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**Exploring the divalent effect in fucosidase inhibition with stereoisomeric pyrrolidine dimers**

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Multi-valent inhibitors offer promise for the enhancement of therapeutic compounds across a range of chemical and biological processes. Here, a significant increase in enzyme-inhibition potencies was observed with a dimeric iminosugar-templated fucosidase inhibitor (IC50=0.108 M) when compared to its monovalent equivalent (IC50=2.0 M). Such a gain in binding is often attributed to a “multivalent effect” rising from alternative recapture of the scaffolded binding epitopes. The use of control molecules such as the the *meso* analogue (IC50=0.365 M) or the enantiomer (IC50=569 M), as well as structural analysis of the fucosidase-inhibitor complex, allowed a detailed analysis of the possible mechanism of action, at a molecular level. Here, the enhanced binding affinity of the dimer over the monomer can be attributed to additional interactions in non-catalytic sites as also revealed in the 3-D structure of a bacterial fucosidase inhibitor complex.

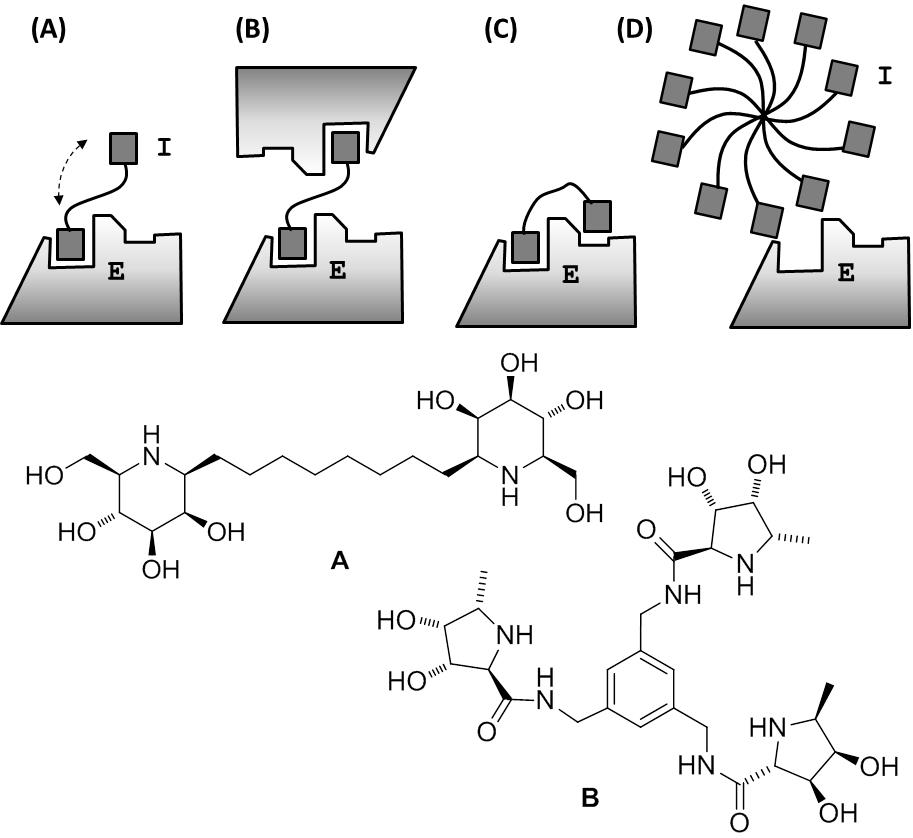
Introduction

Carbohydrate-protein interactions play critical roles in many physiological or pathological events. Indeed, oligosaccharides are adhesion receptors in cell-host recognition and, as such, they are necessary for leucocyte extravasion,1 infection by viruses or bacteria2 and for the development of cancer.3 However, despite their biological relevance, interactions of isolated monosaccharides with proteins are usually weak.4 Typically, d-mannopyranosides bind to concanavalin A, a leguminous carbohydrate-binding protein (lectin) from Jack bean with a dissociation constant *K*d of 0.8 10-3 m.5 In many cases, Nature counter-balances the insufficient affinity of carbohydrate epitopes for their protein target by presenting these epitopes in a multiple fashion; termed avidity.6 Intriguingly, the gain in binding is usually superior to that expected on simple statistic basis, with respect to the number of monovalent ligands, which is referred to as the “cluster glycoside effect” or the “multivalent effect”.7,8 This beneficial interaction might be rationalized on thermodynamic basis, the cooperativity () resulting from n uniform binding elements being defined as :

Gmulti =  . n . Gmono (1)

In expression (1) Gmulti is the binding energy released during the multivalent association of the n ligands and Gmono is the binding energy of one monovalent interaction taken alone.9,10

The structure of glycoenzymes (*ie* enzymes that process oligosaccharides) has been tailored by Nature to assume strong affinities for their transition-states, where the enzyme has evolved to bind the non-standard bond lengths and charges.11 Lectins are usually regarded as multimeric adsorption layers for carbohydrates.12 With enzymes the carbohydrate-protein interactions are optimal in a catalytic site, which is mostly located in the inner space of the protein but might also be shallow in some cases.13 Though there was no natural equivalent of multivalency for simple glycoenzymes, this concept has been recently explored, mainly on glycosidases, with the aim of developing more potent and selective inhibitors.14 Thus, by analogy with lectins, it was assumed that the simultaneous presentation of multiple copies of an inhibitor covalently attached to a molecular scaffold could induce a multivalent effect *via* analogous thermodynamic factors. Several mechanistic hypotheses could account for such an effect (Figure 1).15 The most frequently suggested, in the absence of multiple receptor sites, entails a possible recapture of the tethered molecules of inhibitor **I** after dissociation of the initial E-**I** complex (Figure 1A). In this case, the enhanced affinity results from increased effective concentration (Ceff) of additional epitopes in close proximity of the receptor. The simultaneous ligation of enzyme molecules (Figure 1B) or additional interactions with non-catalytic binding sites (Figure 1C) could also account for the increased inhibition potency of a multimeric compound. Finally, strong inhibition enhancement observed with multimers over their monomeric analogues could also result from simple steric occlusion of the active site (Figure 1D). Such a mode of inhibition has been described with fullerene-based nanoscale inhibitors of carbonic anhydrase and might not be ruled out in the case of high-valent multimers possessing dimensions of the same order of magnitude as the enzyme cavity.16



**Figure 1** : structure of inhibitors **A**,**B** and proposed mechanisms for inhibition of enzyme **E** by dimeric or multimeric molecules **I**, (A) Favoured recapture of the ligand *via* high local concentration; (B) simultaneous ligation of two enzyme molecules (with possible clustering effect and oligomerization of protein); (C) increased strength of binding by additional interactions with an allosteric site; (D) steric occlusion of the active site.

The first application of multivalency to glycosidases was reported by Johns and Johnson in 1998.17a Dimeric deoxymannojirimycin (DMJ) analogue **A** (Figure 1) was prepared and assayed against several glycosidases. Interestingly, while monomeric C-1 substituted DMJ analogues show only poor inhibition of -mannosidase in the mM range, **A** displayed a promising IC50 of 49 M. However, the multivalent effect could not be precisely quantified since the monovalent reference was not assayed in this work. The first accurate measurement of a multivalent effect on glycosidases was provided by Kovensky in 2009.17b Since then, several examples of multivalent glycosidase inhibitors have been prepared and assayed to interrogate the nature of the scaffold (porphyrin,18a fullerene,18b cyclodextrin18c), or the structure of the scaffolded iminosugar taken as the “epitope” (DMJ, deoxygluco-nojirimycin=DNJ, deoxyfuconojirimycin=DFJ and analogues thereof)18d or the number of copies of this binding element (dimers to dodecamers).18e-h Notwithstanding these results, multivalent approaches have not been equally successful with all glycosidases. The most frequent and highest increase in binding has been observed with *Canavalia ensiformis* -mannosidase (Jack bean -mannosidase, JbMan), a multimeric protein from the glycoside hydrolase CAZY family GH38.14c Enzymes that belong to this family present a large and open catalytic site surrounded with non-catalytic carbohydrate binding regions located at the protein surface. Such a configuration is optimal for a recapture mechanism to occur in the easily accessible active site but also for the enhancement of binding forces through additional interactions of the multimeric inhibitor with secondary binding sites or for simple site-occlusion effect. Intriguingly, the most potent multivalent effect on JbMan is produced with *gluco*-configured iminosugar multimers, the monomer of which (DNJ) does not match the catalytic site. Similarly to mannosidases, a number of 3D structures for -fucosidases (AFU) from family GH29 pointed out a favourable environment for multivalency, with a catalytic site maintained near at the surface of the protein. However, contrasting results were observed on -fucosidase with a first series of multivalent inhibitors.19 Trivalent species **B** (Figure 1) showed only modest improvement in binding relatively to the monomeric pyrrolidine, whereas other series of dimers proved significantly more active (statistically corrected). These contrasting observations point to the need of a deeper analysis of the mechanisms in play with multivalent fucosidase inhibitors. To examine this issue, we analyzed herein the possibility for a recapture to occur (Figure 1A) with the aid of chemical tools including dimer **1** and stereoisomers ***meso-*1** and ***ent-*1** as well as monovalent equivalents **2** and **3** or inhibitor **4** (Figure 2). To reinforce our conclusions,the 3D structure of **1** complexed with a representative fucosidase, *Bt*Fuc2970, has also been solved.



**Figure 2** : structures of inhibitors **1**-**4**.

Results and Discussion

**Synthesis of fucosidase inhibitors**

Homodimer **1** and its enantiomer *ent*-**1** were prepared *via* a parallel sequence which started either from 5-deoxy-d-ribose or the enantiomeric l-ribose respectively. The synthetic route is fully described on Scheme 1, for the synthesis of ***ent-*1**, and is based on the nucleophilic addition of an organometallic to a glycosylamine.20a In the case of *ribo* configured glycosylamines such addition is usually highly stereoselective and affords the *anti* adduct as the only isolated isomer.20b-c Thus, glycosylamine ***ent-*6** obtained after amination of hemiacetal ***ent*-5**21with benzylamine was reacted with ethynylmagnesium bromide, which afforded aminoalcohol ***ent-*7** in a complete stereoselective manner (70% yield for the two steps). Intramolecular nucleophilic displacement was effected by activation of the secondary hydroxyl with MsCl and occurred with inversion of configuration at C(OH) to afford pyrrolidine ***ent-*8** (68% yield).



**Scheme 1.** Synthesis of compounds **1**,**3**,***ent-*1** and ***ent-*3**.



**Scheme 2**. Synthesis of inhibitors ***meso-*1** and **4**

Homodimerization was first attempted by using CuI as the promoter,22 but unsatisfactory yield of the corresponding di-yne (16%) was obtained. Nevertheless, ***ent-*9** could be isolated in 84% yield when Pd(PPh3)2Cl2 and CuI were used as co-catalysts,23 in the presence of *i*-PrNH2. Reduction of the alkyne and hydrogenolysis of the benzyl groups, followed by acidolysis gave target homodimer ***ent-*1**. The corresponding monomer ***ent-*3** was obtained from intermediate ***ent-*8** by the same reduction/acidolysis sequence. An analogous synthetic route, when applied to the d-ribose derivative **5** afforded enantiomers **1** and **3**.

The synthesis of the meso analogue ***meso-*1** by a similar 1,3-diyne strategy required a structural discrimination between the two enantiomeric pyrrolidines that have to be coupled. Indeed, hetero-coupling of **8** with its enantiomer ***ent-*8** to give the target meso isomer would also afford a mixture of enantiomers **9**/***ent-*9** via competitive homo-dimerization, a complex mixture from which the targeted isomer would be difficult to isolate. To facilitate the monitoring of the reaction and the separation of the products compound ***meso-*1** was prepared by coupling ***ent-*8** with the known *N*-allyl protected enantiomer **11** (Scheme 2).24 Furthermore, an excess of pyrrolidine **11** was used during the reaction in order to favor the cross-coupling of ***ent-*8** with **11** and minimize homodimerization of alkyne ***ent-*8**. Satisfyingly, this procedure proved successful and afforded hetero-di-yne **13** (61% yield), which was easily separable from the other dimers that were formed ***ent-*9** (25%) and **12**. Cleavage of the *N*-Allyl group in **13** was achieved with the Pd/NDMBA system.25 Optically inactive ***meso-*1** was obtained after hydrogenation and subsequent acidolysis, as described above. Finally, an analogue of **1** which carries a phenyl group in place of the second pyrrolidine moiety was also prepared by hydrogenation of the known di-yne **15**.24

All these compounds were subjected to enzyme kinetic assays in order to determine the half maximal inhibitory concentrations (IC50) and the E-I dissociation constants (*K*i) to uncover the mode of action and the contribution of the dimeric structure to the binding.

**Enzyme Inhibition**

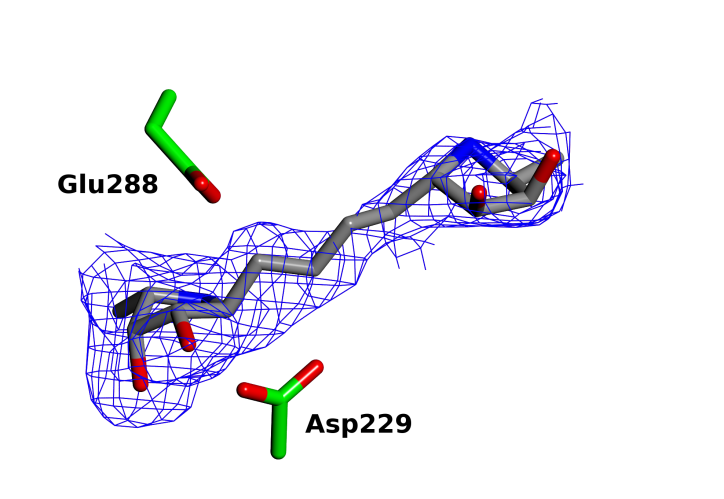
As discussed above, AFU offers a favourable environment for a multivalent effect to occur, since the catalytic site in GH-29 glycosidases might be maintained near at the surface of the protein allowing easy exchange with the solvent, as shown by structural studies conducted on bacterial fucosidases.19,24,26 In the view of the preliminary results obtained with AFU and the increased affinity of multimeric pyrrolidines with short spacers,19b in this work, we targeted low-valent (dimeric) models with a short and flexible linker9c in order to optimize the conditions for recapture to happen (Figure 1A) through spatial proximity of inhibitor molecules and minimal steric hindrance. Such a short linker would also restrain the simultaneous binding of two enzyme molecules or the steric occlusion of the active site, limiting the inhibition enhancement, if any, to mechanism A or C (Figure 1). However, an aggregation mechanism (Figure 1B) should not be totally ruled out, since there is a precedent where divalent mannosides with 4-C linkers are able to cross-link lectins.10 Pyrrolidine-type inhibitors were chosen as models, since it was demonstrated with a large series of previous analogues that these compounds act mainly as competitive inhibitors.27

Here, compounds **2**, **3** or ***ent-*3** are the monovalent equivalents from which dimer **1** and analogues ***ent-*1** or ***meso-*1** derive. Polyhydroxypyrrolidine **2** is a known competitive inhibitor of fucosidase (*K*i = 2.0 M).28 With C2-ethyl substituted **3** and***ent-*3** the binding contribution of the linker could also be evaluated. In a first set of experiments, the IC50 of the prepared compounds were determined to identify, in the simplest manner, an eventual enhancement in enzyme inhibition with the dimers when compared to the monomers (Table 1). As expected, pyrrolidines **1**, ***meso*-1**, **3** and **4** affected significantly enzyme kinetic with IC50 in the low micromolar range. The enantiomeric analogues ***ent*-1** and ***ent*-3** were significantly less potent. Interestingly, a significant increase in enzyme-inhibition potency was observed with **1** (IC50=0.108 M) when compared to its monovalent equivalent **3** (IC50=2.0 M), suggesting a possible multivalent effect. However, the same observation counted for ***meso*-1** (IC50=0.365 M) or the phenyl-substituted **4** (IC50=0.263 M). In addition, an equimolar mixture of monomer **3** and its enantiomer ***ent-*3** was subjected to enzyme inhibition (Table 1, entry 7).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Table 1.** inhibition of Fucosidase from bovine kidney by compounds **1**-**4***.* | | | | |
| entry | Inhibitor | IC50[a] | Ki [a] (inhibition type) | [c] |
| 1 | **1** | 0.108 | 0.023 (competitive) | 7.8[d] |
| 2 | ***meso-*1** | 0.365 | 0.051 (mixed, *K*’i=0.16M) | 3.5[d] |
| 3 | ***ent-*1** | 569 | 178 (mixed, *K*’i=335M) | 0.07[e] |
| 4 | **2** | nd | 2.0 (competitive) [b] |  |
| 5 | **3** | 2.0 | 0.18 (mixed, *K*’i=1.12M) |  |
| 6 | ***ent-*3** | 84 | 12 (mixed, *K*’i=20M) |  |
| 7 | **3** + ***ent-*3** | 4.3 | nd |  |
| 8 | **4** | 0.263 | 0.031(mixed, *K*’i=0.108M) |  |
| [a] M. [b] according to reference 28. [c]  is the affinity enhancement factor , =*K*dmono/*K*dmulti . [d] compound **3** taken as the monomer. [e] compound ***ent-*3** taken as the monomer. | | | | |

This racemic mixture (IC50=4.3 M) behaves like pure **3** (IC50=2.0 M), with an IC50 only affected by the dilution factor. No effect of synergy was observed when both enantiomers were present in the solution and only **3** bound to fucosidase. This result clearly demonstrates that the competition between **3** and ***ent-*3** for binding to AFU is unequivocally won by **3**. Thus, the possibility for the mismatching ***ent-*3** to replace **3** in its binding site seems highly unlikely.

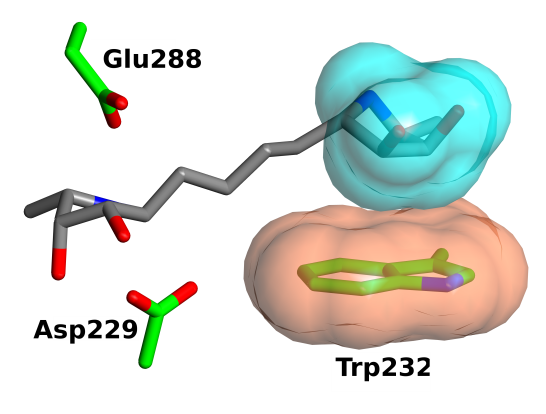
A more detailed analysis of the mechanisms in play required the determination of the dissociation constant *K*i for all our inhibitors (Table 1). Concerning the influence of the linker, the introduction of an ethyl group in the structure of **2** to give **3** improved the affinity for AFU (*K*i = 0.18 M, Table 1, entry 5). Lineweaver-Burk plots (see Supporting Information) suggested a mixed type of inhibition for **3**.29 The enantiomeric 2-ethylpyrrolidine ***ent-*3** proved less potent (*K*i = 12 M, entry 6), as expected, with a loss in the binding strength by a factor of *ca* 60 when compared to **3**. Surprisingly, inhibition potency of the d-fuco configured ***ent-*3** towards AFU compared well with that of other parent l-iminosugars in spite of inadequate configuration. Such strong inhibition potencies of unnaturally configured enantiomers of iminosugars have already been observed in the literature with -d-glucosidase,30 -d-*N*-acetylhexosaminidase31 or -d-galactosidase32 inhibitors. In most of these cases, the mismatching enantiomers display a mixed inhibition, as observed here for ***ent-*3**.The inhibition pattern of dimer **1** was investigated then. As shown by Lineweaver-Burk plots, **1** operates via a competitive mode of action (*K*i = 23 nM), thus interacting with the catalytic site. This result is consistent with our crystallographic studies (see below). As in the case of lectin-carbohydrate multiple interactions, an affinity enhancement factor =*K*dmono/*K*dmulti could be calculated.6 With **1**, the structural dimerization afforded =7.8 when compared to monomer **3**. In any case, these results would suggest the intervention of a “multivalent effect” as defined above. Synergy could occur from the successive binding of two attached pyrrolidines into the catalytic site mediated by enhanced Ceff, as reflected by the rebinding mechanism (Figure 1A). Nevertheless, considering such a mechanistic hypothesis, a “dimer” that is composed of **3** and its enantiomer ***ent-*3** should exhibit no multivalent effect, the recapture of the ***ent-*3** template being highly improbable in the presence of the neighboring **3** as suggested by the results above with the racemic mixture. Intriguingly, the “dimer” ***meso-*1** (*K*i = 51 nM) which features such a structure still proved significantly more active than monomer **3,** displaying an affinity enhancement factor =3.5. In ***meso-*1**, the structural combination of **3** and its enantiomer ***ent-*3** induces synergy, which can be mechanistically rationalized only by fortuitous additional interactions of the mismatching pyrrolidine with allosteric sites as shown in Figure 1C. This was supported by the results with phenyl substituted analogue **4**, a compound which features a “neutral” aglycon unable to induce recapture. The replacement of one pyrrolidine template in dimer **1** with a phenyl group was not detrimental for binding (*K*i = 31 nM) compound **4** being still a better inhibitor than the monomer **3**. Strong inhibition of AFU by **4** could be explained by additional interaction of the hydrophobic moiety with a lipophilic binding pocket other than the active site. Intriguingly, the increase in affinity occurs whatever the nature of the aglycon attached to monomer **3**. Such an improvement in binding strength through dimerization was not observed with the enantiomer ***ent-*3**. In this case, dimerization to give ***ent*-1** was clearly detrimental for binding, with =0.07, the additional pyrrolidine failing to induce either favorable interactions or a prospective recapture process. Thus, the increase in binding strength observed with dimer **1** when compared to monomers **2** or **3** would rather result from additional binding interactions provided by the extra aglycon, as observed with the analogues ***meso-*1**, or **4**. The rebinding effect might not be ruled out here but, if occurring, it is certainly not the only mechanism at play. This result was supported by a structural analysis of the enzyme inhibitor **1** complex (see below).

In parallel, extension of this kinetic study to Jack bean -mannosidase (the standard model used to uncover multivalent signatures on glycosidases) proved unsuccessful. No inhibition of JbMan could be measured at 1 mM concentration with either monomer ***ent-*3** (which features a favourable *manno* configuration but lacks the C-6 hydroxy group of mannose)33 or with the dimeric equivalents ***ent-*1** or ***meso-*1**.

**Structural analysis of the mode of binding**

The 3-D structure of a convenient bacterial family GH29 fucosidase *Bt*Fuc2970, in complex with **1**, was determined at 2.1 Å resolution (3-D structure deposited with PDB code 5I5R). *Bt*Fuc2970 is most likely a dimer in solution, reflected in size-exclusion chromatography, small-angle X-ray scattering (unpublished) and the size and nature of the interfaces revealed in deposited 3-D structures. In such structures, the active centres are usually approximately 60 Å apart and cannot be simultaneously occupied by any of the species described here. Electron density is clear for both pyrrolidines and their linker, Figure 3. The “-1” imino sugar moiety is bound in the catalytic site close to catalytic residues Glu288 and Asp229, essentially as previously observed for diverse fucosidase ligand complexes on this system.19a,24,26 Furthermore, and consistent with proposals (above) the complex is a rare example where the linker and second imino-sugar are visible, and thus strongly bound, reducing the possibility of a recapture to occur. Indeed, the second pyrrolidine unit makes a stacking interaction above Trp232 in the bacterial structure (Figure 4). There may also be the possibility of additional favourable cation-pi interactions from the positively charged pyrrolidine to the delocalised Trp side chain with a distance of around 4.3-4.9 Å. Although it is difficult to extrapolate putative mammalian fucosidase interactions distal to the -1 subsite, it is expected that a similar VDW stacking environment will pertain in the mammalian enzymes24 such as that from bovine kidney, which would explain, at least in part, the significant increase in affinity that was experimentally measured with dimer **1**.

**Figure 3**. Compound **1** lying in the active site of *Bt*Fuc2970; the map shown is a likelihood-weighted *F*o-*F*c map calculated prior to the incorporation of phases from compound **1** in refinement, contoured at 2σ. The catalytic nucleophile Asp229 and acid/base Glu288 residues are annotated. The figure was drawn using CCP4MG.34



**Figure 4**. The second pyrrolidine moiety stacks on top of Trp232 in *Bt*Fuc2970. Van der waals radii for the second pyrrolidine moiety of 1 (cyan) and Trp232 residue of *Bt*Fuc2970 (coral) are drawn as spherical volumes. The figure was drawn using CCP4MG.34

Conclusions

The development of new enzyme inhibitors aspotential therapeutic agents, and biochemical tools, would clearly benefit from ways to improve their inhibition potencies. One option is to mimick Nature's use of avidity. Classically, avidity (in the carbohydrate-binding context) can have many forms - (a) the simultaneous harnessing of multiple natural carbohydrate binding sites on a multimeric glycan receptor - (b) the sequential statistical rebinding to minimize entropic losses and - (c) the harnessing of additional binding surfaces. Several glycosidase inhibitors with multivalent architectures have been described in literature, with contrasting successes. The weakness of this approach lies in our poor knowledge of the mechanism at play. Here, we studied the enhancement in binding affinities of stereoisomeric di-iminosugars towards fucosidase, focusing on the possible mode of action. The structures of the compounds were designed in order to favour the statistical rebinding over other possible mechanisms. As expected, a significant increase in enzyme-inhibition potencies was observed with the dimeric iminosugar **1** when compared to its monovalent equivalent **3**. However, though a rebinding mechanism might not be completely ruled out, we showed that the dimer works either through harnessing of distal surfaces for optimal binding. The 3-D structure of *Bt*Fuc2970 in complex with **1** is a rare example where the linker and the aglycon (here the second imino-sugar) are visible, and thus strongly bound, impairing a possible recapture. Interestingly, besides the interaction of the pyrrolidine moiety with the active site, adventitious interactions might occur in fucosidases either with attached hydrophobic aglycons, as previously observed,24,26,27 but also with non hydrophobic groups like the second pyrrolidine moiety in compounds **1** or ***meso*-1**. The multiplication of contact elements in the structure of the inhibitor, which is gained through dimerization, certainly accounts for the observed improvement in enzyme inhibition. Nevertheless, this work highlights the benefit of a multivalent approach even on systems where a classical multimer avidity is not favourable, but in which a multiple ligand approach may still be useful through multiplication of fortuitous favourable interactions.

Experimental

**Chemistry :**

*General considerations*: All reagents and solvents were commercially available in high purity and used as received. Silica gel F254 (0.2 mm) was used for TLC plates, detection being carried out by spraying with an alcoholic solution of phosphomolybdic acid, *p*-anisaldehyde or an aqueous solution of KMnO4 (2%) / Na2CO3 (4%), followed by heating. Flash column chromatography was performed over silica gel M 9385 (40-63 m) Kieselgel 60. NMR spectra were recorded on Bruker AC 250 (250 MHz for 1H, 62.5 MHz for 13C) or 600 (600 MHz for 1H, 150 MHz for 13C) spectrometers. Chemical shifts are expressed in parts per million (ppm) and were calibrated to the residual solvent peak. Coupling constants are in Hz and splitting pattern abbreviations are: br, broad; s, singlet; d, doublet; t, triplet; q, quartet; qt, quintuplet; m, multiplet. IR spectra were recorded with an IRTM plus MIDAC spectrophotometer and are expressed in cm-1. Optical rotations were determined at 20 °C with a Perkin-Elmer Model 241 polarimeter in the specified solvents. High Resolution Mass Spectra (HRMS) were performed on Q-TOF Micro micromass positive ESI (CV = 30 V).

**General procedure**

Compound **7** (or ***ent-*7**). To a solution of d-ribose acetonide **5**24 (4.059 g, 23.31 mmol) in dichloromethane (26 ml) were added 4 Å molecular sieves (9.2 g) and benzylamine (10.1 ml, 93.2 mmol, 4 equiv.). The mixture was stirred for 4 days at room temperature. Filtration through a pad of Celite® and subsequent evaporation afforded glycosylamine **6** (5.9 g, 97%, yellow oil), which was used in the next step without further purification. Ethynylmagnesium bromide (22 ml of a commercial 0.5 M solution in THF, 11.4 mmol, 3.7 equiv.) was added to a stirred solution of glycosylamine **6** (808 mg, 3.09 mmol) in THF (20 mL) at 0 °C and the resulting mixture was left to react at rt for 7 h. Saturated NH4Cl was then added and the solution was extracted with Et2O. The organic layer was dried (MgSO4), evaporated and aminoalcohol **7** was purified by silica gel chromatography (Et2O/petroleum ether, 6/4) and obtained as a brown oil (633 mg, 70%). *R*f = 0.19 (Et2O/ Petroleum ether : 5/5); []D20 = – 6.8 (*c* 1, CHCl3); IR (film) max, 1075, 1224, 1243, 1372, 1455, 2871, 2936, 2989, 3288; 1H NMR (CDCl3, 600 MHz) 1.19 (d, 3H, *J* = 6.1 Hz, CH3), 1.28 (s, 3H, iPr), 1.35 (s, 3H, iPr), 2.41 (d, 1H, *J* = 2.1 Hz, C≡C*H*), 3.51-3.56 (m, 1H, 6-H), 3.58 (dd, 1H, *J* = 9.6, 2.1 Hz, 3-H), 3.75 (d, 1H, *J* = 11.9 Hz, C*H*2-Ph), 3.85 (dd, 1H, *J* = 9.2, 5.3 Hz, 5-H), 3.98 (dd, 1H, *J* = 5.3, 9.6 Hz, 4-H), 3.99 (d, 1H, *J* = 11.9 Hz, C*H*2-Ph), 7.10-7.34 (m, 5H, Ar).; 13C NMR (151 MHz; CDCl3) 20.3 (CH3), 25.5 (iPr), 28.08 (iPr), 50.2 (3-C), 51.5 (*C*H2-Ph), 64.5 (6-C), 73.5 (C≡*C*H), 78.2 (4-C), 82.2 (*C*≡CH), 82.9 (5-C), 109.0 (iPr), 127.9, 128.8, 129.0 (3xAr), 137.4 (Cq‑Ar); HRMS-ESI+ (*m/z*): [M+H]+ calcd for C17H24NO3: 290.1756; found 290.1756.

Compound ***ent-*7** was prepared froml-ribose acetonide ***ent*-5**21following an identical synthetic route. This sample showed superimposable NMR spectra compared with those of its enantiomer **7** and was used in the subsequent cyclization step without further purification.

Compound **8** (or ***ent-*8**). MsCl (441 µl, 5.36 mmol, 2.45 equiv.) was added to a solution of amino-alcohol **7** (633 mg, 2.19 mmol) in dry pyridine (3 ml) and THF (3 mL) at 0 °C. The mixture was stirred for 2 h at 0 °C and for an additional 2h period at rt. Then H2O and Et2O were successively added and the resulting organic phase was separated. The aqueous layer was washed with Et2O and the combined organic phases were dried over MgSO4 and concentrated. The residue was puriﬁed by silica gel chromatography (Et2O/PE : 1/19) to yield pyrrolidine **8** (403 mg, 68%) as colorless oil. *R*f = 0.30 (Et2O/ Petroleum ether : 1/19); []D20 = + 180.6 (*c* 1.0, CHCl3); IR (film) max 1001, 1074, 1128, 1212, 1379, 1455, 2815, 2936, 2988, 3287; 1H NMR (CDCl3, 500 MHz) 1.22 (d, 3H, *J* = 6.3 Hz, CH3), 1.33 (s, 3H, iPr), 1.54 (s, 3H, iPr), 2.34 (d, 1H, *J* = 2.2 Hz, C≡CH), 2.75-2.85 (dq, 1H, *J* = 6.3, 4.4 Hz, 5-H), 3.48 (d, 1H, *J* = 13.3 Hz, C*H*2-Ph), 3.69 (d, 1H, *J* = 2.2 Hz, 2-H), 3.92 (d, 1H, *J* = 13.3 Hz, C*H*2-Ph), 4.60 (dd, 1H, *J* = 6.2, 4.4 Hz, 4‑H), 4.63 (d, 1H, *J* = 6.2 Hz, 3-H), 7.23 (t, 1H, *J* = 7.4 Hz, Ar), 7.30 (t, 2H, *J* = 7.4 Hz, Ar), 7.38 (d, 2H, *J* = 7.4 Hz, Ar); 13C NMR (126 MHz; CDCl3) 12.9 (CH3), 26.2 (iPr), 26.6 (iPr), 51.8 (*C*H2-Ph), 58.4 (2-C), 59.6 (5-C), 75.7 (C≡*C*H), 78.6 (*C*≡CH), 82.4 (4-C), 83.2 (3-C), 112.0 (Cq-iPr), 127.0, 128.4, 128.8 (3xAr), 139.2 (Cq-Ar); HRMS-ESI+ (*m/z*): [M+H]+ calcd for C17H22NO2: 272.1651; found 272.1640.

Compound ***ent-*8** was prepared from ***ent*-7** and purified by the same method. The spectroscopic data of ***ent-*8** were superimposable with those of **8**; []D20 = – 189 (*c* 0.56, CHCl3).

Dimer **9** (or ***ent-*9**). To a solution of ethynyl-pyrrolidine **8** (75 mg, 0.277 mmol) in THF (3 ml) were added successively Pd(PPh3)2Cl2 (6 mg), CuI (3 mg) and diisopropylamine (0.09 ml). The mixture was stirred at room temperature for 3 h. Then sat. NH4Cl and Et2O were successively added and the resulting organic phase was separated. The aqueous layer was washed with Et2O and the combined organic phases were washed with brine, dried over MgSO4 and concentrated. Purification by chromatography (Et2O/PE: 2/8) yielded dimer **9** (74 mg, 98%) as a yellow oil. *R*f = 0.5 (Et2O/ Petroleum ether : 2/8); []D20 = + 488 (*c* 1, CHCl3); IR (film) max, 1126, 1210, 1372, 1455, 1738, 2811, 2934, 2984; 1H NMR (CDCl3, 250 MHz) 1.23 (d, 6H, *J* = 6.1 Hz, CH3), 1.33 (s, 6H, iPr), 1.54 (s, 6H, iPr), 2.77-2.98 (m, 2H, 5-H), 3.48 (d, 2H, *J* = 13.5 Hz, NC*H*2Ph), 3.75 (s, 2H, 2-H), 3.96 (d, 2H, *J* = 13.5 Hz, NC*H*2Ph), 4.56-4.70 (m, 4H, 3 and 4-H), 7.17-7.54 (m, 10H, Ar); ); 13C NMR (63 MHz; CDCl3) 12.86 (CH3), 26.15 (iPr), 26.56 (iPr), 51.91 (NBn), 59.19, 59.92 (2 and 5‑C), 71.82, 74.61 (*C*≡*C*), 82.34, 82.87 (3 and 4-C), 112.15 (iPr), 127.11, 128.43, 128.74 (3xAr-C), 138.88 (Cq-Ar); HRMS-ESI+ (*m/z*): [M+H]+ calcd for C34H41N2O4: 541.3066; found 541.3046.

Dimer ***ent-*9** was prepared from ***ent*-8** and purified by the same method. The spectroscopic data of ***ent-*9** were superimposable with those of **9**; []D20 = – 502 (*c* 0.56, CHCl3).

Dimer **1** (or ***ent-*1**). Hydrogenation of **9** (37 mg, 0.07 mmol) was effected overnight in MeOH (2 mL) in the presence of Pd/C (37 mg) under 1 atm H2. The resulting suspension was filtered over a pad of celite washed several times with MeOH. Evaporation of the solvent gave a colourless oil (17 mg), which was diluted in MeOH (0.5 mL) and 1 *M* HCl (0.5 mL) and stirred for 16 h at rt. After evaporation, the crude product was purified by ion exchange chromatography on Dowex 50WX8 (H+) to give dimer **1** (12 mg, 63%) as a colourless solid. []D20 = – 60.4 (*c* 0.3, H2O); 1H NMR (CD3OD, 500 MHz) δ 1.19 (d, 6H, *J* = 6.6 Hz, CH3), 1.38-1.58 (m, 6H, 3xC*H*2), 1.60-1.74 (m, 2H, C*H*2), 3.01-3.12 (m, 2H, 2-H), 3.26 (dq, 2H, *J* = 6.6, 3.5 Hz, 5-H), 3.78 (dd, 2H, *J* = 7.9, 3.5 Hz, 3-H), 3.83 (t, 2H, *J* = 3.5 Hz, 4-H); 13C NMR (CD3OD, 126 MHz) δ 14.39 (CH3), 27.90 (Pyr-CH2-*C*H2), 35.04 (Pyr-*C*H2-CH2), 56.35 (5-C), 62.44 (2-C), 75.01 (4-C), 79.66 (3-C); HRMS-ESI+ (*m/z*): [M+Na]+ calcd for C14H28NaN2O4: 311.1947; found 311.1953.

Dimer ***ent-*1** was prepared from compound ***ent*-9** and purified by the same method. The spectroscopic data of ***ent-*1** were superimposable with those of **1**; []D20 = +75 (*c* 0.29, H2O).

Pyrrolidine **3** (or ***ent-*3**). Hydrogenation and acid hydrolysis of **8** (42 mg, 0.15 mmol) was effected as described above for **9**. After purification by ion exchange chromatography on DOWEX 50WX8 (H+) pyrrolidine **3** was obtained as a colourless oil (22 mg, 96%). []D20 = – 61.2 (*c* 0.33, MeOH); 1H NMR (D2O, 500 MHz) δ 0.96 (t, 3H, *J* = 7.5 Hz, CH2C*H*3), 1.12 (d, 3H, *J* = 6.8 Hz, CH3); 1.38-1.47 (m, 1H, C*H*2CH3), 1.60-1.71 (m, 1H, C*H*2CH3), 2.95 (dt, 1H, *J* = 4.9 8.3 Hz, 2-H), 3.24 (dq, 1H, *J* = 4.1 6.8 Hz, 5-H), 3.89 (dd, 1H, *J* = 4.1 8.3, Hz, 3-H), 3.93 (bt, 1H, , *J* = 4.1 Hz, 4-H); 13C NMR (D2O, 126 MHz) δ 10.58 (CH2*C*H3), 14.54 (CH3), 26.49 (*C*H2CH3), 53.85 (5-C), 61.78 (2-C), 74.20 (4-C), 77.73 (3-C); HRMS-ESI+ (*m/z*): [M+H]+ calcd for C7H16NO2: 146.1181; found 146.1185.

Pyrrolidine ***ent-*3** was prepared from ***ent*-8** and purified by the same method. The spectroscopic data of ***ent-*3** were superimposable with those of **3**; []D20 = +48 (*c* 0.4, MeOH).

Bis-pyrrolidine **13**. To a solution of ethynyl-pyrrolidine ***ent*-8** (67 mg, 0.247 mmol) and **11** (124 mg, 0.561 mmol) in THF (6 ml) were added successively Pd(PPh3)2Cl2 (12 mg), CuI (6 mg) and diisopropylamine (0.186 ml). The mixture was stirred at room temperature for 3 h. Then sat. NH4Cl and Et2O were successively added and the resulting organic phase was separated. The aqueous layer was washed with Et2O and the combined organic phases were washed with brine, dried over MgSO4 and concentrated. Purification by preparative TLC (Et2O/PE/CH2Cl2: 1/5/4) yielded dimer ***ent-*9** (*R*f = 0.6, 18 mg), dimer **12** (*R*f = 0.3, 78 mg) and the targeted bis-pyrrolidine **13** (*R*f = 0.45, 74 mg, 61%) as a yellow oil. []D20 = – 185 (*c* 0.16, CHCl3); 1H NMR (CDCl3, 250 MHz) 1.07 (d, 3H, *J* = 6.3 Hz, CH3), 1.14 (d, 3H, *J* = 6.3 Hz, CH3); 1.25 (s, 6H, iPr), 1.42 (s, 3H, iPr), 1.45 (s, 3H, iPr), 2.62 (quint, 1H, *J* = 6.3 Hz, 5-H), 2.70-2.77 (m, 1H, 5’-H), 2.91 (dd, 1H, *J* = 7.9 13.4 Hz, NC*H*2CH=CH2), 3.26-3.35 (m, 1H, NC*H*2CH=CH2), 3.38 (d, 1H, *J* = 13.4 Hz, NC*H*2Ph), 3.65 (s, 1H, 2-H), 3.83 (d, 1H, *J* = 13.4 Hz, NC*H*2Ph), 3.90 (s, 1H, 2’-H), 4.48-4.63 (m, 4H, 3-H, 3’-H, 4-H, 4’-H), 5.03-5.10 (m, 1H, NCH2CH=C*H*2), 5.19-5.30 (m, 1H, NCH2CH=C*H*2), 5.68-5.86 (5.03-5.18 (m, 1H, NCH2C*H*=CH2), 7.12-7.40 (m, 5H, Ar-H); 13C NMR (63 MHz; CDCl3) 12.5 (CH3), 12.7 (CH3), 25.7 (CH3), 26.0 (CH3), 26.2 (CH3), 26.4 (CH3), 50.8 (CH2), 51.7 (CH2), 59.0, 59.3, 59.7 and 59.7 (2-C, 2’-C, 5-C and 5’-C), 71.4, 71.6, 74.4 and 74.5 (2x-*C*≡*C-*), 82.0, 82.2, 82.6 and 82.7, (3-C, 3’-C, 4-C and 4’-C), 111.8 and 112.0 (Cq-iPr), 117.3 (NCH2CH=*C*H2), 126.9, 128.2 and 128.5 (Ar-C), 135.3 (NCH2*C*H=CH2), 138.7 (Ar-Cq); HRMS-ESI+ (*m/z*): [M+H]+ calcd for C30H39N2O4: 491.2910; found 491.2911.

Compound ***meso-*1**. Deprotection of **13** was effected in a 3-step procedure by treating first a solution of **13** (100 mg, 0.2 mmol) in CH2Cl2 (3 mL) with N,N’-dimethylbarbituric acid (129 mg) and Pd(PPh3)4 (23 mg) for 10 h at 40 °C. Water was then added and the solution was extracted with CH2Cl2. The organic layer was dried (MgSO4), evaporated and intermediate **14** was purified by silica gel chromatography (Et2O/petroleum ether, 5/5) and obtained as a yellow oil (45 mg, 50%). For **14**, []D20 = – 271 (*c* 0.9, CHCl3). Hydrogenation and acidolysis of **14** (43 mg) was effected as described above for the synthesis of **1** to give dimer ***meso-*1** as a colourless powder (10 mg, 37%). []D20 = 0 (*c* 0.2, MeOH); 1H NMR (CD3OD, 500 MHz) δ 1.16 (d, 6H, *J* = 6.7 Hz, CH3); 1.38-1.55 (m, 6H, 3xC*H*2), 1.62-1.71 (m, 2H, C*H*2), 3.02 (dt, 2H, *J* = 5.3 7.5 Hz, 2-H), 3.20 (dq, 2H, *J* = 4.0 6.6 Hz, 5-H), 3.75 (dd, 2H, *J* = 4.0 7.5, Hz, 3-H), 3.93 (bt, 1H, *J* = 4.0 Hz, 4-H); 13C NMR (CD3OD, 126 MHz) δ 14.62 (CH3), 28.04 and 35.46 (*C*H2), 56.17 (5-C), 62.50 (2-C), 75.22 (4-C), 79.88 (3-C); HRMS-ESI+ (*m/z*): [M+Na]+ calcd for C14H28N2O4Na: 311.1947; found 311.1940.

Compound **4**. Hydrogenation of **15**24(11 mg, 0.045 mmol) was effected as described above for **1**. After purification by chromatography on silica gel (EA/MeOH, 8/2) **4** was obtained as a yellow oil (10.6 mg, 93%). []D20 = – 38 (*c* 0.35, MeOH); 1H NMR (CD3OD, 500 MHz) δ 1.37 (d, 3H, *J* = 6.9 Hz, CH3); 1.43-1.57 (m, 2H, CH2), 1.65-1.77 (m, 3H, CH2), 1.83-1.92 (m, 1H, CH2), 2.60-2.70 (m, 2H, CH2), 3.34-3.40 (m, 1H, 2-H), 3.67 (dq, 1H, *J* = 2.6 6.9, Hz, 5-H), 3.96-3.99 (m, 2H, 3-H, 4-H), 7.12-7.28 (m, 5H, Ar); 13C NMR (CD3OD, 126 MHz) δ 12.23 (*C*H3), 27.22, 32.18, 32.24 and 36.57 (4🞨 -*C*H2-), 57.92 (5-C), 62.45 (2-C), 73.25 (4-C), 77.83 (3-C), 126.80, 129.33, 129.40 (3xAr) and 143.39 (Cq-Ar); HRMS-ESI+ (*m/z*): [M+H]+ calcd for C15H24NO2: 250.1807; found 250.1811.

**Glycosidase inhibition assays :**

**IC50 determination on fucosidase from bovine kidney.** Compounds were assayed according to a reported procedure.24 Enzyme activity was determined at 35 °C (acetate buffer, pH = 5.6) after incubation of 2 mM *p*-nitrophenyl fucoside for 15 min and quenching the reaction by addition of 0.4 M sodium carbonate. The p-nitrophenolate formed was quantified at 410 nm. Inhibitors were pre-incubated at 35°C for 5 min with the fucosidase before addition of the substrate. At least five concentrations of each compound were tested and IC50’s were determined using Dixon plots.

**General procedure for the *K*i determination on fucosidase from bovine kidney**. Inhibition constants (*K*i) were determined spectrophotometrically by measure of the absorbance at 405 nm of the *p*-nitrophenolate resulting from the hydrolysis of the *p*-nitrophenyl α-L-fucopyranoside (substrate) by α-fucosidase from bovine kidney, in the presence of the corresponding inhibitor. Each assay was performed at pH 6 (phosphate-citric acid buffered solution). For each data point, the experiments were run by addition of the enzyme to a solution of the substrate in the absence or presence of varying concentration of inhibitor (inhibitor concentrations straddling the *K*i). Different concentrations for the substrate were also used. The mixtures were incubated for 20 min at 37 ºC and each reaction was stopped by addition of sodium borate solution (pH 9.8). The experiment for each data point was performed by duplicate. The inhibition type and *K*i values were determined from the Lineweaver-Burk plots (See supporting information).

***K*i determination of compound 1 on *Bt*Fuc2970.** Substrate 2-chloro-4-nitrophenyl-α-l-fucopyranoside (CNP-fucoside) was purchased from Carbosynth Ltd. Experiments were run over a time course of 5 minutes during which absorbance at 405 nm was detected. For each data point, a solution of 50 mM HEPES buffer pH 7.4, 100 mM NaCl, 250 nM *Bt*Fuc2970 was equilibrated thermally (37 ºC) in the presence of a varying concentration (inhibitor concentrations straddling the *K*i) of inhibitor. To each of these solutions 50 µM CNP-fucoside was added to initiate hydrolysis. *K*i was determined by analysing initial enzyme rates in the absence of inhibitor and comparing with the rate in the presence of increasing concentrations of inhibitor. The *K*i was calculated as the reciprocal of the gradient of the plot (see Supporting Information).

**Mannosidase inhibition assay**. Compounds were assayed at 1 mM concentration, according to the reported procedure.35 Enzyme activity was determined at 35 °C (acetate buffer, pH = 5.6) after incubation of 2 mM *p*-nitrophenyl mannoside for 15 min and quenching the reaction by addition of 0.4 M sodium carbonate.

**X-ray crystal structure determination**. A gene encoding *Bacteroides thetaiotaomicron* α-ʟ-fucosidase Fuc2970 was expressed and *Bt*Fuc2970 protein was purified as described previously.[36](#_ENREF_2) *Bt*Fuc2970 crystals were obtained in similar conditions to those described previously. *Bt*Fuc2970 crystals were transferred to a 1 µL solution (20% w/v PEG 3350, 0.1 M ammonium sulphate, 0.1 M imidazole pH 7.0) To this solution, 1 µL **1** (10 mM in 10 mM HEPES, 100 mM NaCl, pH 7.0) was added for ligand complexation. After incubation for *ca*. 30 minutes, crystals were transferred to solutions containing mother liquor supplemented with 20% glycerol, and cryo-cooled using liquid N2. Crystals were tested for diffraction in-house and diffraction images for diffracting crystals collected at Diamond Light Source beamline I03. Diffraction data were indexed and integrated using iMOSFLM37 and scaled and merged using AIMLESS38. The diffracting crystal of *Bt*Fuc2970:**1** was observed in a space group almost isomorphous with PDB entry 2WVV; model coordinates from the protein atoms of 2WVV were used directly to obtain initial phases for the model. Rfree sets were assigned as in PDB entry 2WVV to maintain the integrity of the cross-validation data set. The structural model was refined iteratively using COOT39 and REFMAC5[40](#_ENREF_6). A coordinate set and geometrical restraints for inhibitor **1** were kindly created manually by Garib Murshudov (Medical Research Council Laboratory of Molecular Biology, Cambridge); these were used to introduce inhibitor **1** to the structural model. The final model for *Bt*Fuc2970:**1** was validated using MOLPROBITY[41](#_ENREF_7), after which X-ray data were deposited with the RCSB Protein Data Bank ([www.rcsb.org](http://www.rcsb.org)42, see Supporting Information).

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**Graphical Abstract :**



The possible mechanisms of action of a dimeric fucosidase inhibitor are discussed through enzymatic assays of a series of analogues and crystallographic analysis of the enzyme-inhibitor complex.