E_1, ^1E, E_2, ^2E, E_3, ^3E, E_4, ^4E, and E_5). The fact that **2a** can adopt a larger number of envelope conformations indicates that its ring structure is more flexible than that of **1a**. The difference in ring flexibility is probably due to the differences in the ring substitutions, which are known to determine the pseudorotational itineraries of five-membered rings.[33]

Table 2. *Relative gas-phase and solution-phase energies for envelope conformers of mannostain A (**1a**) and aminocyclopentitetrol **2a**. Energies are only given for those conformers that were docked in the active site of dGMII.*

Low energy conformers of **1a**	Relative energy [kcal mol^{-1}] gas phase	solution phase	Low energy conformers of **2a**	Relative energy [kcal mol^{-1}] gas phase	solution phase
E_1	0.0	0.0	E_1	0.0	1.8
^1E	2.8	1.5	^1E	1.2	3.1
E_2	8.6	4.6	E_2	6.4	3.4
E_3	5.2	1.7	^2E	1.6	3.5
^3E	8.9	2.8	E_3[a]	6.4	3.4
^4E	5.4	0.5	^3E[a]	1.6	3.5
			E_4[a]	0.0	1.8
			^4E[a]	1.2	3.1
			E_5	0.3	0.0

[a] Conformers E_3, ^3E, E_4, and ^4E are mirror images of conformers E_2, ^2E, E_1, and ^1E, respectively, with the same relative energies. These conformers were also docked in the binding site of dGMII.

Each of the low-energy conformers of **1a** and **2a** was subsequently docked into the binding site of DGMII. The six envelope conformers of **1a** could be complexed in three different binding modes (Figure 1), with conformer ^{1}E exhibiting the most favorable binding energy ($\Delta G_{binding} = -9.1$ kcal mol^{-1}). In this binding mode the five-membered ring of **1a** stacks against the aromatic side chain of Trp95, a type of interaction seen in many carbohydrate–protein complexes.[40] The 3- and 4-hydroxy oxygen atoms of **1a** coordinate with the zinc ion in the active site to give a T_6-octahedral coordination geometry. Furthermore, the nitrogen atom of the exocyclic amino group of **1a** forms hydrogen bonds with the carboxylate oxygen atom of the putative general acid/base residue Asp341 and with the hydroxy oxygen atom of Tyr269. In addition, the thiomethyl moiety of **1a** makes favorable hydrophobic interactions with Arg228 and Tyr727. In the crystal structure of DGMII with swainsonine[9] (Figure 1), the Zn^{2+} ion has T_6-octahedral coordination geometry in complexing the 3- and 4-hydroxy oxygen atoms. However, the bridgehead nitrogen atom of swainsonine forms a hydrogen bond with the catalytic nucleophile Asp204. Thus, the two complexes may differ in the interactions of their amines with acidic residues in the binding site.

Conformers E_2 and ^{3}E of **1a**, which were predicted to bind with only slightly smaller computed binding energies than ^{1}E ($\Delta G_{binding} = -8.7$ and -8.8 kcal mol^{-1}, respectively), displayed a binding mode very similar to that of swainsonine. In this case, the exocyclic amine of **1a** formed a hydrogen bond with the catalytic nucleophile Asp204. Furthermore, the 3- and 4-hydroxy oxygen atoms of **1a** coordinated with the zinc ion in T_6-octahedral coordination geometry. The somewhat-lower computed binding energies may be due to loss of hydrogen-bonding interactions between the 4-hydroxy group and the amino hydrogen atoms of His90 and His471 but may also reflect limitations in the computational method.

Conformers E_1, E_3, and ^{4}E displayed yet another complexation mode of **1a**. In this case, the amine of **1a** coordinated with the zinc ion in the T_5 coordination geometry. Pentavalent (T_5) coordination is energetically more favorable than the hexavalent (T_6) coordination of zinc[41] and this interaction may therefore be an important contributor to the stabilization of these complexes. Further stabilization came from hydrogen bonds between the nitrogen atom of the amine and the carboxylic oxygen atom of the catalytic nucleophile Asp204. The interactions between the thiomethyl group and Trp727 and Arg228 were lost in this binding mode, which may account for the slightly lower binding energies.

It has been suggested that the coordination of the oxygen atoms on positions 3 and 4 of swainsonine with Zn^{2+} mimics a similar chelation of the 2- and 3-hydroxy oxygen atoms of the mannosyl oxycarbenium ion. This coordination is an important determinant of the α-mannoside specificity of the enzyme. The binding of swainsonine is stabilized by an important hydrogen bond between the bridgehead nitrogen atom and the catalytic nucleophilic residue Asp204. In the case of the mannosyl oxycarbenium ion, its flattened ring

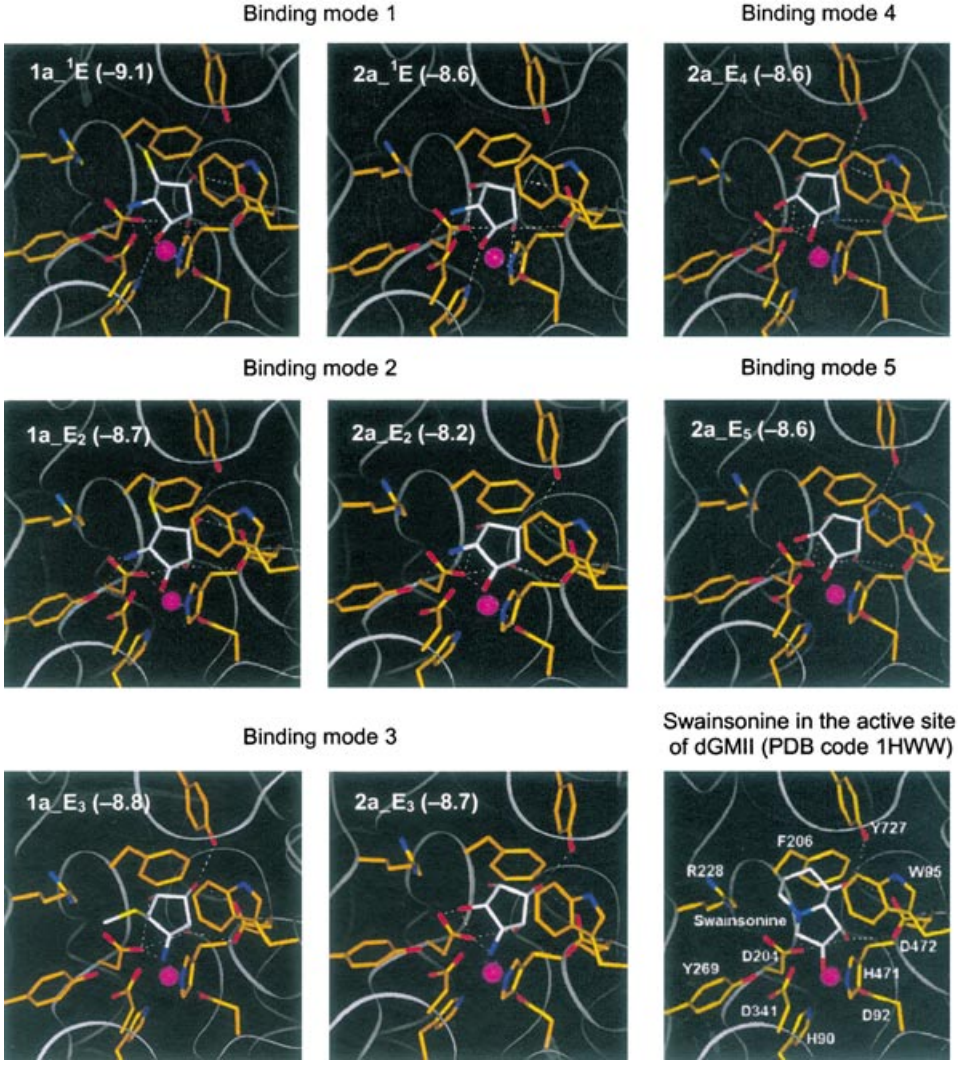


Figure 1. *Different binding modes observed for conformers of **1a** and **2a**. Binding modes 1, 2, and 3 were observed for **1a** and **2a**, while modes 4 and 5 were observed only for **2a**. The calculated free energies of binding ($\Delta G_{binding}$) are given in the parentheses (in kcal mol^{-1}) and the H-bond interactions are indicated by white dashed lines. For comparison, Swainsonine bound in the active site of dGMII, as seen in the crystal structure (PDB code 1HWW), is also shown in lower right corner.*

structure may place the anomeric center in close proximity to nucleophile Asp204 for favorable interactions. Furthermore, swainsonine, and probably the oxycarbenium ion, can make van der Waals stacking interactions with Trp95 and Phe206. The conformers E_2 and ^3E of **1a** displayed a very similar binding mode, in which the exocyclic amino group formed a hydrogen bond with Asp204 and the 3- and 4-hydroxy oxygen atoms coordinated with the Zn^{2+} ion. However, due to the flexibility of its five-membered ring, mannostatin can bind in other low-energy modes. For example, the small change in the ring conformation of ^1E places the exocyclic amine in a position to form hydrogen bonds with Asp341 and Tyr269. Other ring conformers allowed the amine of **1a** to coordinate with the Zn^{2+} ion in a favorable T_5 coordination geometry.

In addition to the three binding modes observed for **1a**, aminocyclopentitretrol **2a** could complex in two additional ways (Figure 1). All the five binding modes displayed very similar computed binding energies. Conformers ^3E and E_4 of **2a** complexed in a unique mode, in which the amine and 2-hydroxy oxygen atom coordinate with the zinc ion to give a T_6 coordination geometry. The ring stacked against Trp95 and the amine formed hydrogen bonds with the catalytic nucleophiles Asp204, Asp472, and Asp92. In the case of E_5, the amine formed hydrogen bonds with the hydroxy groups of Tyr727 and Asp472. This binding mode is further stabilized by coordination of the 2- and 3-hydroxy oxygen atoms with the zinc ion. All the hydroxy groups formed hydrogen bonds with Asp204 in addition to hydrogen-bond interactions with Tyr269, Asp92, and Asp472.

Mannostatin (**1a**) is a significantly more potent inhibitor of HGMII than aminocyclopentitretrol **2a** (Table 1). Overall, the computational studies show that **2a** is more flexible and allows additional binding modes. Furthermore, due to inversion of configuration at C-2 and replacement of the thiomethyl moiety with a hydroxy group, the hydrophobic interactions of thiomethyl moiety of **1a** cannot be made by **2a**. Although the 2-hydroxy group of **2a** can make hydrogen bonds with Asp204 and Asp341, it is unlikely that these interactions can compensate for the loss of hydrophobic interactions.[42,43]

Modification of the amine of mannostatin with benzylic moieties did not improve the inhibitory potential of the resulting compounds (Table 1). In order to rationalize these observations, docking studies were also performed with compound **1b**. Benzyl moieties were attached to the low-energy conformations of **1a** and the resulting derivatives were docked in the binding site of DGMII to give three different binding modes. In the case of conformer ^1E (Figure 2), typical interactions were observed, such as a hydrogen bond between the amine and general acid/base residue Asp341 and coordination of the 3- and 4-hydroxy oxygen atoms with the zinc ion. Furthermore, the phenyl ring of **1b** made van der Waals interactions with the aromatic moiety of Tyr269. The orientation of both the phenyl rings was observed to be similar to the preferred off-centered parallel-displaced arrangement with averages of $R_{cen} = 4.4$ Å, $\gamma = 19.9°$, and $\theta = 41.5°$ as reported by McGaughey et al.[44] (R_{cen} = the average distance between the centroids of the two phenyl rings, γ = the angle between the planes of the two rings, and θ = the normal–centroid angle). The latter π–π stacking required, however, a tilting of the five-membered ring structure of **1b** that disrupted a hydrogen bond between the 4-hydroxy group and the Nε2 nitrogen atom of His90, a bond that is observed in the complex with the parent compound. The phenyl ring in conformers E_2, E_3, and ^4E of **1b** made similar stacking interactions with Tyr269. For all three conformers, the amine formed a hydrogen bond with the catalytic nucleophile Asp204 and the hydroxy oxygen atom of Tyr269. As in ^1E, the stacking interactions required a tilting of the five-membered ring of **1b** that led to a loss of hydrogen bonds. Thus, the computational studies indicate that the favorable interactions made between the phenyl ring of **1b** and Tyr269 may be offset by loss of hydrogen bonds due to a tilting of the five-membered ring of the inhibitor.

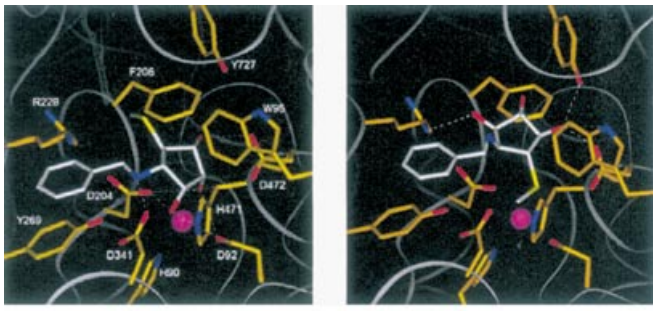


Figure 2. *Binding modes observed for conformers of* ***1b****.*

Conformers E_1 and ^3E of **1b** display a very different binding mode to that of the similar conformers of parent compound **1a** (Figure 2) In these cases the orientation of the inhibitor is flipped in the catalytic site, thereby allowing the thiomethyl group to make hydrophobic interactions with Trp95. Nevertheless, stacking interactions of the phenyl ring of **1b** with Tyr269, as well as hydrogen bonds with Arg228 and Tyr727, further stabilize these complexes.

Docking of the conformers of **2b** in the binding site of DGMII resulted in two binding modes, which mainly differ in the interactions of the amine with either Asp204 (E_1, E_2, E_3, ^3E, and E_5) or Asp341 (^1E, ^2E, E_4, and ^4E). A stacking interaction of the phenyl ring of **2b** with Tyr269 (average $R_{cen} = 4.3$ Å, $\gamma = 13.0°$, and $\theta = 41.5°$) plays an important role for positioning the inhibitor in the binding site of the enzyme. In the case of conformers E_3, ^3E, E_4, ^4E, and E_5, the five-membered ring structure of **2b** was tilted to allow the stacking interactions. As a result, the hydrogen-bond patterns observed in **2a** were altered. However, conformers E_1, ^1E, E_2, and ^2E did not require a tilt of the five-membered ring and therefore no loss of hydrogen bonds was observed. In this case, the interactions made by the phenyl moiety might be responsible for the observed improved affinity of compounds **2b**–**g** for GMII enzymes.

Although, the molecular docking analysis predicted that each of the inhibitors had a significant affinity for the enzyme, the method could not distinguish between stronger and weaker binding. The benzylated analogues would be expected

to pay a proportionally larger entropic penalty upon binding than that associated with the simpler, more rigid inhibitors. Autodock does not explicitly consider conformational entropy and so would be expected to overestimate the strength of binding of compounds **1b** and **2b**. While a quantitative ranking of affinities was not achieved, much information could be gained from inspection of the predicted binding modes.

Conclusion

Mannostatin analogues and structurally simpler aminocyclopentitetrols have been synthesized, which have various benzylic moieties at their amino functions. It was observed that mannostatin A (**1a**) is a markedly better inhibitor than the corresponding aminocyclopentitetrol **2a**. Furthermore, the substitutions of the aminocyclopentitetrols led to significant improvements of inhibitory activity for both HGMII and HLM, whereas similar substituents of mannostatin A had only a marginal effect. Computational studies have been performed to provide a rationale for these observations. First, the conformational properties of mannostatin and aminocyclopentitetrol were studied by geometry optimizations of the ten possible envelope conformations with ab initio molecular orbital calculations. Subsequently, the low-energy conformers of each compound were docked in the binding site of DGMII and the results were compared with interactions of swainsonine observed in a crystal structure with the same enzyme. It has been shown that mannostatin and aminocyclopentitretrol could bind to DGMII in a similar mode to that observed with swainsonine. The latter compound probably mimics the mannosyl oxycarbenium ion, which is a putative intermediate in the hydrolysis of mannosidases. Thus, it appears that mannostatin and aminocyclopentitretrol can inhibit GMII enzymes in a similar fashion. However, due to the flexibility of the five-membered rings of **1a** and **2a**, additional low-energy binding modes could be adopted. It is conceivable that the ring structure of **1a** and **2a** remains flexible within the binding site of GMII enzymes, thereby allowing transitions between different binding modes. The thiomethyl moiety of mannostatin could make favorable hydrophobic interactions with Arg228 and Tyr727. These interactions are not present in complexes with aminocyclopentitretrol **2a** and they therefore provide a rationale for the lower inhibitory potential of this compound. It was also observed that the five-membered ring of **2a** is significantly more flexible than that of **1a** and allows additional binding modes. Attachment of benzyl moieties to mannostatin led to stacking interactions with the aromatic moiety of Tyr269. These π–π interactions required, however, a tilting of the ring structure of **1b** that resulted in a disruption of the hydrogen-bonding network observed. Due to the greater flexibility of aminocyclopentitretrol **2a**, attachment of the benzyl moiety led to conformers that could make the stacking interactions without disrupting hydrogen bonds. This observation may provide a rationale for the improved activities of benzyl-substituted aminocyclopentitretrols.

It is to be expected that further combinatorial extension of the benzyl derivatives will lead to compounds that can interact with the HGMII extended binding pocket for the aglycone of the natural substrate, thereby accentuating the selectivity of this enzyme. In this respect, the different binding modes computed for both compounds may provide an important opportunity for the design of targeted libraries.

Experimental Section

General procedure for reductive amination: A solution of NaOH in MeOH (0.1 M, 0.2 equiv) was added under argon at room temperature to a solution of compound **1a** or **2a** in MeOH to give a solution with pH 7. A methanolic solution of benzaldehyde (2.5 M, 1.0 equiv) and molecular sieves (3 Å) was added to this solution, then a methanolic solution of $NaCNBH_3$ (1.75 M, 0.6 equiv) was added. After 20 h, the reaction was diluted with MeOH and filtered through celite. The solution was acidified to pH 1 with 1 N HCl and concentrated in vacuo. The residue was purified by flash chromatography (Iatro beads; CH_3CN/HOAc/H_2O, 10:0.5:1) to give the expected product.

(1*R*,2*R*,3*R*,4*R*,5*S*)-1-(Methylthio)-2,3,4-trihydroxy-5-(*N*-benzylamino)cyclopentane (1b): Yield: 98%; $R_f = 0.35$ (CH_3CN/H_2O/HOAc, 10:2:1); $[\alpha]_D = +21.4°$ ($c = 0.45$, MeOH), lit. $= +20.1°$ [23] ($c = 0.43$, MeOH); ^{1}H NMR (300 MHz, D_2O): $\delta = 7.44$ (s, 5H), 4.37–4.26 (m, 3H), 4.02 (t, $J = 4.5$ Hz, 1H), 3.96 (t, $J = 6.0$ Hz, 1H), 3.39 (t, $J = 7.8$ Hz, 1H), 3.13 (t, $J = 7.8$ Hz, 1H), 1.99 (s, 3H) ppm; ^{13}C NMR (75 MHz, D_2O): $\delta = 130.6$, 130.2, 130.0, 129.6, 74.6, 71.5, 68.8, 59.7, 51.3, 50.6, 12.1 ppm.

(1*R*,2*R*,3*R*,4*R*,5*S*)-1-(Methylthio)-2,3,4-trihydroxy-5-(*N*-(*p*-fluorobenzyl)amino)cyclopentane (1c): Yield: 75%; $R_f = 0.38$ (CH_3CN/H_2O/HOAc, 10:2:1); $[\alpha]_D = +33.0°$ ($c = 0.58$, MeOH); ^{1}H NMR (300 MHz, D_2O): $\delta = 7.51$–7.47 (m, 2H), 7.20–7.1 (m, 2H), 4.37–4.26 (m, 3H), 4.06–3.97 (m, 2H), 3.38 (t, $J = 7.2$ Hz, 1H), 3.14 (t, $J = 7.2$ Hz, 1H), 2.03 (s, 3H) ppm; ^{13}C NMR (75 MHz, D_2O): $\delta = 132.4$, 132.3, 126.8, 126.7, 116.5, 116.2, 74.7, 71.6, 68.8, 59.7, 51.4, 49.9, 12.1 ppm.

(1*R*,2*R*,3*R*,4*R*,5*S*)-1-(Methylthio)-2,3,4-trihydroxy-5-(*N*-(*p*-chlorobenzyl)amino)cyclopentane (1d): Yield: 64%; $R_f = 0.40$ (CH_3CN/H_2O/HOAc, 10:2:1); $[\alpha]_D = +15.3°$ ($c = 0.28$, MeOH); ^{1}H NMR (300 MHz, D_2O): $\delta = 7.46$ (s, 4H), 4.34–4.28 (m, 3H), 4.05–3.99 (m, 2H), 3.42 (t, $J = 7.2$ Hz, 1H), 3.15 (t, $J = 7.2$ Hz, 1H), 2.03 (s, 3H) ppm; ^{13}C NMR (75 MHz, D_2O): $\delta = 131.7$, 129.5, 74.6, 71.6, 68.7, 59.8, 51.2, 49.9, 12.0 ppm.

(1*R*,2*R*,3*R*,4*R*,5*S*)-1-(Methylthio)-2,3,4-trihydroxy-5-(*N*-(*p*-bromobenzyl)amino)cyclopentane (1e): Yield: 80%; $R_f = 0.37$ (CH_3CN/H_2O/HOAc, 10:2:1); $[\alpha]_D = +33.4°$ ($c = 0.44$, MeOH); ^{1}H NMR (300 MHz, D_2O): $\delta = 7.61$ (d, $J = 8.4$ Hz, 2H), 7.37 (d, $J = 8.4$ Hz, 2H), 4.31–4.20 (m, 3H), 4.04–3.96 (m, 2H), 3.40 (t, $J = 6.2$ Hz, 1H), 3.11 (t, $J = 6.6$ Hz, 1H), 2.03 (s, 1H) ppm; ^{13}C NMR (75 MHz, D_2O): $\delta = 132.4$, 131.8, 74.8, 71.6, 68.9, 59.9, 51.7, 49.9, 12.0 ppm.

(1*R*,2*R*,3*R*,4*R*,5*S*)-1-(Methylthio)-2,3,4-trihydroxy-5-(*N*-(*p*-methoxybenzyl)amino)cyclopentane (1f): Yield: 99%; $R_f = 0.38$ (CH_3CN/H_2O/HOAc, 10:2:1); $[\alpha]_D = +28.5°$ ($c = 0.63$, MeOH); ^{1}H NMR (300 MHz, D_2O): $\delta = 7.43$ (d, $J = 8.4$ Hz, 2H), 7.02 (d, $J = 8.4$ Hz, 2H), 4.67–4.22 (m, 3H), 4.05–3.96 (m, 2H), 3.38 (t, $J = 7.2$ Hz, 1H,), 3.13 (t, $J = 7.2$ Hz, 1H), 2.00 (s, 1H) ppm; ^{13}C NMR (75 MHz, D_2O): $\delta =$ 131.9, 123.1, 114.9, 74.6, 71.6, 68.7, 59.4, 55.7, 51.3, 50.1, 12.0 ppm.

(1*R*,2*R*,3*R*,4*R*,5*S*)-1-(Methylthio)-2,3,4-trihydroxy-5-(*N*-(*p*-allyl ester benzyl)amino)cyclopentane 1g: Yield: 59%; $R_f = 0.49$ (CH_3CN/H_2O/HOAc, 10:2:1); $[\alpha]_D = +19.3°$ ($c = 0.21$, MeOH); ^{1}H NMR

(300 MHz, D_2O): $\delta = 8.08$ (d, $J = 7.8$ Hz, 2H), 7.61 (d, $J = 8.1$ Hz, 2H), 6.11–5.98 (m, 1H), 5.39 (d, $J = 17.4$ Hz, 1H), 5.29 (d, $J = 10.5$ Hz, 1H), 4.65–4.30 (m, 3H), 4.07–3.99 (m, 2H), 3.45 (t, $J = 7.2$ Hz, 1H), 3.17 (t, $J = 7.5$ Hz, 1H), 2.00 (s, 1H) ppm; ^{13}C NMR (75 MHz, D_2O): $\delta = 132.1$, 130.5, 130.3, 118.7, 74.6, 71.6, 68.7, 66.7, 60.1, 51.2, 50.1, 49.1, 12.1 ppm.

(1*R*,2*R*,3*R*,4*S*,5*S*)-1,2,3,4-Tetrahydroxy-5-(*N*-benzylamino)cyclopentane (2b): Yield: 97%; $R_f = 0.14$ (CH_3CN/H_2O/HOAc, 10:2:1); $[\alpha]_D = +1.2°$ ($c = 0.59$, MeOH); ^1H NMR (300 MHz, D_2O): $\delta = 7.47$–7.40 (m, 5H), 4.30–4.23 (m, 4H), 3.99–3.97 (m, 2H), 3.58 (t, $J = 6.3$ Hz, 1H) ppm; ^{13}C NMR (75 MHz, D_2O): $\delta = 130.8$, 130.3, 130.0, 129.5, 71.0, 69.0, 58.0, 50.8 ppm.

(1*R*,2*R*,3*R*,4*S*,5*S*)-1,2,3,4-Tetrahydroxy-5-(*N*-(*p*-fluorobenzyl)amino)cyclopentane (2c): Yield: 73%; $R_f = 0.25$ (CH_3CN/H_2O/HOAc, 10:2:1); $[\alpha]_D = +1.0°$ ($c = 0.69$, MeOH); ^1H NMR (300 MHz, D_2O): $\delta =$ 7.51–7.46 (m, 2H), 7.20–7.14 (m, 2H), 4.28–4.24 (m, 4H), 3.98 (s, 2H), 3.58 (t, $J = 6.3$ Hz, 1H) ppm; ^{13}C NMR (75 MHz, D_2O): $\delta = 132.5$, 132.4, 71.0, 69.0, 58.0, 50.1 ppm.

(1*R*,2*R*,3*R*,4*S*,5*S*)-1,2,3,4-Tetrahydroxy-5-(*N*-(*p*-chlorobenzyl)amino)cyclopentane (2d): Yield: 48%; $R_f = 0.40$ (CH_3CN/H_2O/HOAc, 10:2:1); $[\alpha]_D = +10.0°$ ($c = 0.18$, MeOH); ^1H NMR (300 MHz, D_2O): $\delta = 7.37$–7.34 (m, 4H), 4.17–4.08 (m, 4H), 3.90 (s, 2H), 3.43 (t, $J =$ 6.3 Hz, 1H) ppm; ^{13}C NMR (75 MHz, D_2O): $\delta = 131.6$, 129.3, 70.9, 69.0, 58.0, 50.0 ppm.

(1*R*,2*R*,3*R*,4*S*,5*S*)-1,2,3,4-Tetrahydroxy-5-(*N*-(*p*-bromobenzyl)amino)cyclopentane (2e): Yield: 65%; $R_f = 0.42$ (CH_3CN/H_2O/HOAc, 10:2:1); $[\alpha]_D = +1.4°$ ($c = 0.06$, MeOH); ^1H NMR (300 MHz, D_2O): $\delta =$ 7.62–7.59 (m, 2H), 7.38–7.35 (m, 2H), 4.22–4.18 (m, 4H), 3.98 (s, 2H), 3.68 (t, $J = 6.6$ Hz, 1H) ppm; ^{13}C NMR (75 MHz, D_2O): $\delta = 131.6$, 129.3, 70.9, 69.0, 58.0, 50.0 ppm.

(1*R*,2*R*,3*R*,4*S*,5*S*)-1,2,3,4-Tetrahydroxy-5-(*N*-(*p*-methoxybenzyl)amino)cyclopentane (2f): Yield: 79%; $R_f = 0.45$ (CH_3CN/H_2O/HOAc, 10:2:1); $[\alpha]_D = +3.2°$ ($c = 0.78$, MeOH); ^1H NMR (300 MHz, D_2O): $\delta =$ 7.42–7.39 (m, 2H), 7.02–7.00 (m, 2H), 6.05–6.00 (m, 1H), 5.41–5.27 (m, 2H), 4.23 (s, 2H), 3.97 (s, 2H), 3.52 (t, $J = 6.3$ Hz, 1H) ppm; ^{13}C NMR (75 MHz, D_2O): $\delta = 131.8$, 123.3, 114.8, 70.9, 68.9, 57.7, 55.6, 50.1 ppm.

(1*R*,2*R*,3*R*,4*S*,5*S*)-1,2,3,4-Tetrahydroxy-5-(*N*-(*p*-allyl ester benzyl)amino)cyclopentane 2g: Yield: 87%; $R_f = 0.52$ (CH_3CN/H_2O/HOAc, 10:2:1); $[\alpha]_D = +5.1°$ ($c = 0.62$, MeOH); ^1H NMR (300 MHz, D_2O): $\delta =$ 8.07–8.05 (m, 2H), 7.60–7.57 (m, 2H), 6.05–6.00 (m, 1H), 5.41–5.27 (m, 2H), 4.36 (s, 2H), 4.24 (s, 2H), 3.97 (s, 2H), 3.58 (t, $J = 6.3$ Hz, 1H) ppm; ^{13}C NMR (75 MHz, D_2O): $\delta = 168.1$, 136.2, 132.1, 131.0, 130.4, 118.7, 71.0, 68.9, 66.7, 58.5, 50.3 ppm.

Inhibition studies: Assays with HLM were assembled at 4°C in a reaction volume (50 μL) containing 4-methylumbelliferyl α-D-mannopyranoside (3 mM; Sigma), sodium acetate (100 mM; pH 4.5), and purified recombinant HLM that was expressed and purified from HEK293 cells. (Detailed methods will be published separately.) Assays with HGMII were prepared in a similar reaction volume containing 4-methylumbelliferyl α-D-mannopyranoside (2.5 mM; Sigma), sodium acetate (83.3 mM; pH 5.6), ZnCl (83.3 μM), and purified recombinant HGMII that was expressed and purified from HEK293 cells. (Detailed methods will be published separately.) Reactions were incubated for one hour at 37°C and stopped with the addition of sodium carbonate solution (150 μL) at a final concentration of 150 mM. Fluorescence was quantified on a Spectramax Gemini XS fluorescence reader. All fluorescence values are compared to a 4-methylumbelliferone standard curve. Inhibitors were prepared as stock solutions in water (1–10 mM depending on the amount of compound) and IC_{50} data were obtained in triplicate over a concentration range of 10 nM–1 mM and then plotted as percentage inhibition versus inhibitor concentration. K_i determinations were performed under similar conditions to IC_{50} determinations, except that 4-methylumbelliferyl α-D-mannopyranoside concentrations were also varied from 160 μM–3 mM. Dixon plots were used to transform the kinetic data into K_i values.

Computational studies: All the geometry optimizations were performed with the Gaussian 94 program[45] by using density functional theory (B3LYP[46–48]) and the 6-31G*[49] basis set. The low-energy unique conformers of **1a** and **2a** were determined by optimizing ten possible envelope conformers (^1E, E_1, ^2E, E_2, ^3E, E_3, ^4E, E_4, ^5E, and E_5; the abbreviated nomenclature used here is similar to the one suggested for the furanose ring system[50]) by constraining a specific torsion angle to 0° and allowing all other parameters to optimize at the BLYP/6-31G* level of theory. The hydroxy and amino groups of the resulting structures were placed in three different orientations to give different rotamers, which were reoptimized without any restraints at the B3LYP/6-31G* level. The solvation effects on the energies of the resulting conformers were approximated by using the Poisson–Boltzmann treatment[37,38] provided in the Jaguar program.[39] The unique envelope conformers obtained were docked in the binding site of DGMII.

Preparation of ligand and receptor molecules for docking: The crystal structure of *Drosophila* GMII complexed with swainsonine (PDB file code: 1HWW) was used as a model for the macromolecule in docking experiments. The protein target (DGMII) and the ligands were prepared for docking by using AutoDock Version 3.0.5.[51,52] Charges were assigned by using the Kollman algorithm.[53] Atomic solvation parameters and fragmental volumes were determined by using the Addsol programs available in AutoDock. AutoTors was used to define torsional angles in the ligand. Polar hydrogen charges of Gasteiger type[54] were assigned and the nonpolar hydrogen atoms were merged with the carbon atoms. The macromolecule was kept rigid in all the docking simulations.

Docking simulations: Grid maps for docking simulations were generated, with 40 grid points (with 0.375 Å spacing) in the *x*, *y*, and *z* directions, by the Autogrid program. The center of the grid was positioned at the Zn^{+2} ion (Zn1102). Lennard–Jones parameters 12–10 and 12–6 (supplied with the program package) were used for modeling hydrogen bonds and van der Waals interactions, respectively. The distance-dependant dielectric permitivity of Mehler and Solmajer[55] was used for the calculations of the electrostatic grid maps. The genetic algorithm (GA) and Lamarckian genetic algorithm with the pseudo-Solis and Wets modification (LGA/pSW) methods were used with the default parameters. For all simulations, the populations in the genetic algorithm were 50 and each simulation comprised 2.5×10^5 energy evaluations. Each docking experiment consisted of a series of 100 simulations.

The π–π stacking interactions between the phenyl ring in certain **1b** and **2b** conformers and the phenyl ring in Tyr269 were evaluated by determining the average distance between the centroids of the two phenyl rings (R_{cen}), the angle between the planes of the two rings (γ), and the normal–centroid angle (θ), as discussed in ref. [44].

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