Review

Recent development of two chitinase inhibitors, Argifin and Argadin, produced by soil microorganisms

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Abstract: Chitin, the second most abundant polysaccharide in nature, occurs in fungi, some algae and many invertebrates, including insects. Thus, chitin synthesis and degradation could represent specific targets for fungicides and insecticides. Chitinases hydrolyze chitin into oligomers of N-acetyl-D-glucosamine at key points in the life cycles of organisms, consequently, chitinase inhibitors have become subject of increasing interest. This review covers the development of two chitinase inhibitors of natural origin, Argifin and Argadin, isolated from the cultured broth of microorganisms in our laboratory. In particular, the practical total synthesis of these natural products, the synthesis of lead compounds via computer-aided rational molecular design, and discovery methods that generate only highly-active compounds using a kinetic target (chitinase)-guided synthesis approach (termed in situ click chemistry) are described.

Keywords: Argadin, Argifin, chitinase inhibitor, solid-phase synthesis, rational molecular design, in situ click chemistry

1. Introduction

Chitinases catalyze the hydrolysis of chitin, a linear homopolymer of N-acetyl-D-glucosamine (GlcNAc), which is present in a wide range of organisms, including bacteria, fungi, insects, viruses, higher plants and animals.1)–5) Chitinases can be classified as endochitinases or exochitinases. Endochitinases cleave chitin at internal sites to generate multimers of GlcNAc. Exochitinases catalyze the hydrolysis of chitin progressively to produce GlcNAc, chitobiose or chitotriose, respectively (Fig. 1).5) Chitinases are currently classified into two different families of glycosyl hydrolases, namely family-18 and family-19, on the basis of amino acid sequence similarities.6)–8) Family-18 contains chitinases from various organisms, whereas family-19 chitinases are only found in plants and Streptomyces species. Family-18 chitinases have been well studied, with information available on their three-dimensional (3-D) structure and the biochemistry of the enzyme reaction.9)

Chitin is a major constituent of fungal cell walls, the exoskeletons of crustaceans and insects, and of the microfilarial sheaths of parasitic nematodes.2)–3) Chitin has, so far, not been found in mammals. Accumulation of chitin by organisms is modulated by chitin synthase-mediated biosynthesis and by chitinase-mediated hydrolytic degradation. Thus, chitinases are expected to be specific targets for antifungal, insecticidal and antiparasitic agents.9) Paradoxically, while chitin does not exist in mammals, human chitinase family members, such as acidic mammalian chitinase, have recently been described.2)–10)–12) Acidic mammalian chitinase is a member of the family-18 chitinases, and highly expressed in the stomach and at a lower level in the lung. The endogenous substrates and physiological functions for acidic mammalian chitinase are currently unknown. Inhibition of acidic mammalian chitinase results in decreased airway inflammation and airway hyperresponsiveness in a mouse asthma model, suggesting that the acidic mammalian chitinase activity is a part of the mecha-
nism of Th2 cytokine-driven inflammatory response in asthma. Therefore, it offers significant potential for the treatment of asthma and other related diseases in humans.

2. Naturally-occurring chitinase inhibitors

To date, at least six naturally occurring inhibitors of family-18 chitinases (exochitinases) have been reported on articles from other research groups (Fig 2). The most studied and most potent chitinase inhibitor is allosamidin (1), which was isolated in 1986 by Sakuda and Suzuki et al. from Streptomyces sp. No1713, and identified as a potent chitinase inhibitor in the silkworm, Bombyx mori, in vitro, as well as preventing larval ecdysis in vivo. Research on this natural product has divulged many details with respect to the mode of action of 1, as well as the structure and function of the enzymes via crystal structure analysis of chitinase-1 complexes.

Styloguanidines (2-4), unique hexacyclic bisguanidin alkaloids, were isolated from a sponge, Stylotella aurantium, collected in the Yap sea by Kato and colleagues in 1995. Styloguanidines showed inhibitory activity against a bacterial chitinase from Schwanella sp. at 2.5 μg/disk. The in vivo results indicate that moulting of cyprid larvae of barnacles was inhibited by these compounds at a concentration of 10 ppm, implying that these inhibitors have possibilities as an antifouling agent.

Cl-4 (cyclo-L-Arg-D-Pro) (5) was isolated from the culture broth of a marine bacterium, Pseudomonas sp. IZ208, by Izumida and colleagues in 1996 and found to exhibit potent inhibitory activity against chitinase from Bacillus sp. Using the agar plate method and the chitin-degrading bacterium, EY410, Cl-4 exhibited moderate chitinase inhibition at a concentration of 50 μg/disk. Chitinase inhibitory activity of the related analogue, cyclo-D-Arg-L-Pro (enantiomer of 5) was weaker than 5, but simpler analogs (L-, D-Arg, L-, D-Pro and cyclo-Gly-Gly) showed no inhibition. Cyclo-L-Arg-L-Pro and 5 also showed 18% and 17% inhibition at 1.0 mM concentration, respectively,

Fig. 2. Structures of naturally-occurring chitinase inhibitors and their inhibitory activities.
the enzyme method (using *Bacillus* sp. chitinase). Moreover, 5 was found to inhibit cell separation in *Saccharomyces cerevisiae* and blocked morphological changes in *Candida albicans*, presumably through inhibition of chitinases in these organisms. 19)

Psammaplin A (6) was isolated from the Fijian marine sponge, *Aplysinella rhax*, during a chitinase inhibition bioassay guided isolation protocol using *Bacillus* sp. by Jaspars and colleagues in 2002. 20) Psammaplins were originally isolated from a marine sponge, *Psammaplysilla purpurea*, collected in the region of Tonga by Crews et al. in 1987, and identified as a cytotoxic materials. 21), 22) Only compound 6 among the range of psammaplin natural products showed significant inhibitory activity against *Bacillus* sp. chitinase, with an IC$_{50}$ value of 68 μM. Gooday and colleagues also measured the activity of 6 using the chitinase bioassay. 23) Results showed inhibition of endochitinase enzymes, in particular, against the bacterial enzymes from *Streptomyces*, with an IC$_{50}$ value of 50 μM.

3. Argadin and Argifin, produced by soil microorganisms

A novel class of natural product chitinase inhibitors was reported by our research group in 2000. During screening for family-18 chitinase (exochitinases) inhibitors from 11,900 extracts of soil microorganisms, two cyclic pentapeptides, argadin (7) 24) and argifin (8), 25), 26) 27) were isolated from the cultured broths of *Clonostachys* sp. FO-7314 and *Gliocladium* sp. FTD-0668, respectively, and found to be potent chitinase inhibitors of blowfly (*Lucilia cuprina*). Inhibitory activity of these cyclopeptide compounds against *L. cuprina* chitinase was studied and compared with that of allosamidin (1) (Fig. 3). These compounds (7 and 8) inhibited *L. cuprina* chitinase with IC$_{50}$ values of 150 nM at 37°C and 3.4 nM at 20°C, and 3.7 μM at 37°C and 0.10 μM at 20°C, respectively. Allosamidin (1) showed inhibition with IC$_{50}$ values of 2.3 nM at 37°C and 0.4 nM at 20°C. Therefore, 7 showed better potency than that of 8, and was only nine times weaker than 1 at 20°C. A subsequent bioassay using American cockroach (*Periplaneta americana*) revealed that 7 and 8 have the ability to inhibit molting. Notably, 7 and 8 (20 μg each) were injected into cockroach larvae, and mortality of 60% and 73%, respectively, were observed after 5 to 23 days after injection. The larvae killed showed new cuticle formation below the partially-opened old cuticle and so were unable to leave the old exuvia, leading to their death shortly after

Fig. 3. Structures of naturally-occurring chitinase, argadin (7) and argifin (8), and photomicrographs of their producing strains.
sclerotization of the new cuticle, the damage presumably resulting from inhibition of chitinases in the molting process. A subsequent expanded bioassay revealed surprisingly inhibitory activities against *Serratia marcescens*, *Aspergillus fumigatus*, and human chitinases in the nanomolar to micromolar range for the IC$_{50}$ values shown in Fig. 3.

The three-dimensional (3-D) structure of 7 and 8, in complex with *Aspergillus fumigatus* chitinase B1, *Serratia marcescens* chitinase B, human chitotriosidase and acidic mammalian chitinase, were resolved by X-ray crystallography.

Hence, 7 and 8 could be good lead compounds to develop novel and practical drugs for use as sub-nanomolar chitinase inhibitors, as these compounds (and related analogs) seem to be synthetically more accessible using standard peptide chemistry than the structurally-complex allosamidin (1).

4. **Total synthesis of argadin**

Establishment of the total synthesis of argadin (7) appears to be a very important objective to facilitate development of novel chitinase inhibitors, as the original source does not produce 7 in sufficient quantity, as well as for supplying its analogues for biological tests. Indeed, the total synthesis of 7, involving hybrid approaches of solid- and liquid-phase reaction sequences, was reported by Eggleston and colleagues in 2006.

Consequently, our primary goal for argadin synthesis was to achieve a more efficient and highly-practical process. We subsequently accomplished the solid-phase total synthesis of 7 in 2009.

Our synthetic route for 7 is outlined in Scheme 1. The cyclic peptide structure of 7 allowed us to adopt a solid-phase strategy based on application of a 9-fluorenylmethoxy carbonyl (Fmoc) protective
group for the amine of amino acid fragments, with allyl protection on the carboxylic acid of the L-aspartic-β-semialdehyde (9)\textsuperscript{34} unit. This strategy enables cyclization of the linear precursor (still attached to a solid support) via the side-chain of an L-aspartic-β-semialdehyde residue. Our synthetic strategy for 7 offers many advantages, including (i) anchorage of the first residue (C-terminal) to resin through the side-chain aldehyde function, (ii) the selected glycerol polystyrene resin acts as a protective group of the aldehyde to prevent the formation of the sensitive cyclic hemiaminal, (iii) on-resin cyclization includes stepwise selective deprotection of the C- and N-terminal, followed by intramolecular condensation, (iv) single-step operation to convert to the N\textsuperscript{α}-acetlyarginine residue from ornithine by acetylguanylation, and (v) the whole reaction sequence can be carried out on resin, except for cleavage from resin and hemiaminal formation at the final step. At first, the aldehyde in 9 was loaded onto (±)-glycerol polystyrene resin to give 10. To confirm the loading yield or conversion yields for every step, cleavage from the resin with TFA, followed by treatment with morpholinomethyl polystyrene resin (PS-NMM), gave the desired compounds, which were monitored by LC-UV-MS analysis. The resin-bound amino acid 10 was subsequently submitted to four deprotection-coupling cycles to build the linear pentapeptide by standard Fmoc SPPS (solid-phase peptide synthesis). After synthesis of the linear pentapeptide (17) was accomplished, deprotection on both the C- and N-terminal was carried out to afford the precursor of the cyclic peptide compound (19). Optimally, macro-lactamization of 19 was carried out under the O-(7-azabenzotriazol-1-yl)-N, N, N', N'-tetramethyluronium hexafluorophosphate (HATU) activation condition over a 2-cycle repetition, followed by deprotection of 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) group, acetylguanylation using 1-H-pyrazole-1-[N-(tert-butoxycarbonyl)]-N'-acetylcarnboxamidine (22),\textsuperscript{35} and finally formation of hemiaminal accompanied by total deprotection, including resin cleavage to yield argadin (7). Thus, we completed the solid-phase total synthesis of 7 using a supported acetal resin. This synthesis was concise requiring 15 steps in the longest linear sequence from (±)-glycerol polystyrene resin, with HPLC separation after cleaving from the resin, to give 7 in 4.7% overall yield. The process allows us to easily generate a variety of analogs for structure-activity-relationship (SAR) studies. Furthermore, evaluation of peptide analogs, exploiting a combination of our synthetic protocol and computer-aided rational molecular calculations, will provide more potent analogs of 7.

5. Total synthesis of argifin

Design and development of practical and efficient strategies for argifin (8) synthesis has become an important since the original source, Gliocladium sp. FTD-0668, no longer produces this cyclic peptide. A rapid and diverse synthesis pathway for 8 is required to supply the compound for biological tests as well as the SAR studies. The first total synthesis of 8 was actually developed by Eggleston and coworkers in 2005 utilizing solid phase peptide synthesis.\textsuperscript{36} Their strategy, however, included liquid phase reaction sequences for introduction of the N\textsuperscript{α}-methyl-carbamoyl group during the final stages, with two-time HPLC separation due to its hydrophilicity, indicating that an efficient strategy was still required, especially enable rapid synthesis of analogs. Subsequently, in 2009, they reported an improved route for synthesis of 8 and its analogs using an all-solid phase approach.\textsuperscript{37} At the same time, we independently reported the total synthesis of 8 accomplished by solid-phase synthetic protocols in 2009.\textsuperscript{38}

Our synthetic route of 8 is outlined in Scheme 2. To avoid complications of the liquid phase reactions, we envisaged solid phase total synthesis for all reaction sequences, except for the final cleavage step from the resin. The precess began with the loading of carboxylic acid 24 onto 2-chlorotrityl resin. To confirm the loading yield (or conversion yields) for each step, cleavage from the resin with TFA, provided the desired compounds, which were monitored by LC-UV-MS analysis. The resin-bound amino acid 25 was subsequently submitted to deprotection-coupling cycles to build the linear pentapeptide by standard Fmoc SPPS as in the synthesis of argadin (7). The macrolactamization of 32 with HATU, in a 2-cycle repetition on resin, furnished the corresponding cyclic compound without oligomerization. To complete the total synthesis, after Dde deprotection, N\textsuperscript{α}-methylcarbamoylguanidino formation with 33, followed by deprotection and cleavage with 90% TFA in DCM from the resin, furnished 8, in overall 13% yield after HPLC purification. For our synthetic protocols, 8 could be prepared from fully-protected argifin as a solid intermediate, with deprotection and cleavage from the resin under acidic conditions.
at final step. This means that the process has no liquid-phase steps. Additionally, 1-H-pyrazole-1-\(\text{N}^\prime\)-(tert-butoxycarbonyl)-\(\text{N}^\prime\)-\(\text{N}^\prime\)-\(\text{p}\)-methoxybenzylcarbamoyl-N-methyl)carboxamidine (34) was designed to simply introduce the \(\text{N}^\circ\)-methylcarbamoylguanidine onto the NH\(_2\) group of Orn for solid phase synthesis. Actually, the \(\text{N}^\circ\)-methylcarbamoylguanidine formation being effectively introduced using primary amines with 34 at room temperature in good yields, suggested that this method can not only be utilized for synthesis of 8, but also synthesis of similar products containing the \(\text{N}^\circ\)-methylcarbamoyl group, such as the naturally-occurring Banyasin A.\(^{39}\)

6. Computer-aided rational molecular design from argifin

As mentioned above, we achieved efficient solid-phase total synthesis of argifin (8), which could be applied to enable synthesis of analogs. In addition, the 3-D structure of 8, in complex with chitinase B from Serratia marcescens (SmChiB), was resolved by X-ray analysis (Fig. 4).\(^{28}\) And an inhibitory activity of 8 against SmChiB is shown as an IC\(_{50}\) value of 6.4 \(\mu\text{M}.\)\(^{30}\) These factors and observations stimulated us to design argifin-derivatives with more potent inhibitory activity, leading us to undertake rational molecular design of argifin-derivatives and test them against SmChiB.\(^{40}\) The work involved molecular dynamics (MD) simulation with explicit water molecules, the molecular docking calculation, and free energy analysis using the molecular mechanics Poisson-Boltzmann surface area (MM-PBSA) method.\(^{41}\) To obtain its solution structure, the MD simulation with explicit water molecules for 1700 ps length, using the crystal structure of SmChiB-8 complex, was performed.\(^{42}\) Consequently, a total of 200 snapshots derived from the MD trajectory were used for the 2D root-mean-square deviation (RMSD) analysis on the binding site, which included 8 and 26 residues from SmChiB, by ptraj module of AMBER8.\(^{43}\) This allowed us to group a number of snapshots into several different kinds of conformations. As a result, two representative solution structures of argifin-SmChiB were obtained (referred to as S890 and S1505 in reference 42).

The two structures were used to obtain guides
for designing argifin derivatives. From the X-ray analysis of argifin-SmChiB, the L-Arg(1), N-methyl-L-Phe(2) and L-Asp(3) of 8 (Fig. 5) appear to be essential for binding (Fig. 4). In addition, the available space around the N-methyl-L-Phe(2) seems to be too small to accommodate a more bulky residue such as Trp. Therefore, our attention shifted to two other residues of 8, that is, L-Asp(4) and D-Ala(5) (Fig. 5).

As shown in Fig. 6A, three acidic residues of SmChiB, that is, E315, D316 and D336, were located very close to the L-Asp(4) of 8, suggesting the presence of unfavorable electrostatic interaction. Therefore, an analog for the modification of carboxyl group of L-Asp(4) to 4-benzylpiperidine group could be envisaged, due to the prospect of synthetic accessibility. Further, we found an interesting deep groove close to L-Asp(4) of 8 in pose S890, which is formed by D316, P317, Y318, P319, K335, D336 and R338 of SmChiB (as shown in Fig. 6A). The 4-benzylpiperidine group of one derivative (35) could be expected to form additional interactions within this groove (Fig. 6B). A wide space to accommodate more bulky side chains around D-Ala(5) of 8 was also found in pose S890 (Fig. 6A). Therefore, five derivatives (36-40), in which D-Ala(5) was converted to more bulky D-amino acids, were designed in order to make additional constants with F12, F51, Y98 and W403 of SmChiB (docking model of D-Leu(5) derivative 37 with SmChiB is shown in Fig. 6C).

The interaction mode of each derivative with SmChiB was predicted as follows: conformational analysis of the ring structure was first performed using the CAMDAS2.1 (Conformational Analyzer with Molecular Dynamics and Sampling) programme. Next, the molecular docking calculation of conformers obtained by CAMDAS was performed against the binding sites of poses S890 and S1505, using Glide version 4.0 (SP mode), and the generated poses were ranked according to GlideScore. On the top 100 poses, the MM-PPSA method was applied to estimate the free energies of their complex structures (Gcomplex). Finally, a pose with the lowest Gcomplex was selected as the interaction model. As expected, the interaction model of 35 located its 4-
benzylpiperdinyl group in the target groove to make an additional interaction with SmChiB in pose S890 (Fig. 6B). The calculations also predicted that all side-chains for d-amino acid moiety (36-40) could occupy the available space in the 8-SmChiB complex (Fig. 6C; as an example, the model for 37).

The binding affinity ($\Delta G_{\text{bind}}$) of each derivative was approximated by applying the single-point MM-PBSA calculation. The arginin based relative binding affinity ($\Delta G_{\text{bind}}$) was estimated using the $G_{\text{bind}}$ value of 8 in pose S890 as a reference (Table 1). All derivatives showed greater $\Delta G_{\text{bind}}$ values than that of 8, indicating that they might have better potency. Then, all six derivatives (35-40) were synthesized according to our strategy from 2-chlorotrityl resin-L-Asp(3) for 14 total sequences in 7.4 to 37% overall yields, after HPLC purification at final cleavage from resin (Scheme 3), with IC$_{50}$ values determined against SmChiB (Table 1). As expected, 35, 37 and 40 were found to possess better inhibitory activity, with IC$_{50}$ values of 1.3, 1.9 and 4.5 $\mu$M, respectively, than 8 (6.4 $\mu$M). Furthermore, a hybrid derivative (41), effectively a combination of 35 and 37, was prepared. As expected, 41 showed lowest $\Delta G_{\text{bind}}$, and was found to be the most potent inhibitor against SmChiB, with an IC$_{50}$ value of 0.23 $\mu$M, about a 30-fold increase over that of 8.

Our rational molecular design of derivatives based on the structure of 8 led to production of a derivative (41), which showed great potential for inhibitory activity against SmChiB.
7. Evaluation of analogs of acyclic peptide of argin

Having achieved the efficient solid-phase total synthesis of argin (8), we also prepared acyclic peptide compounds, based on peptide fragments from the framework of 8 to search for novel and potent chitinase inhibitors.\(^{38}\) We undertook investigation of analogs by examining nine acyclic derivatives bearing the \(\text{N}_\text{o}\)-methylcarbamoylguanidino group to elucidate structure-activity-relationships (SAR) against each \(\text{SmChi}\) isozyme. For synthesis of acyclic peptides, we used the solid phase peptide synthesis strategy using 2-chlorotrityl chloride resin, that was successfully applied in the total synthesis of 8. From the synthetic perspective, elaboration of the acyclic analogs is outlined in Scheme 4. The carboxylic acids at each amino acid (L-Asp, \(N\)-methyl-L-Phe and L-Orn) were loaded onto 2-chlorotrityl chloride resin, followed by elaboration of appropriate amino acids (\(N\)-methyl-L-Phe, L-Orn, D-Ala and L-Asp) and acetylation of the terminal NH\(_2\) to furnish the acetylated products. Deprotection of the Dde group and introduction of the \(\text{N}_\text{o}\)-methylcarbamoylguanidino moiety afforded fully-functional compounds. Finally, cleavage from the resin, followed by deprotection of the Boc and PMB group, readily furnished nine acyclic analogs (42–44a, b, and c) in 21% to 78% yields (see Table 2).

For determination of IC\(_{50}\) values against each
SmChi isozyme, the nine acyclic compounds were subjected to a competition assay (Table 2). Interestingly, L-Asp-(N-methyl-L-Phe)-L-Arg (42a) and L-Asp-(N-methyl-L-Phe)-L-Arg-D-Ala (42b) exhibited approximately 50~70-fold more potent activity against SmChiB (with IC$_{50}$ values of 0.13 µM and 0.091 µM, respectively) than that of 8, suggesting that the D-Ala moiety is not a crucial function for ex-
pression of competent inhibitory activity with respect to SmChiB, except for L-Arg-series 44a–c (Table 2). In contrast, possessing the D-Ala moiety increases activity in terms of SmChiA. Furthermore, the addition of L-Asp moiety next to D-Ala weakens both activities. These correlations for all-series were clearly demonstrated. Conversely, lack of the L-Asp unit next to N-methyl-L-Phe (in 43a–c and 44a–c) decreases activity of both SmChiA and B, suggesting the L-Asp(OAllyl) plays a key role in the activity. Likewise, the N-methyl-L-Phe moiety is also an important amino acid, indicated by the finding that the L-Arg series display weak activity against both SmChiA and B. Unfortunately, all of the acyclic compounds, including 8, exhibit no activity against SmChiC1. It is, however, notable that the novel acyclic peptide 42b exhibits 70-fold more potent activity against SmChiB than 8, indicating that the cyclic form is not necessary for anti-chitinase activity. This means that we have identified not only a simplified structure with potent inhibitory activity but also a new scaffold, derived from the natural product, which possesses potent inhibitory activity.

8. The active framework of argifin and use of in situ click chemistry

The studies of argifin (8) and its analogs by X-ray crystallography with various chitinases revealed that there are at least four conserved hydrogen-bond interactions between the N\textsuperscript{\alpha}-methylcarbamoyl-L-arginine moiety and the polar groups arrayed in the hydrolytic pocket of the family 18 chitinases. \textsuperscript{28,29,31,37} The remarkable fidelity of the hydrogen-bonding network between the chitinases and the argifin ligand implicates its critical role in revealing the micromolar to nanomolar range of inhibition. In fact, van Aalten and co-workers revealed though X-ray analysis that the ability of the N\textsuperscript{\alpha}-methylcarbamoyl group to penetrate fully into the active site pocket of chitinases strongly correlated with the inhibition of chitin hydrolysis. \textsuperscript{31} From our SAR studies, the N\textsuperscript{\alpha}-methylcarbamoyl group was obviously a crucial component for expression of inhibitory activity on chitinases. \textsuperscript{38} Hence, we concluded that the N\textsuperscript{\alpha}-methylcarbamoyl-L-arginine core represents an ideal anchor to derivatize and elaborate better chitinase inhibitors.

Our work thus focused on the design and simplification to azide-bearing N\textsuperscript{\alpha}-methylcarbamoyl-L-arginine substrate, as a smaller analogs of macrocyclic peptide natural product 8, and the use of target-guided synthesis (TGS) (for reports of TGS see Rideout, \textsuperscript{46} Rideout \textit{et al.}\textsuperscript{47} Inglese and Benkovic, \textsuperscript{48} Boger \textit{et al.}\textsuperscript{49} Maly \textit{et al.}\textsuperscript{50} Nicolaou \textit{et al.}\textsuperscript{51} Greasley \textit{et al.}\textsuperscript{52} Nguyen and Huc, \textsuperscript{53} Nicolaou \textit{et al.}\textsuperscript{54} Kehoe \textit{et al.}\textsuperscript{55} Poulin-Kerstien and Devan, \textsuperscript{56} and Hu \textit{et al.}\textsuperscript{57}) for the screening of novel and more potent chitinase inhibitors employing the 1,3-dipolar cycloaddition \textsuperscript{58} between an azide ligand and a library of acetylenes. \textsuperscript{30} In situ click chemistry for drug discovery is dependent on irreversibly reacting reagents that are inert under physiological conditions, \textsuperscript{59} as previously demonstrated by the discovery of highly-potent inhibitors of acetylcholine esterase, \textsuperscript{60–63} carbonic anhydrase II, \textsuperscript{64} and HIV-1 protease. \textsuperscript{65} Click chemistry is an application of covalent bond formation, especially 1,3-dipolar cycloaddition, which has been increasingly applied over the last several years in biology and material science because it is perfectly orthogonal to the acid-base reactivity phenomena. The reaction between azide and alkyne only occurs if the both functions meet each other under just the right conditions. As shown in Fig. 7 for \textit{in situ} click chemistry, at first, azide or alkyne building blocks are incubated in the presence of target protein. The protein binds initially the building
blocks with the highest affinity. The enforced pro-
pinquity of the azide and alkyne accelerates triazole
formation as a covalent bond to link two building
blocks. The newly-generated triazole compound shows
higher affinity compared with corresponding mono-
valent building blocks.

Initially, we investigated appropriate inhibitors
to develop in situ click chemistry from the Nο-
methylcarbonyl-L-Arg sca/C11old. A simple Arg-derived
inhibitor (45), discovered by van Aalten and co-
workers had been reported independently,31) it
showed low inhibitory activity against SmChi in con-
trast to 8, which was examined by our group (Fig.
8). We consequently synthesized the azide-bearing inhibitor 46 as a reactive sca/C11old for capturing com-
plementary acetylenic reagents to form triazole-
linked inhibitors by TGS. Elucidations of IC50 values
against each SmChi isozyme showed that this azide-
bearing inhibitor 46 expressed a low inhibitory activity similar to that of the azide-lacking inhibitor 45,
which are in striking contrast to the potency of the
natural product 8. Hence, amide derivatives of azide
46 with amines other than methylamine were made
and tested to see whether the binding could be
restored to a level that would make azide 46 a
sufficiently good anchor at the active site, to be used
for capture of alkyne-bearing candidates through in
situ triazole formation. Fortunately, the dibenzyla-
mide analog 48 of azide 46 emerged as a potent in-
hibitor (0.045 μM and 0.58 μM IC50 values against
SmChiA and B, respectively). Interestingly, the IC50
value against SmChiB of 48 was 10-fold stronger
than that of parent 8. The monobenzylamide 47 was
also active but less so than 48. As seen in Fig. 8, the
compounds 45 to 48 can be ranked by inhibition constants, i.e. 48 > 47 > 46 > 45. We therefore used
the potent azide analogue 48 as a target ‘anchor’
molecule for in situ click chemistry.

The in situ click chemistry experiments were
performed in parallel in 96-well microtiter plates to
explore the chitinase-accelerated reaction, using a
mixture of SmChiA, B, and C1. Utilization of the
mixed SmChi has the advantage of accelerating the
identification of novel inhibitors against each iso-
zyme of chitinase through a one-off screening. Al-
though a singular isozyme or multiple isozymes of
chitinase may participate in the formation of tria-
zoles under this particular screening condition, the
identification of the actual templating isozyme or iso-
zymes can be determined in a follow-up assay using
separate isozymes. Consequently, azide 48 (100 μM)
and 71 structurally-diverse alkynes (300 μM) (struc-
tures are shown in supplementary information of
reference 30) were incubated in the presence of
SmChiA, B and C1 (9.6 mUnit/mL) in 10% MeOH
containing phosphate buffer solution at pH 7.0
(Scheme 5). Formation of the triazole products was
monitored by HPLC and mass spectrometry in

![Figure 8](image)

**Fig. 8.** Structures and IC50 values of Nο-methylcarbamoyl-L-
Arg derived inhibitors.

![Scheme 5](image)

**Scheme 5.** SmChi templated in situ click chemistry protocol and the guided triazole analog.
selected ion recording detection (LCMS-SIR, also known as HPLC and mass spectrometry in selected ion monitoring LCMS-SIM) after 20 h at 37 °C.

After analysis of each reaction mixture, only alkyne 49 (IC50 > 30 μM) had been sufficiently accelerated in its cycloaddition with azide 48 in the presence of SmChi (9.6 mUnit/mL); c) Without SmChi (background reaction).

Fig. 9. Results of in situ click chemistry between 48 and 49, monitored by LCMS-SIR. a) Authentic sample of 50 from thermal reaction (100 °C, 12 h), apparently single peak (4.9 min) of 50 (anti: syn = 3:2) was observed; b) Reaction (37 °C, 20 h) between 48 (100 μM) and 49 (300 μM) in the presence of SmChi (9.6 mUnit/mL); c) Without SmChi (background reaction).

subjected to copper(I)-catalyzed azide-alkyne cycloaddition conditions (CuAAC)66–68 along with ruthenium-catalyzed reaction conditions (RuAAC)69,70 to prepare pure regioisomers of 50, allowing identification of the regiochemistry of the triazole formed by the enzymes. As expected, the pure 1,4- and 1,5-disubstituted triazole products (anti-50 and syn-50) were obtained (Scheme 6). Having both pure triazoles in hand, we turned our attention to identification of the generated triazole analogue by TGS and the participated isozymes of enzyme for in situ click chemistry. As shown in Table 3, the inhibitory activities of both-regioisomers of 50 against SmChiA and C1 were almost the same as that of 48. On the other hand, syn-50 displayed high inhibitory activity against SmChiB (IC50 value of 0.022 μM), which is approximately 30-fold stronger than that of 48 (approximately 300-fold potency compared with natural 8). These results strongly indicate that syn-50 is most likely formed in situ by the SmChiB isozyme in the enzyme mixture.

Analysis of syn-anti selection for the in situ screening by LCMS-SIR revealed that a combination of azide 48 and alkyne 49 had led to the accelerated formation of syn-50 in the presence of pure (His)6-SmChiB in an enzyme-dose dependent manner (Fig. 10). Moreover, no syn-triazole formation was observed in the control incubation containing SmChiB and the same azide and alkyne in the presence of argadin natural product 7 (IC50 values against SmChiB;
Table 3. IC$_{50}$ values of 48, anti-, and syn-50 against SmChiA, B and C

| SmChi | 48 | anti-50 | syn-50 |
|-------|----|---------|--------|
| SmChiA | 0.045 ± 0.01 | 0.050 ± 0.002 | 0.061 ± 0.01 |
| SmChiB | 0.58 ± 0.04 | 1.0 ± 0.09 | 0.022 ± 0.002 |
| SmChiC | >30 | >30 | >30 |

Fig. 10. Identification of syn-selectivity for in situ click chemistry between 48 (100 μM) and 49 (300 μM), monitored by LCMS-SIR. a) Authentic 50 from thermal reaction: syn- and anti-50 (2 : 3 ratio); b) Reaction with pure (His)$_6$-SmChiB (192 μg/mL; 37°C, 20 h); c) (His)$_6$-SmChiB (96 μg/mL; 37°C, 20 h); d) (His)$_6$-SmChiB (48 μg/mL; 37°C, 20 h); e) (His)$_6$-SmChiB (192 μg/mL), argadin 7 (100 μM; 37°C, 20 h); f) Without enzyme (background reaction).

33 ± 2.8 nM, thereby validating syn-50 as an in situ ‘hit’ and confirming that its formation required the enzyme active site to be accessible. Interestingly, the regioisomer anti-50, is less active against SmChiB than the ‘anchor’ molecule 48, which probably presents the -CH$_2$-N$_3$ group in a unique position when 48 and the protein form their complex. At this point, the chitinase–48 complex is most likely a single entity properly presenting the azide to the ‘well suited’ alkyne ligand correctly binding to the complex so that a syn-triazole selectively clicks into its existence.

Through our in situ click chemistry research, we have discovered a highly-active chitinase inhibitor. Our strategy employed an azide substituent appended to an active domain excised, as it were, from the more complex natural macrocyclic peptide 8. The SmChi, which in this case was specifically SmChiB, served as both mold and template for triazole formation between a unique pair of azide and alkyne fragments. Indeed, a number of analogs, based on bioactive molecules, need to be synthesized to fully reveal the SAR and affinity of any specific target molecule (e.g. enzymes) and to identify superior materials for traditional lead discovery. In the process of in situ click chemistry, the highly exergonic nature of triazole formation makes the process completely irreversible, and thereby locks in unique information, a kind of embedded message of the encounter. More practically, it allowed us to discover a lead template for the discovery of a selective chitinase inhibitor directed toward the functions of SmChi, without the need for lengthy and costly analog syntheses.

9. Conclusion

To date, no practical use for chitinase inhibitors has been identified. Nevertheless, naturally-occurring as well as synthetic non-natural chitinase inhibitors still hold great promise as antifungal, insecticidal or antiparasitic agents, as well retaining promise as possible therapeutics for asthma and other related diseases in humans. In addition, specific inhibitors might provide powerful tools to help investigate and explain novel phenomena. We believe that newly-discovered chitinases inhibitors will, in future, proved to be good lead compounds for development into highly-effective agricultural chemicals, medicines and/or biological reagents.

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