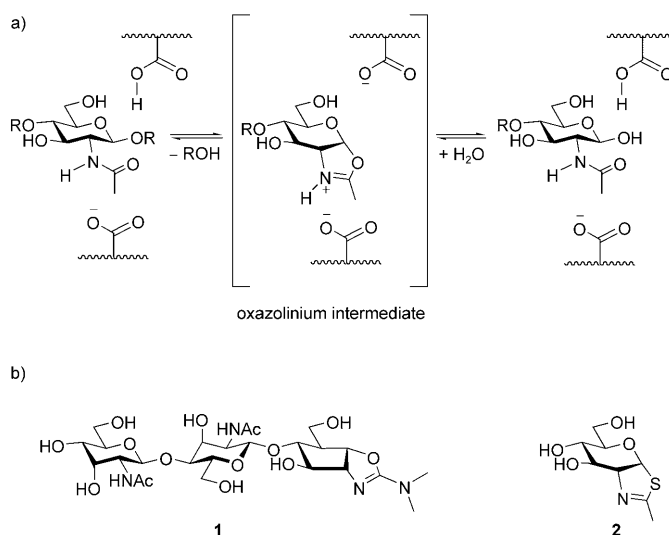


Chitinase Inhibition by Chitobiose and Chitotriose Thiazolines**

James M. Macdonald, Chris A. Tarling, Edward J. Taylor, Rebecca J. Dennis, David S. Myers, Spencer Knapp, Gideon J. Davies, and Stephen G. Withers*

Chitinases (EC 3.2.1.14; glycosidase families GH 18 and 19)^[1] are widely distributed enzymes that hydrolyze the glycosidic linkage of the repeating β -1,4-linked *N*-acetylglucosamine-containing polymer chitin. Inhibitors of chitinases have a number of potential applications, including use as insecticides and fungicides,^[2] the prevention of human-malaria-parasite transmission,^[3] and the treatment of human asthma^[4] as well as the sexually transmitted disease human trichomoniasis.^[5] Known chitinase inhibitors include cyclic dipeptides,^[6] cyclopentapeptides,^[7] pseudo-sugars, such as glycosylamides,^[8] a chitobionoxime,^[9] and allosamidin (**1**, and its analogues).^[10] Allosamidin (**1**) is the most potent broad-spectrum inhibitor of chitinases ($K_i = 1 \text{ nM}$ – $1 \text{ }\mu\text{M}$); however, it remains very difficult to synthesize in useful quantities.^[10]

The generally accepted mechanism of action of the retaining chitinases of family 18 involves neighboring-group participation with the formation of an oxazolinium intermediate within the -1 subsite (Scheme 1 a).^[11] Indeed, the powerful inhibitory effect of **1** (Scheme 1 b) is ascribed to the (protonated) cyclic-urea moiety, which resembles the oxazolinium-ion intermediate. The *N*-acetyl- β -hexosaminidases from GH 20 and 84 are exo-acting hydrolases that share the same substrate-assisted mechanism^[12] but cleave a single sugar at a time. The *N*-acetylglucosamine thiazoline **2** was designed as a stable mimic of the oxazolinium intermediate and/or closely related transition states, and was shown to be a potent, competitive inhibitor of enzymes from these families.^[12]



Scheme 1. a) Mechanism of chitin hydrolysis by family-18 chitinases. R = β -1,4-linked *N*-acetylglucosamine residue(s). b) Structures of allosamidin (**1**) and the *N*-acetylglucosamine thiazoline **2**.

In keeping with their endoglucosaminidase activity, chitinases are not significantly inhibited by the monosaccharide thiazoline **2**. However, it seemed logical that chitobiose or chitotriose thiazolines (e.g. **3** and **4**) might well function as good inhibitors, as observed analogously in the inhibition of the *N*-glycoprotein-degrading endo-*N*-acetylglucosaminidase endo-A.^[13] A major concern with the use of oligosaccharide-derived inhibitors as biological probes is degradation by exo- and endohexosaminidases. This problem is obviated in allosamidin by the attachment of *allo*-configured HexNAc residues that are not cleaved by exohexosaminidases and yet do not deleteriously affect chitinase binding. Herein we describe efficient and scaleable syntheses of **3** and **4**, as well as their thioamide analogues, **9** and **10**, which we show to be metabolically stable. Kinetic and X-ray crystallographic analysis of their binding to the model enzyme chitinase A (ChiA) from *Serratia marcescens* (ATCC 990) confirmed that these compounds are potent inhibitors and provided structural insight into their high affinities and the toleration of thioamide groups.

The synthesis of the di- and trisaccharide thiazolines **3** and **4** started with octaacetylchitobiose (**5**) and undecaacetylchitotriose (**6**), respectively (Scheme 2). Both **5** and **6** are commercially available; they can also be synthesized from the naturally abundant polymer chitin by chemical^[14] (acetylation reaction) or chemoenzymatic methods^[14d,15] (enzymatic hydrolysis followed by conventional acetylation). The

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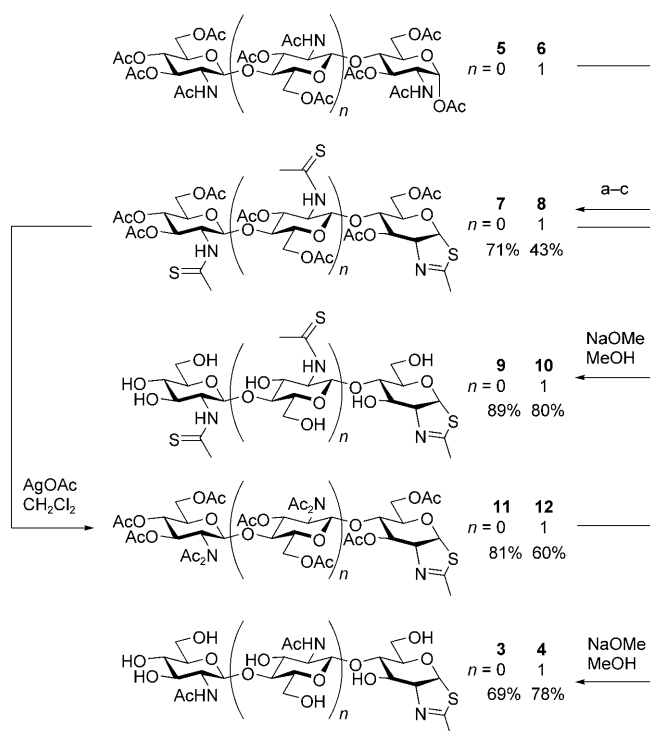
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Scheme 2. Synthesis of chitobiose and chitotriose thiazolines **3** and **4** and their thioamide analogues **9** and **10**. a) HCl, AcOH; b) AgOAc, AcOH; c) Lawesson reagent, Cl(CH₂)₂Cl.

anomeric configurations of the α -acetoxy groups of **5** and **6** were inverted to give the corresponding β anomers by initial treatment with HCl and AcOH to give the anomeric chlorides, followed by treatment with AgOAc in AcOH. Next, treatment with the Lawesson reagent effected both the conversion of the amides into thioamides as well as intramolecular displacement of the anomeric β -acetoxy group by the sulfur atom of the adjacent thioamide to afford thiazolines **7** and **8** in 71 and 43% yield, respectively (3 steps from compounds **5** and **6**). Portions of each of the per-O-acetylated thiazolines **7** and **8** were deacetylated to give two additional chitinase inhibitors: the chitobiose thiazoline thioamide **9** (89% yield) and chitotriose thiazoline dithioamide **10** (80% yield). To reach **3** and **4**, we first converted the thioamides **7** and **8** into the diacetylimides **11** and **12** with silver acetate in dichloromethane^[16] (in 81 and 60% yield, respectively) without damage to the thiazoline moiety. Finally, imides **11** and **12** were O-deacetylated and mono-N-deacetylated by using sodium methoxide in methanol to give the targets analogous to **2**: the chitobiose thiazoline derivative **3** (69% yield) and the chitotriose thiazoline derivative **4** (78% yield).

Inhibitor **3** was also synthesized by a more protracted route comprising the coupling of 3-O-acetyl-6-O-benzoyl-GlcNAc thiazoline with *N*-trichloroacetyl-protected, per-O-acetyl-protected glucosamine trichloroacetimidate, followed by deprotection, as described in detail in the Supporting Information. This latter route offers flexibility for the attachment of modified sugar moieties; however, it is less efficient than the chemoenzymatic route for the synthesis of the parent compound and would be challenging for longer congeners.

Kinetic analysis of the inhibition of chitinase A from *S. marcescens* was carried out with 4-nitrophenyl-*N,N'*-diacetyl- β -chitobioside as the substrate. To a certain extent, this enzyme exhibits non-Michaelian kinetic behavior, with activation at low substrate concentrations and substrate inhibition at high concentrations.^[17] The substrate concentrations, [S], chosen for the inhibition studies (25–150 μ M) were in a region of the $v/[S]$ curve in which the effects of allosteric behavior and substrate inhibition are minimal.

Given the ambiguities in the literature concerning the pH dependence of ChiA, a pH profile of k_{cat}/K_M was first determined by the substrate-depletion method, which yielded a classic bell-shaped curve defined by $\text{p}K_a$ values of 7.9 and 4.7 and an optimum enzyme activity at pH 6.2 (see Figure 1 in the Supporting Information). All subsequent inhibition studies were performed at this pH value. Well-behaved competitive inhibition was observed in all cases (as exemplified for **3** in Figure 2 in the Supporting Information). K_i values determined in this way are presented in Table 1. The

Table 1: K_i values for the inhibition of chitinase Chi18A from *S. marcescens* by chitothiazolines.

Chitothiazoline inhibitor	K_i [μ M]
chitobiose thiazoline 3	25
chitobiose thiazoline thioamide 9	30
chitotriose thiazoline 4	0.25
chitotriose thiazoline dithioamide 10	0.15

monosaccharide version, GlcNAc thiazoline **2**, is a very poor inhibitor of ChiA, with $K_i > 1$ mM (results not shown). However, the addition of one GlcNAc residue, as in **3**, improved binding at least 40-fold, and the second GlcNAc residue provided a further 100-fold increase in affinity to bring down the K_i value for the pseudotrisaccharide **4** well below that measured for the inhibition of ChiA by allosamidin ($K_i = 0.6$ μ M). This result stands in contrast to the recent report that a disaccharide thiazoline with an interresidue sulfur linkage was not a significant inhibitor of ChiA; the lack of inhibition observed in that study is presumably due to a different geometry imposed by the thioglycosidic linkage.^[18] Importantly, the presence of thioamides rather than amides as the C2 substituents in the appended GlcNAc moieties had no deleterious effect upon binding, as seen in the K_i values for **9** and **10**. There is, however, a spectacular effect on degradation by exohexosaminidases: no degradation of **10** was observed upon extended incubation with high concentrations of the *Streptomyces plicatus* hexosaminidase.

To gain structural insight into the source of the huge affinity increases found upon inhibitor homologation, as well as to illuminate the reason for the lack of discrimination against the thioamides, we solved the structures of complexes of *S. marcescens* Chi18A (*Sm*Chi18A) at resolutions from 2.4 to 1.9 Å (see Table 1 in the Supporting Information). Electron density was clear and unambiguous for all four ligands (Figure 1). We found that both “disaccharide” thiazolines bind in the –2 and –1 subsites, as expected. In the case of the trisaccharide thiazolines, in both their amide and thioamide

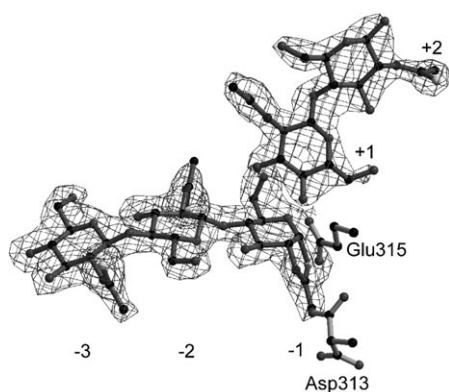
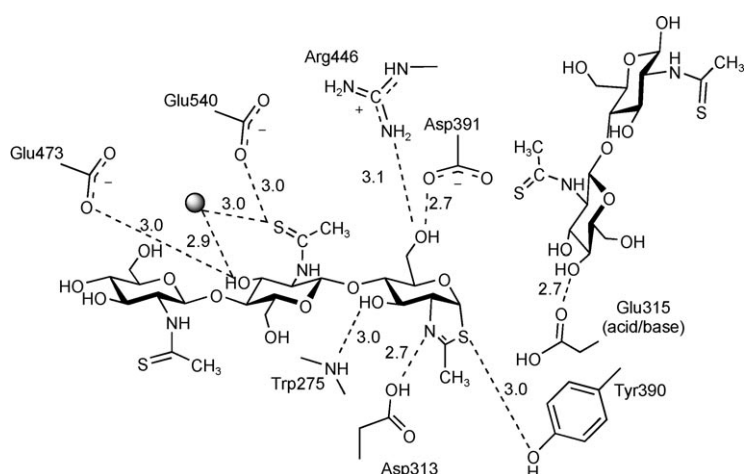


Figure 1. Electron density map determined for the complex of SmChi18A with **10**. The density map was prepared from a $2F_{\text{obs}} - F_{\text{calc}}$ synthesis and is contoured at approximately 1σ (ca. 0.4 electrons/Å). The two molecules of **10** in the -3 to -1 and $+1$ to $+2$ subsites are shown with gray bonds, along with the catalytic acid/base (Glu315) and the oxazoline stabilizer Asp313. Coordinates for complexes with **3**, **4**, **9**, and **10** are all deposited on the PDB (see Table 1 in the Supporting Information.)

forms, one molecule binds in the -3 to -1 subsites (as expected), and a second molecule binds in the “leaving-group subsites” $+1$ and $+2$ (with the third moiety of the ligand disordered in solvent; see Figure 3 in the Supporting Information). The electron density was unambiguous in the assignment of the pyranoside ring of the thiazoline as a 4C_1 chair conformation as observed previously for hexosaminidase/thiazoline complexes on families GH 20,^[19] 84,^[20] and 85.^[21] The complexes with **3**, **4**, **9**, and **10** enabled mapping of all the enzyme–substrate interactions within the -3 to $+2$ subsites, which are known to be the kinetically productive subsites of SmChi18A (Scheme 3). Notable is the double occupancy of Asp313, the residue that stabilizes the oxazolinium-ion intermediate through electrostatic and hydrogen-bonding effects, as recently shown for the *Streptomyces plicatus* hexosaminidase by computation.^[22] Dual occupancy in this case may reflect different protonation states of the carboxylic acid moiety.



Scheme 3. Representation of the interactions of SmChi18A with **10**.

The chitobiose and chitotriose thiazolines, particularly in their thioamide forms, are potent new mechanism-based hexosaminidase inhibitors that should therefore exhibit broad-spectrum inhibitory effects on chitinases. The synthesis of these inhibitors, especially in their thioamide forms, is practical and readily scalable. The stability of the thioamide derivatives towards degradation by exohexosaminidases is particularly noteworthy. As has been shown previously,^[12] the presence of thioamide groups dramatically slows enzyme-catalyzed cleavage. Furthermore, the potent GlcNAc thiazoline inhibitor **2** is produced directly upon cleavage; thus, further degradation is minimized. This design for enzymatic stability is simpler for the synthetic chemist than incorporation of the *N*-acetylglucosamine residues of **1**. Indeed, the thioamide is key to the formation of the thiazoline moiety itself. These properties, along with the demonstrated bio-availability of sugar thiazolines,^[23] make these compounds particularly effective reagents for the probing or modulation of chitinase activity in biological systems.

Experimental Section

Details of all synthetic steps and product characterization are provided in the Supporting Information, as are full details of cloning, expression, kinetic analysis, and structure solution of the SmChi18A complexes, along with additional information in the respective PDB headers. Briefly, the gene encoding ChiA was cloned and subsequently expressed by using a pET22b vector in *Escherichia coli* BL21 cells. Inhibition analyses were performed in 50 mM phosphate buffer (pH 6.3) with 4-nitrophenyl-*N,N'*-diacetyl- β -chitobioside as the substrate. The pH dependence of k_{cat}/K_M was determined by the substrate-depletion method. SmChi18A crystals were grown at 20 °C from a mixture (at pH 8.0) composed of 1.0 M sodium citrate, 10 mM sodium borate, and 10% (v/v) dioxane with protein at a concentration of 10 mg mL⁻¹. Complexes were obtained either by soaking with powdered **3**, **4**, **9**, or **10** for 24 h prior to data collection or by cocrystallization with approximately 5 mM ligand. Structures were solved and refined by using programs from the CCP4 suite.^[24]

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- [1] B. L. Cantarel, P. M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, B. Henrissat, *Nucleic Acids Res.* **2009**, *37*, D233–D238.
- [2] a) M. Londershausen, A. Turberg, B. Bieseler, M. Lennartz, M. G. Peter, *Pestic. Sci.* **1996**, *48*, 305–314; b) K. Shiomi, N. Arai, Y. Iwai, A. Turberg, H. Kolbl, S. Omura, *Tetrahedron Lett.* **2000**, *41*, 2141–2143; c) O. A. Andersen, M. J. Dixon, I. M. Eggleston, D. M. F. van Aalten, *Nat. Prod. Rep.* **2005**, *22*, 563–579.
- [3] J. M. Vinetz, J. G. Valenzuela, C. A. Specht, L. Aravind, R. C. Langer, J. M. C. Ribeiro, D. C. Kaslow, *J. Biol. Chem.* **2000**, *275*, 10331–10341.
- [4] Z. Zhu, T. Zheng, R. J. Homer, Y. K. Kim, N. Y. Chen, L. Cohn, Q. Hamid, J. A. Elias, *Science* **2004**, *304*, 1678–1682.

- [5] P. M. Loiseau, C. Bories, A. Sanon, *Biomed. Pharmacother.* **2002**, *56*, 503–510.
- [6] D. R. Houston, B. Synstad, V. G. H. Eijssink, M. J. R. Stark, I. M. Eggleston, D. M. F. van Aalten, *J. Med. Chem.* **2004**, *47*, 5713–5720.
- [7] F. V. Rao, D. R. Houston, R. G. Boot, J. Aerts, M. Hodgkinson, D. J. Adams, K. Shiomi, S. Omura, D. M. F. van Aalten, *Chem. Biol.* **2005**, *12*, 65–76.
- [8] A. Rottmann, B. Synstad, V. Eijssink, M. G. Peter, *Eur. J. Org. Chem.* **1999**, 2293–2297.
- [9] G. Vaaje-Kolstad, A. Vasella, M. G. Peter, C. Netter, D. R. Houston, B. Westereng, B. Synstad, V. G. H. Eijssink, D. M. F. van Aalten, *J. Biol. Chem.* **2004**, *279*, 3612–3619.
- [10] A. Berecibar, C. Grandjean, A. Siriwardena, *Chem. Rev.* **1999**, *99*, 779–844.
- [11] a) D. M. F. van Aalten, D. Komander, B. Synstad, S. Gaseidnes, M. G. Peter, V. G. H. Eijssink, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 8979–8984; b) I. Tews, A. C. Terwisscha van Scheltinga, A. Perrakis, K. S. Wilson, B. W. Dijkstra, *J. Am. Chem. Soc.* **1997**, *119*, 7954–7959; c) A. C. T. van Scheltinga, S. Armand, K. H. Kalk, A. Isogai, B. Henrissat, B. W. Dijkstra, *Biochemistry* **1995**, *34*, 15619–15623.
- [12] a) S. Knapp, D. Voadlo, Z. N. Gao, B. Kirk, J. P. Lou, S. G. Withers, *J. Am. Chem. Soc.* **1996**, *118*, 6804–6805; b) G. E. Whitworth, M. S. Macauley, K. A. Stubbs, R. J. Dennis, E. J. Taylor, G. J. Davies, I. R. Greig, D. J. Voadlo, *J. Am. Chem. Soc.* **2007**, *129*, 635–644.
- [13] B. Li, K. Takegawa, T. Suzuki, K. Yamamoto, L. X. Wang, *Bioorg. Med. Chem.* **2008**, *16*, 4670–4675.
- [14] a) S. A. Barker, A. B. Foster, M. Stacey, J. M. Webber, *J. Chem. Soc.* **1958**, 2218–2227; b) T. Osawa, *Carbohydr. Res.* **1966**, *1*, 435–443; c) E. W. Thomas, *Carbohydr. Res.* **1973**, *26*, 225–226; d) S. I. Nishimura, H. Kuzuhara, Y. Takiguchi, K. Shimahara, *Carbohydr. Res.* **1989**, *194*, 223–231.
- [15] a) M. Yalpani, D. Pantaleone, *Carbohydr. Res.* **1994**, *256*, 159–175; b) K. Matsuoka, Y. Matsuzawa, K. Kusano, D. Terunuma, H. Kuzuhara, *Biomacromolecules* **2000**, *1*, 798–800.
- [16] M. Avalos, R. Babiano, C. J. Durán, J. L. Jiménez, J. C. Palacios, *Tetrahedron Lett.* **1994**, *35*, 477–480.
- [17] Y. Honda, M. Kitaoka, K. Tokuyasu, C. Sasaki, T. Fukamizo, K. Hayashi, *J. Biochem.* **2003**, *133*, 253–258.
- [18] A. Fettke, A. Peikow, M. G. Peter, E. Kleinpeter, *Tetrahedron* **2009**, *65*, 4356–4366.
- [19] B. L. Mark, D. J. Voadlo, S. Knapp, B. L. Triggs-Raine, S. G. Withers, M. N. G. James, *J. Biol. Chem.* **2001**, *276*, 10330–10337.
- [20] R. J. Dennis, E. J. Taylor, M. S. Macauley, K. A. Stubbs, J. P. Turkenberg, S. J. Hart, G. N. Black, D. J. Voadlo, G. J. Davies, *Nat. Struct. Mol. Biol.* **2006**, *13*, 365–371.
- [21] D. W. Abbott, M. S. Macauley, D. J. Voadlo, A. B. Boraston, *J. Biol. Chem.* **2009**, *284*, 11676–11689.
- [22] I. R. Greig, F. Zahariev, S. G. Withers, *J. Am. Chem. Soc.* **2008**, *130*, 17620–17628.
- [23] a) M. S. Macauley, G. E. Whitworth, A. W. Debowski, D. Chin, D. J. Voadlo, *J. Biol. Chem.* **2005**, *280*, 25313–25322; b) S. A. Yuzwa, M. S. Macauley, J. E. Heinonen, X. Y. Shan, R. J. Dennis, Y. A. He, K. A. Stubbs, E. A. McEachern, G. J. Davies, D. J. Voadlo, *Nat. Chem. Biol.* **2008**, *4*, 483–490.
- [24] Collaborative Computational Project Number 4, *Acta Crystallogr. Sect. D* **1994**, *50*, 760.