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Inhibition of Two Family 18 Chitinases by Various Allosamidin Derivatives

Margarethe Spindler-Barth,[1]* Regine Blattner,[2] Constantine E. Vorgias[3] & Klaus Dieter Spindler[4]

[1] Lehrstuhl für Hormon-und Entwicklungsphysiologie, Heinrich-Heine Universität Düsseldorf, Germany
[2] Industrial Research Limited, Gracefield Research Center, Lower Hutt, New Zealand
[3] Division of Biochemistry and Molecular Biology, Dept of Biology, University of Athens, Greece
[4] Abteilung Allgemeine Zoologie (Biologie I), Universität Ulm, Germany

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Abstract: The inhibitory activities of several allosamidin derivatives on two family 18 chitinases, an insect enzyme from the epithelial cell line from *Chironomus tentans*, and a bacterial enzyme, chitinase A from *Serratia marcescens*, were evaluated. The following structural requirements are necessary for inhibition of the *Chironomus* enzyme:

1. One *N*-acetylallosamine residue can be omitted without impairment of enzyme inhibition.
2. At least one *N*-acetylallosamine sugar must be present.
3. Glucosamine can replace the allosamine moiety without a negative effect on the inhibitory activity.
4. The spatial arrangement of the allosamizoline moiety is important for inhibition.
5. If one sugar is omitted and the arrangement of the cyclitol residue is changed, the inhibitory effect is diminished further.

For purified chitinase A from *Serratia marcescens* the arrangement of the aglycone moiety is equally important, but recognition of the sugar is different:

1. Omission of one allosamine residue decreases the inhibitory activity considerably.
2. Inhibition is improved if the remaining *N*-acetylallosamine is replaced by the epimer *N*-acetylglucosamine.

Only endochitinase activity is affected, since chitin formation (up to 10^{-4} M) and *N*-acetylglucosaminidase activity (up to 10^{-3} M) are not impaired, at least in *Chironomus* cells. © 1998 SCI.

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Key words: structure–activity relationship; insecticide cell line; *Chironomus tentans*; *Serratia marcescens*

1 INTRODUCTION

Allosamidin, a fermentation product of *Streptomyces sp.*[1] inhibits chitinases from various phyla with considerable differences in sensitivity.[2–6] However, within insects, the effect of allosamidin on chitinases from the lepidopteran *Bombyx mori* L.,[2] the mite *Boophilus microplus* Can., the dipteran *Lucilia cuprina* (Weid.) (unpublished results) and from culture supernatant of the dipteran cell line from *Chironomus tentans* Walk.,[2] are rather similar with K_i values below 1 μM, although different methods for determination of enzymatic activity were used. This similarity means that the convenient source of enzyme culture supernatant of the epithelial

* To whom correspondence should be addressed.

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cell line from *Chironomus tentans* can be used to represent a range of insect preparations in determining the essential structural requirements of the allosamidin molecule necessary for inhibition of chitin degradation.

The catalytic centre of insect chitinases characterized so far consists of a highly conserved $(\beta a)_8$ barrel, typical for members of family 18 glycosylhydrolases[7] which includes also enzymes from other eukaryotes and from prokaryotes, like chitinase A from *Serratia marcescens* Bizio[8] and the plant enzyme hevamine.[9]

Since the essential amino acids of the catalytic domain of chitinases from *Chironomus* and *Serratia* are identical, whereas other domains are different (Feix, M., pers. comm.), we have compared inhibition by various allosamidin derivatives to determine the influence of the non-catalytic chitinase domains.

2 EXPERIMENTAL METHODS

2.1 Enzymes and enzyme preparation

The epithelial cell line from *C. tentans*[10] was propagated as described earlier.[2] Since about 90% of chitinase activity is secreted into the culture medium,[11] cells were separated by low-speed centrifugation (1000*g*, 4 min); 10–20 μl culture supernatant was sufficient for determination of chitinase activity. Purified chitinase A from *S. marcescens*[12] was diluted to 4 μg ml^{-1} and used immediately (10 μl) for the tests.

2.2 Enzyme assays

Enzyme activity was determined using a fluorimetric test with methylumbelliferyl-labelled substrates (Sigma, Deisenhofen) as described previously.[2] Chitin formation was measured as already described.[13]

2.3 Inhibitors

Allosamidin was a generous gift of Eli Lilly AG, compound **VIII** was kindly provided by Dr Vasella (ETH Zürich, Switzerland). All other derivative were synthesized as described elsewhere.[14–16] Compounds were added to the reaction mixture of the enzyme assays without preincubation. Inhibition curves were presented as log-logit transformations. K_i values were calculated from the corresponding IC_{50} values according to Cheng and Prusoff.[17]

3 RESULTS AND DISCUSSION

3.1 Enzyme from *Chironomus tentans*

Structural formulae of all substances tested are presented in Figs 1 and 2. A comparison of the influence of allosamidin and its analogues on inhibition of chitinase activity in *Chironomus* cell culture supernatant (Figs 3 and 4; Table 1) revealed the following structural requirements:

- One *N*-acetyl-allosamine residue can be omitted without decrease of the inhibitory effect (compound **II**), but one sugar must be present; allosamizoline alone (compound **III**) is a very weak inhibitor.
- Replacement of the *N*-acetylallosamine moiety by the epimeric compound *N*-acetylglucosamine impairs chitinase inhibition only slightly (compound **I**) compared with allosamidin.
- Although omission of one sugar alone has no effect if the steric configuration of the oxazoline ring is correct, as in compound **II** and allosamidin, if loss of one sugar and a change in the aglycone moiety is combined, as in compounds **V** and **VI**, inhibition is diminished further.
- The arrangement of the hydroxyl and hydroxymethyl groups around the ring of the aglycone moiety determines the level of inhibition. Changing the orientation of the oxazoline group (compounds **IV** and **V**), varying the position of the glycosidic linkage to the allosamizoline moiety (compounds **VI** and **VII**), or changing the absolute stereochemistry of the allosamizoline ring (**VIII**), reduces the enzyme inhibition considerably.
- The oxazoline moiety seems to be the most sensitive part of the molecule. It may mimic a transition state, in which the oxo-carbonium intermediate is stabilized by the carbonyl oxygen of the neighbouring *N*-acetyl group.[9]

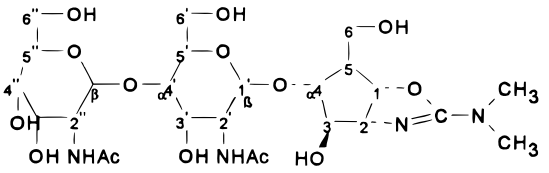
3.2 Enzyme from *Serratia marcescens*

As above, the arrangement of groups around the aglycone moiety determines the level of inhibition of the

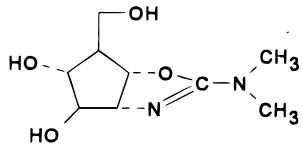
TABLE 1
Inhibition of Chitinase Activity from *Chironomus tentans* Cell Culture and from *Serratia marcescens* Chitinase A by Allosamidin and its Derivatives

	K_i (μM)[a]	
Compound	Chironomus tentans	Serratia marcescens
Allosamidin	0·22	0·1
I	0·67	0·87
II	0·35	53·1
III	234	182
IV	>5500	717
V	30·8	70·9
VI	16·9	77·5
VII	62·9	>5500
VIII	267	n.d.

[a] Determined according to Cheng and Prusoff.[17]



Allosamidin



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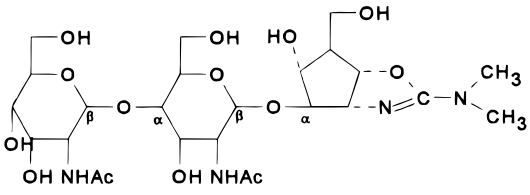
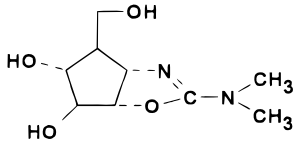
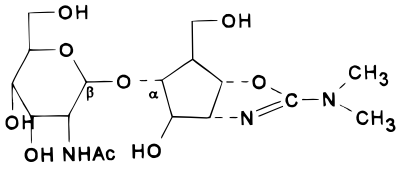


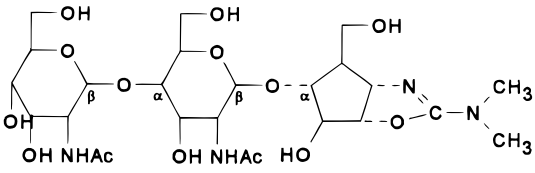
Compound I



Compound IV



Compound II



Compound V

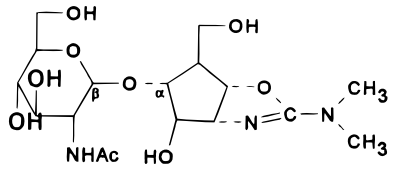
Fig. 1. Structural formulae for allosamidin and derivatives **I**–**V**.

purified chitinase A from *Serratia marcescens* (Figs 5 and 6), as in compounds **IV**–**VIII**. However, here the sugar moiety is recognized differently. Omission of one *N*-acetylallosamine impairs the inhibitory effect (compound **II**), exchange of the remaining *N*-acetylallosamine for *N*-acetyl-glucosamine improves inhibition (compound **I**).

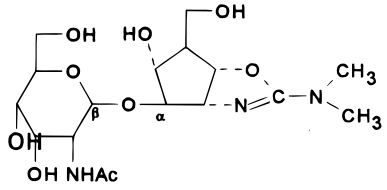
3.3 Other species

Only a minor decrease in the inhibitory action is reported for *N*,*N*′-diacetyl-*β*-chitobiosyl allosamizoline (Fig. 1) for chitinases from *Bombyx mori* and *Saccharomyces* sp., whereas for the *Trichoderma* enzyme a 30-fold decrease in the inhibitory activity has been observed.[5,18] X-ray data from hevamine, a plant chitinase of family 18 hydrolases[9] has revealed that the OH-group at C3, whether equatorial or axial, is too far away for interaction with amino acid residues of the catalytic domain. These reports and our data indicate that the orientation of the hydroxyl group at C3 of the sugar moiety is recognized differently by various chitinases.

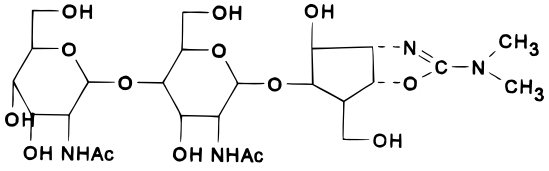
Species-specific differences are also pronounced in the dimethylamino-group of the cyclitol, since removal of one methyl group at the oxazoline ring is without effect on inhibition of chitinases from *B. mori* and *Trichoderma sp.*, but increases the inhibitory potential in *Saccharomyces cerevisiae* Meyer ex Hansen and *Candida albicans* (Robin) Berkhout.[3,4] Our own unpublished results with a chitinase from the bacterium *Serratia marcescens*, a crustacean (*Artemia salina* L.) and an insect cell line (*C. tentans*) revealed that removal of one methyl group has only a slight effect on chitinase inhibition, whereas the removal of the second methyl group considerably reduces inhibition.[19] *N*-Acetylglucosaminidase in the culture supernatant of *Chironomus* cells is not affected by allosamidin or compound **VII** up to concentrations of 1 mM.[2] The same is also true for compounds **I**–**VII** (data not shown).



Compound VI



Compound VII



Compound VIII

Fig. 2. Structural formulae for derivatives **VI–VIII** and *N,N*-diacetyl-*β*-chitobiosyl allosamizoline.

3.4 Other enzymes

Chitinase is secreted mainly into the culture medium, whereas chitin synthase is membrane-bound. Therefore the two enzyme activities can be measured separately.[13] None of the compounds tested affects chitin synthesis in homogenates of *Chironomus* cells up to a concentration of 5×10^{-4} M. The failure of allosamidin and its derivatives to impair *N*-acetylglucosaminidase activity or to inhibit chitin synthesis is a further indication that recognition of *N*-acetylglucosamine or its oligomers, which must take place in these enzymes also, is not essential for inhibition.

3.5 Binding mechanisms

Since the essential parts of the catalytic domain of chitinase A from *S. marcescens*[20] and from *Chironomus* cells (Feix, M., pers. comm.) are highly conserved, our results suggest that other domains of the enzyme influence substrate binding and inhibition by allosamidin and its derivatives, as proposed earlier.[20] Isolation of the full-length clone of the *Chironomus* enzyme is in progress in our laboratory, which may then allow elucidation of the three-dimensional structure of an insect chitinase, which may help to explain species-specific differences.

4 CONCLUSIONS

The structure–activity relationship of allosamidin derivatives reveals that the molecule can be simplified without loss of inhibitory action on chitinases. The directed design of potential insecticides will be further facilitated by the three-dimensional structure of the insect enzyme.

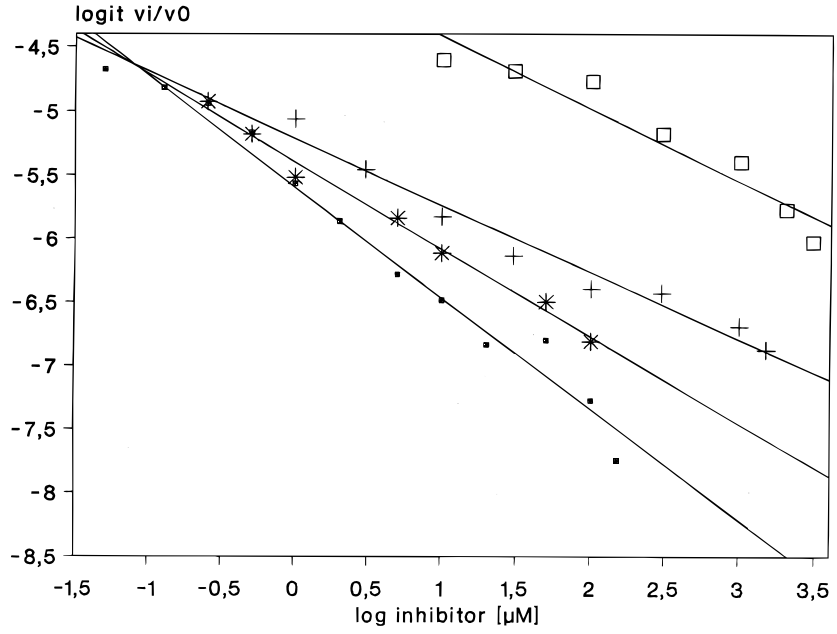


Fig. 3. Inhibition of chitinase activity from *Chironomus tentans* cell culture by (■) allosamidin, (+) **I**, (✳) **II** and (□) **III**.

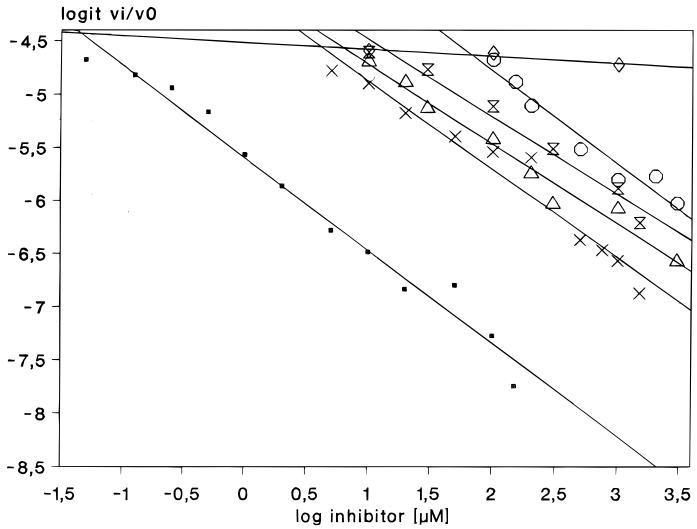


Fig. 4. Inhibition of chitinase activity from *Chironomus tentans* cell culture by (■) allosamidin, (◇) **IV**, (△) **V**, (×) **VI**, (⧖) **VII** and (⬡) **VIII**.

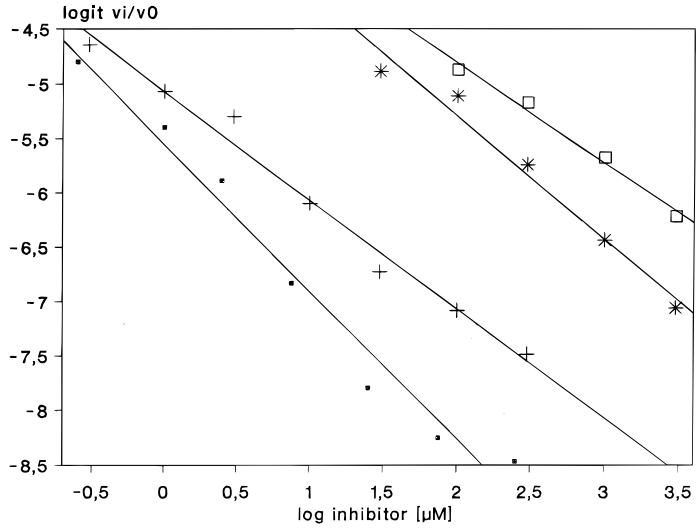


Fig. 5. Inhibition of chitinase A from *Serratia marcescens* by (■) allosamidin, (+) **I**, (✳) **II** and (□) **III**.

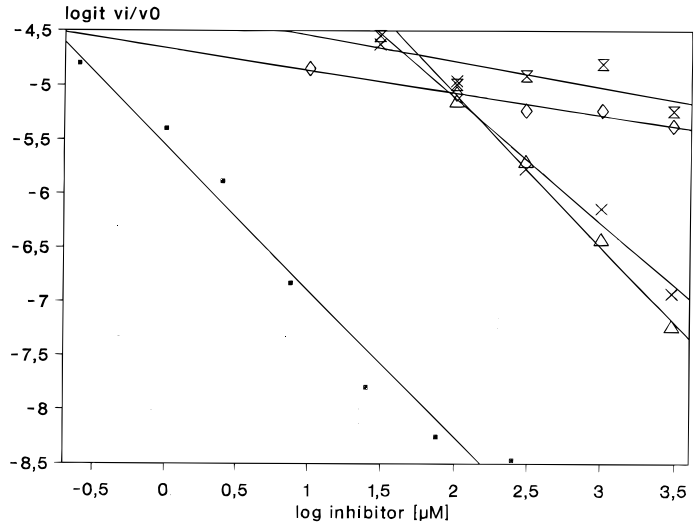


Fig. 6. Inhibition of chitinase A from *Serratia marcescens* by (■) allosamidin, (◇) **IV**, (△) **V**, (×) **VI** and (⧖) **VII**.

REFERENCES

1. Sakuda, S., Isogai, A., Matsumoto, S., Suzuki, A. & Koseki, K., The structure of allosamidin, a novel insect chitinase inhibitor, produced by *Streptomyces sp. Tetrahedron Lett*., **27** (1986) 2475–8.
2. Spindler, K.-D. & Spindler-Barth, M., Inhibition of chitinolytic enzymes from *Streptomyces griseus* (Bacteria), *Artemia salina* (Crustacea) and a cell line from *Chironomus tentans* (Insecta) by allosamidin and isoallosamidin. *Pestic. Sci*., **16** (1994) 113–20.
3. Nishimoto, Y., Sakuda, S., Takayama, S. & Yamada, Y., Isolation and characterization of new allosamidins. *J. Antibiotics*, **44** (1991) 716–22.
4. Kinoshita, M., Sakuda, S. & Yamada, Y., Preparation of *N*-monoalkyl and *O*-acyl derivatives of allosamidin, and their inhibitory action. *Biosci. Biotech. Biochem*., **57** (1993) 1699–703.
5. Terayama, H., Kuzuhara, H., Takahashi, S., Sakuda, S. & Yamada, Y., Synthesis of a new allosamidin analog, *N*,*N*′-diacetyl-*β*-chitobiosyl allosamizoline, and its inhibitory activity against some chitinases. *Biosci. Biotech. Biochem*., **57** (1993) 2067–9.
6. Overdijk, B. & Van Steijn, G. J., Human serum contains a chitinase: Identification of an enzyme, formerly described as 4-methylumbelliferyl-tetra-*N*-acetylchitotrioside hydrolase (MU-TACT hydrolase). *Glycobiology*, **4** (1994) 797–803.
7. Henrissat, B., A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J*., **280** (1991) 309–16.
8. Perrakis, A., Tews, I., Dauter, Z., Oppenheim, A. B., Chet, I., Wilson, K. S. & Vorgias, C. E., Crystal structure of a bacterial chitinase at 2·3 Å resolution. *Structure*, **2** (1994) 1169–80.
9. Terwisscha van Scheltinga, A. C., Armand, S., Kalk, K. H., Isogai, A., Henrissat, B. & Dijktra, B. W., Stereochemistry of chitin hydrolysis by a plant chitinase/lysozyme and X-ray structure of a complex with allosamidin: Evidence for substrate assisted catalysis. *Biochemistry*, **34** (1995) 15619–23.
10. Wyss, Ch., *Chironomus tentans* epithelial cell lines sensitive to ecdysteroids, juvenile hormones, insulin and heat shock. *Exp. Cell Res*., **139** (1982) 309–19.
11. Spindler-Barth, M., Hormonal regulation of chitin metabolism in insect cell lines. In *Chitin enzymology*, ed. R. A. A. Muzzarelli. Eur. Chitin Society, Ancona 1993, 75–82.
12. Brurberg, M. B., Eijsink, V. C. & Nes, I. F., Characterization of a chitinase gene (*chiA*) from *Serratia marcescens* BJL200 and one-step purification of the gene product. *FEMS Microbiology Lett*., **124** (1994) 399–404.
13. Ludwig, M., Spindler-Barth, M. & Spindler, K.-D., Properties of chitin synthase in homogenates from *Chironomus* cells. *Arch. Insect Biochem. Physiol*., **18** (1991) 251–63.
14. Blattner, R., Furneaux, R. H. & Lynch, G. P., The use of 2-deoxy-2-trichloracetamido-*D*-glycopyranose derivatives in the synthesis of oligosaccharides. *Carbohydrate Res*., in press.
15. Blattner, R., Furneaux, R. H., Kemmitt, T., Tyler, P. Ferrier, R. J. & Tiden, A.-K., Syntheses of the fungicide/insecticide allosamidin and a structural isomer. *J. Chem. Soc*., *Perkin Trans*., **1** (1994) 3411–21.
16. Blattner, R., Gerard, P. J. & Spindler-Barth, M., Synthesis and biological activity of allosamidin and allosamidin analogues. *Pestic. Sci*., **50** (1997) 312–18.
17. Cheng, Y.-C. & Prusoff, W. H., Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzyme reaction. *Biochem. Pharmacol*., **22** (1973) 3099–108.
18. Takahashi, S., Terayama, H., Kuzuhara, H., Sakuda, S. & Yamada, Y., Preparation of demethylallosamidin isomer having an *N*,*N*′-diacetylchitobiosyl moiety and its potent inhibition against yeast chitinase. *Biosci. Biotech. Biochem*., **58** (1994) 2301–2.
19. Spindler, K.-D., Sakuda, S. & Spindler-Barth, M., Effect of demethylation on the chitinase inhibitory activity of allosamidin. *Arch. Insect Biochem. Physiol*. **36** (1997) 223–227.
20. Perrakis, A., Ouzounis, Ch, Wilson, K. S. & Vorgias, C. E., Implications of the 3-D structure determination of family 18 chitinases. Similarities with FnIII domains, oviductal proteins and narbonins. *Adv. Chitin Sci*., **1** (1996) 34–41.