Review

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

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(Contributed by Satoshi ŌMURA, M.J.A.)

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 chitine 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-

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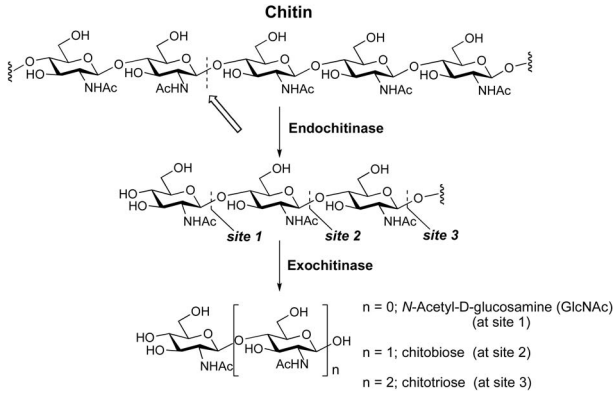


Fig. 1. The structure of chitin and the products of chitinase hydrolysis.

nism of Th2 cytokine-driven inflammatory response in asthma.[12] 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*.[13),14)] 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.[11),15),16)]

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.[17)] 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.[18)] Using the agar plate method and the chitin-degrading bacterium, EY410, **5** and cyclo-L-Arg-L-Pro (diastereomer of **5**) 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, by

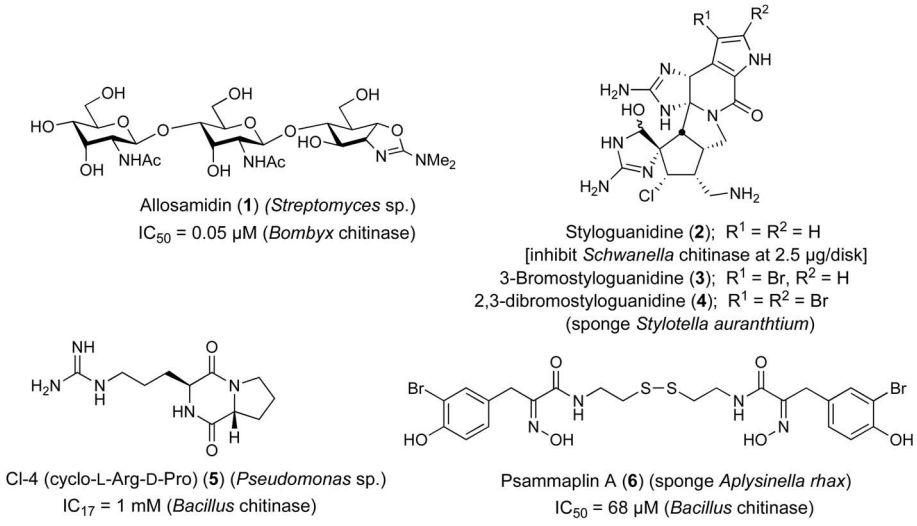
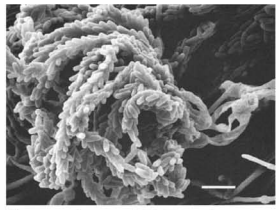
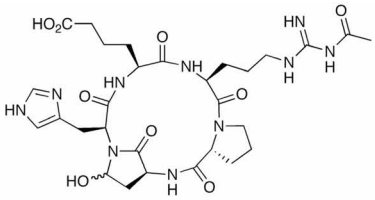


Fig. 2. Structures of naturally-occurring chitinase inhibitors and their inhibitory activities.

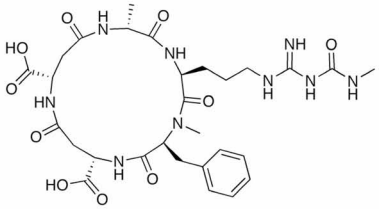


Clonostachys sp. FO-7314; bar 10 μm



Argadin (**7**)

G*liocladium* sp. FTD-0668; bar 20 μm



Argifin (**8**)

	IC$_{50}$ values (μM)							Ki value (μM)
	*Sm*ChiA	*Sm*ChiB	*Sm*ChiC$_1$	*Lucilia cuprina* Chi (37 °C)	*Lucilia cuprina* Chi (20 °C)	*Aspergillus fumigatus* ChiB1	Human chitotriosidase	*SmChiB*
Argadin (**7**)	0.016	0.033	>30	0.150	0.0034	0.5	0.013	0.02
Argifin (**8**)	0.025	6.4	>30	3.70	0.100	1.1	4.5	33
Allosamidin (**1**)	–	–	–	0.0023	0.0004	—	—	0.45

(*Sm* = *Serratia marcescens*; Chi = chitinase)

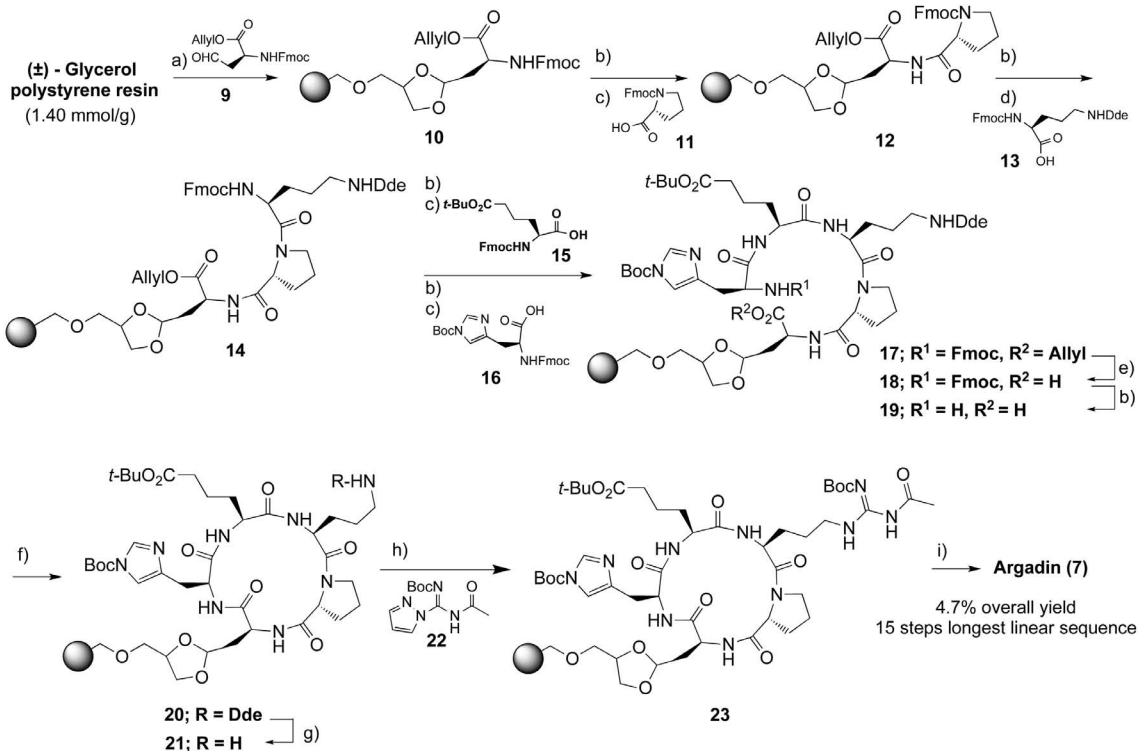
Fig. 3. Structures of naturally-occurring chitinase, argadin (**7**) and argifin (**8**), and photomicrographs of their producing strains.

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)–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 lavae, 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 exuva, leading to their death shortly after



Scheme 1. Solid-phase synthesis of argadin **7**; a) *p*-TsOH, 1,4-dioxane, reflux, 6 h; b) 20% piperidine/DMF, 1h; c) PyBOP, DIPEA, DCM/DMF (4/1), 2 h; d) HBTU, DIPEA, DCM/DMF (4/1), 2 h; e) $Pd(PPh_3)_4$, dimedone, THF, 1 h; f) HATU, DIPEA, DCM/DMF (4/1) 1 h × 2 (two-cycle repetition); g) 2% hydrazine/DMF, 1 h; h) DIPEA, DCM/DMF (4/1), 1 h; i) 95% TFA/H_2O, 3 h, then HPLC separation. Ts = *p*-toluenesulfonyl, Fmoc = 9-fluorenylmethoxycarbonyl, PyBOP = (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate, DIPEA = diisopropylethylamine, HBTU = *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate, HATU = *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate, Dde = 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl, Boc = *t*-butoxycarbonyl, TFA = trifluoroacetic acid.

sclerotization of the new cuticle, the damage presumably resulting from inhibition of chitinases in the molting process. A subsequent expanded bioassay of **7** and **8** 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.[28)–30)] The three-dimensional (3-D) structure of **7** and **8**, in complex with *Aspergillus fumigatus* chitinase B1, *Serratia marcesens* chitinaese B, human chitotriosidase and acidic mammalian chitinase, were resolved by X-ray crystallography.[28),29),31)] 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.[32)] 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.[33)]

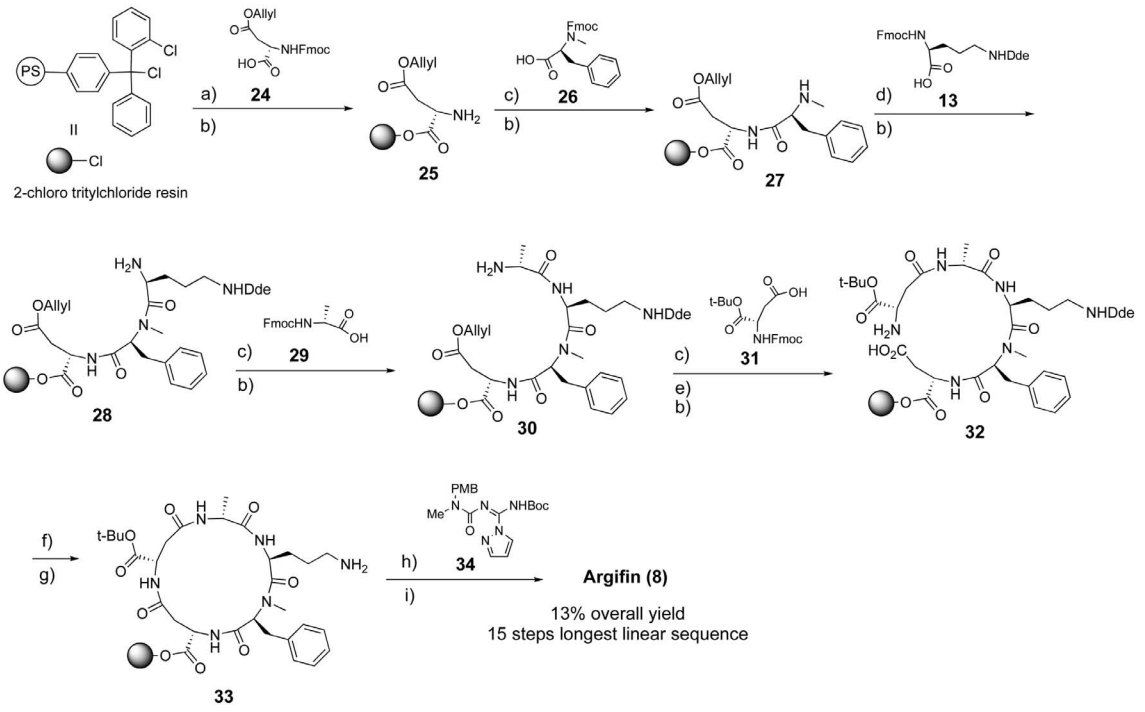
Our synthetic route for **7** is outlined in Scheme 1. The cyclic peptide structure of **7** allowed us to adopt a solid-phase strategy based on application of a 9-fluorenylmethoxycarbonyl (Fmoc) protective

group for the amine of amino acid fragments, with allyl protection on the carboxylic acid of the L-aspartic-β-semialdehyde (**9**)[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^{ω}-acetylarginine 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, macrolactamization 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'*-acetylcarboxamidine (**22**),[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 cyclicpeptide. 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.[36] Their strategy, however, included liquid phase reaction sequences for introduction of the N^{ω}-methylcarbamoyl 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.[37] At the same time, we independently reported the total synthesis of **8** accomplished by solid-phase synthetic protocols in 2009.[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^{ω}-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



Scheme 2. Solid-phase synthesis of argadin **8**; a) DIPEA, DCM, 2 h; b) 20% piperidine/DMF, 1 h; c) PyBOP, DIPEA, DCM/DMF (4/1), 2 h; d) HATU, DIPEA, DCM/DMF (4/1), 2 h; e) $Pd(PPh_3)_4$, dimedone, THF, 1 h; f) HATU, DIPEA, DCM/DMF (4/1) 1 h × 2 (two-cycle repetition); g) 2% hydrazine/DMF, 1 h; h) DIPEA, DCM/DMF (4/1), 1 h; i) 90% TFA/DCM 1 h, then HPLC separation. *t*-Bu = *t*-butyl, PMB = *p*-methoxybenzyl.

at final step. This means that the process has no liquid-phase steps. Additionally, 1-*H*-pyrazole-1-[*N*-(*tert*-butoxycarbonyl)-*N*'-(*N*-*p*-methoxybenzylcarbamoyl-*N*-methyl)]carboxamidine (**34**)[38] was designed to simply introduce the N^{ω}-methylcarbamoylguanidine onto the NH_2 group of Orn for solid phase synthesis. Actually, the N^{ω}-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 N^{ω}-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* (*Sm*ChiB), was resolved by X-ray analysis (Fig. 4).[28] And an inhibitory activity of **8** against *Sm*ChiB is shown as an IC_{50} value of 6.4 μM.[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 *Sm*ChiB.[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 *Sm*ChiB-**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 *Sm*ChiB, 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-*Sm*ChiB were obtained (referred to as S890 and S1505 in reference 42).

The two structures were used to obtain guides

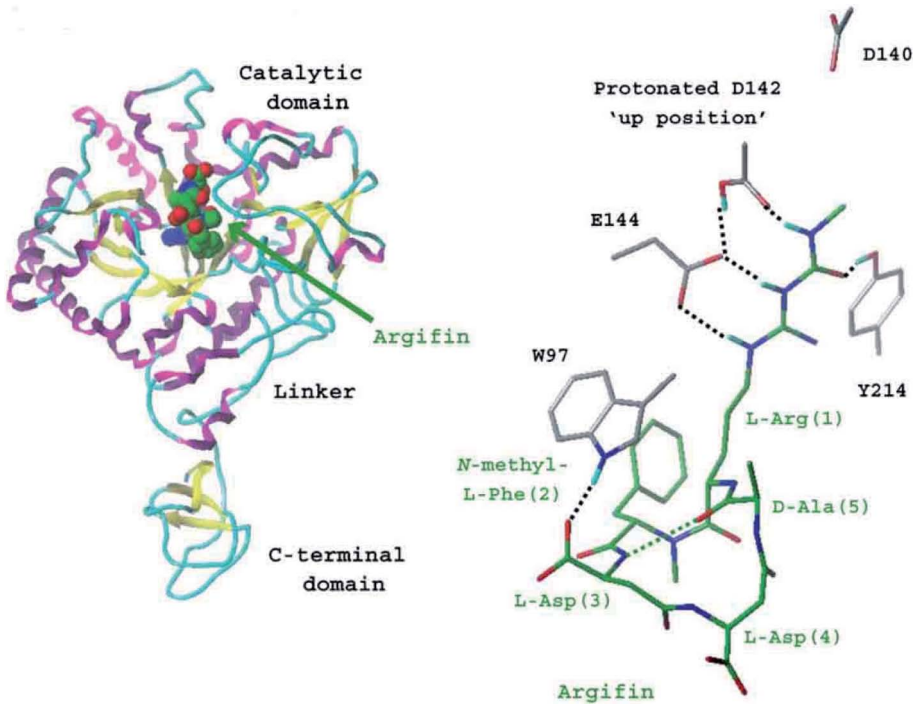
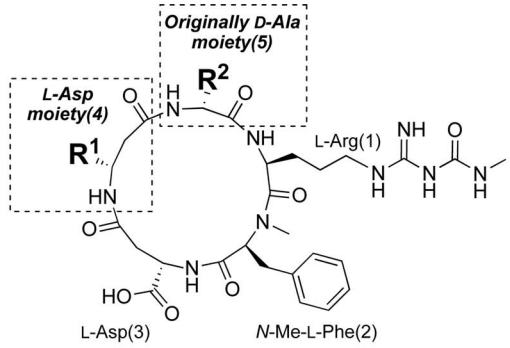


Fig. 4. X-ray crystal structure of argifin-*Sm*ChiB complexes. (Left) Stereo figure of the *Sm*ChiB fold, shown as a ribbon drawing. Argifin is shown in a space-filling display with carbon (green), oxygen (red) and nitrogen (blue) atoms. (Right) Stereo figure of the *Sm*ChiB active site together with argifin molecule. The critical amino acid residues of *Sm*ChiB to interact to argifin are selected. The hydrogen-bonding interactions between argifin-*Sm*ChiB are indicated on the right by black dashed lines. Intra-molecular hydrogen bond is indicated by green dashed line.

for designing argifin derivatives. From the X-ray analysis of argifin-*Sm*ChiB, 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 *Sm*ChiB, 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-benzylpiperdine 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 *Sm*ChiB (as shown in Fig. 6A). The 4-benzylpiperdine 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 *Sm*ChiB (docking model of D-Leu(5) derivative **37** with *Sm*ChiB is shown in Fig. 6C).

The interaction mode of each derivative with *Sm*ChiB 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.[44] 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),[45] 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 ($G_{complexS}$). Finally, a pose with the lowest $G_{complex}$ was selected as the interaction model. As expected, the interaction model of 35 located its 4-



Derivatives	R^1 in L-Asp moiety(4)	D-amino acids(5) (including R^2)
Argifin (**8**)	-COOH	D-Ala
35	-CO(4-Benzylpiperdinyl)	D-Ala
36	-COOH	D-Val
37	-COOH	D-Leu
38	-COOH	D-Met
39	-COOH	D-Phe
40	-COOH	D-Trp
41	-CO(4-Benzylpiperdinyl)	D-Leu

Fig. 5. Argifin (**8**) and artificial derivatives (**35**–**41**).

benzylpiperdinyl group in the target groove to make an additional interaction with *Sm*ChiB in pose S890 (Fig. 6B). The calculations also predicted that all side-chains for D-animo acid moiety (**36**–**40**) could occupy the available space in the **8**-*Sm*ChiB complex (Fig. 6C; as an example, the model for **37**).

The binding affinity (ΔG_{bind}) of each derivative was approximated by applying the single-point MM-PBSA calculation. The argifin based relative binding affinity ($\Delta\Delta G_{bind}$) was estimated using the G_{bind} value of **8** in pose S890 as a reference (Table 1). All derivatives showed greater ΔG_{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 *Sm*ChiB (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 μM, respectively, than **8** (6.4 μM). Furthermore, a hybrid derivative (**41**), effectively a combination of **35** and **37**, was prepared. As expected, **41** showed lowest ΔG_{bind}, and was found to be the most potent inhibitor against *Sm*ChiB, with an IC_{50} value of 0.23 μM, about a 30-fold increase over that of **8**.

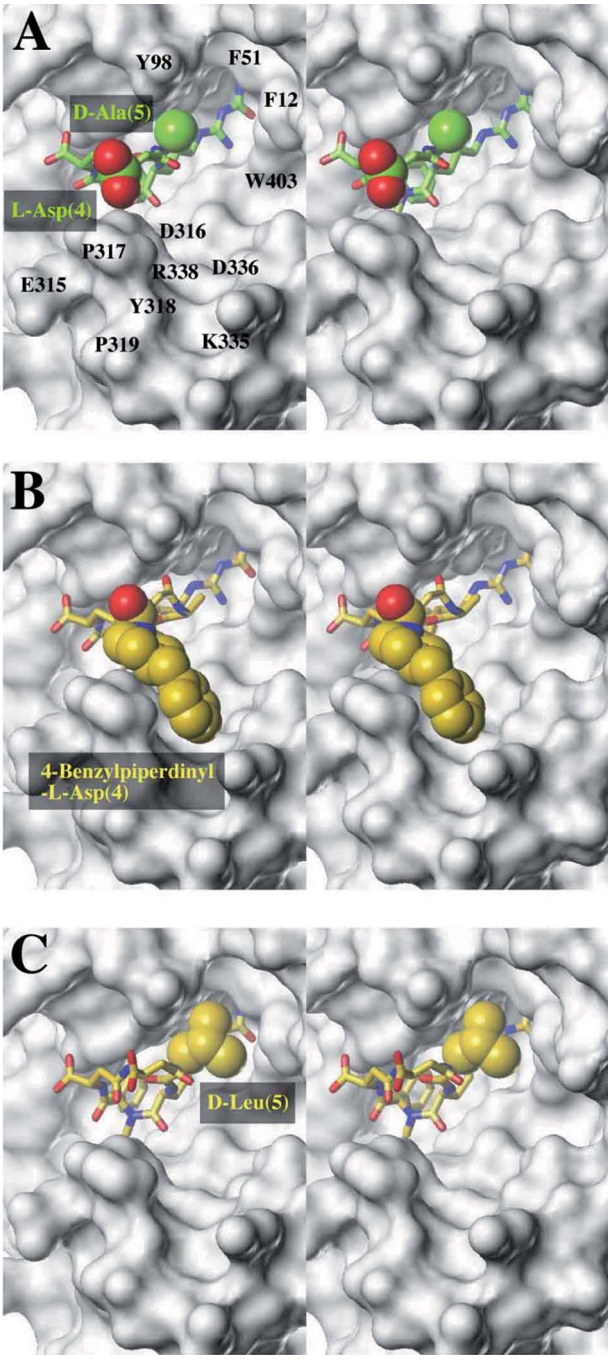
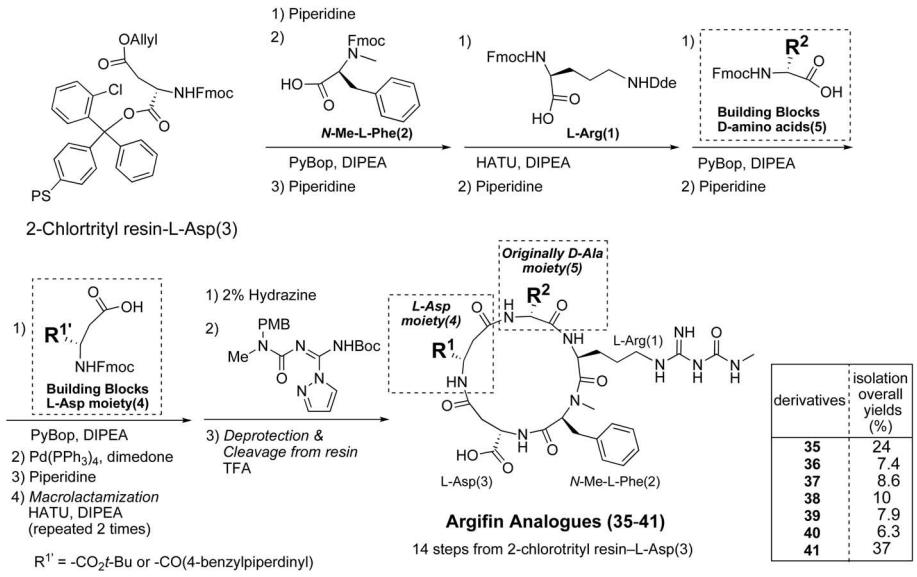


Fig. 6. Stereoview of interaction of argifin **8** in pose S890 (A), and those of derivatives **35** (B) and **37** (C) obtained by our procedure. L-Asp(4) and D-Ala(5) of 8, 4-benzylpiperdinyl-L-Asp(4) of derivative **35**, and D-Leu(5) of derivative **37**, are shown in space-filling display.

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 *Sm*ChiB.

Table 1. ΔG_{bind}, and $\Delta\Delta G_{bind}$ estimated by the MM-PBSA method, and inhibitory activities against *Sm*ChiB for argifin (**8**) and derivatives **35**–**41**

Derivatives	ΔG_{bind} (kcal/mol)	ΔG_{bind} (kcal/mol)	IC_{50} (μM) against *Sm*ChiB
Argifin (**8**)	− 45.81	0.00	6.4 ± 1
35	− 55.54	− 9.73	**1.3 ± 0.1**
36	− 46.61	− 0.80	6.1 ± 0.4
37	− 49.33	− 3.52	**1.9 ± 0.3**
38	− 47.89	− 2.08	10.0 ± 0.6
39	− 49.31	− 3.50	9.9 ± 1
40	− 53.72	− 7.91	4.5 ± 0.6
41	− 57.34	− 11.53	**0.23 ± 0.05**

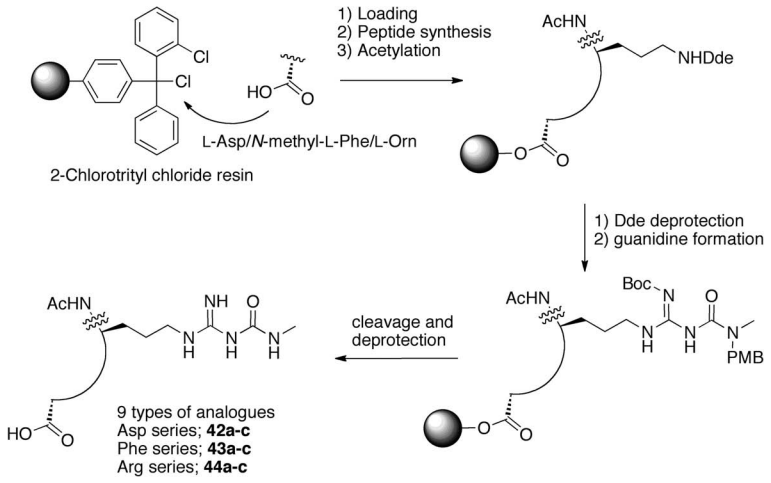


Scheme 3. Synthesis of the argifin library.

7. Evaluation of analogs of acyclic peptide of argifin

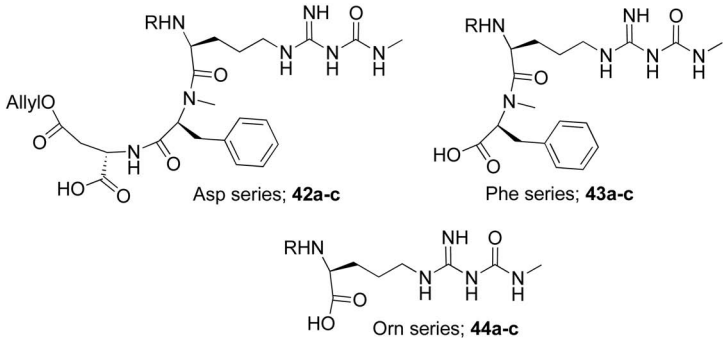
Having achieved the efficient solid-phase total synthesis of argifin (**8**), we also prepared acyclic peptide compounds, based on peptide fragments from the framework of **8** to search for novel and potent chitinase inhibitors.[38] We undertook investigation of analogs by examining nine acyclic derivatives bearing the N^{ω}-methylcarbamoylguanidino group to elucidate structure-activity-relationships (SAR) against each *Sm*Chi 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 N^{ω}-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



Scheme 4. General procedure of nine types of linear analogs.

Table 2. IC_{50} results for nine types of linear analogs



	R	IC_{50} (μM)			yields (%)[b]
		*Sm*ChiA	*Sm*ChiB	*Sm*ChiC	
42a	Ac	0.14 ± 0.01	0.13 ± 0.05	>30	78
42b	Ac-D-Ala	**0.025 ± 0.002**	**0.091 ± 0.004**	>30	44
42c	Ac-Asp-D-Ala	1.1 ± 0.06	1.0 ± 0.2	>30	20
43a[a]	Ac	0.75 ± 0.07	3.7 ± 0.7	>30	22
43b	Ac-D-Ala	0.090 ± 0.01	2.7 ± 0.6	>30	40
43c	Ac-Asp-D-Ala	8.4 ± 1	30 ± 7	>30	30
44a	Ac	31 ± 1	260 ± 55	>30	58
44b	Ac-D-Ala	6.6 ± 0.6	64 ± 12	>30	35
44c	Ac-Asp-D-Ala	32 ± 8	110 ± 17	>30	21
8	Argifin	0.025 ± 0.002	6.4 ± 1	>30	13

[a] ref. 37. [b] isolated yields by HPLC purification.

*Sm*Chi 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 *Sm*ChiB (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-

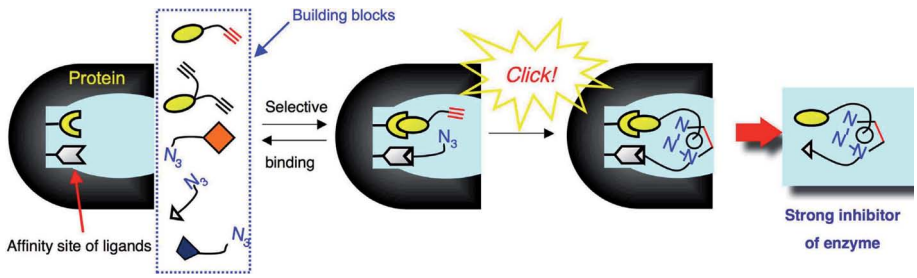


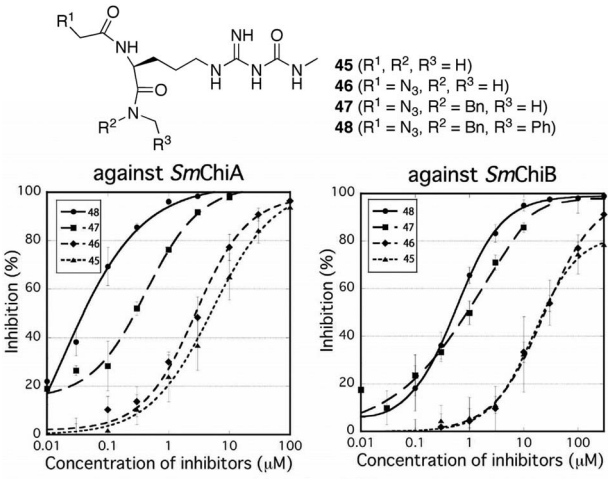
Fig. 7. An illustration of *in situ* click chemistry.

pression of competent inhibitory activity with respect to *Sm*ChiB, except for L-Arg-series **44a–c** (Table 2). In contrast, possessing the D-Ala moiety increases activity in terms of *Sm*ChiA. 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 *Sm*ChiA 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 *Sm*ChiA and B. Unfortunately, all of the acyclic compounds, including **8**, exhibit no activity against *Sm*ChiC$_1$. It is, however, notable that the novel acyclic peptide **42b** exhibits 70-fold more potent activity against *Sm*ChiB 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 arfigin (**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^{ω}-methylcarbamoyl-L-arginine moiety and the polar groups arrayed in the hydrolytic pocket of the family 18 chitinases.[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^{ω}-methylcarbamoyl group to penetrate fully into the active site pocket of chitinases strongly correlated with the inhibition of chitin hydrolysis.[31)] From our SAR studies, the N^{ω}-methylcarbamoyl group was obviously a crucial component for expression of inhibitory activity on chitinases.[38)] Hence, we concluded that the N^{ω}-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^{ω}-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,[46)] Rideout *et al.*[47)] Inglese and Benkovic,[48)] Boger *et al.*[49)] Maly *et al.*[50)] Nicolaou *et al.*[51)] Greasley *et al.*[52)] Nguyen and Huc,[53)] Nicolaou *et al.*[54)] Kehoe *et al.*[55)] Poulin-Kerstien and Dervan,[56)] and Hu *et al.*[57)]) for the screening of novel and more potent chitinase inhibitors employing the 1,3-dipolar cycloaddition[58)] between an azide ligand and a library of acetylenes.[30)] *In situ* click chemistry for drug discovery is dependent on irreversibly reacting reagents that are inert under physiological conditions,[59)] as previously demonstrated by the discovery of highly-potent inhibitors of acetylcholine esterase,[60)–63)] carbonic anhydrase II,[64)] and HIV-1 protease.[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 *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



IC_{50} values (μM)

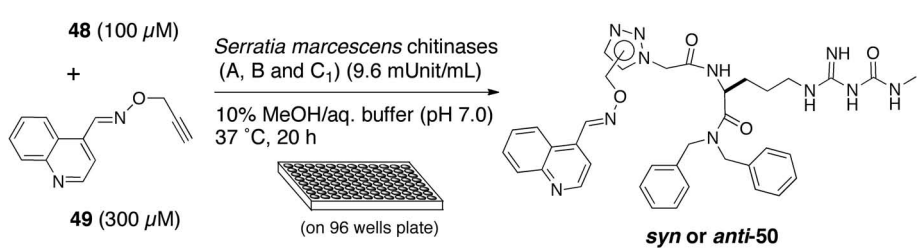
	45	46	47	48
*Sm*ChiA	5.2±1	3.0±0.7	0.46±0.1	0.045±0.01
*Sm*ChiB	16 ±3	24 ±4	0.90±0.4	0.58 ±0.04
*Sm*ChiC$_1$	>30	>30	>30	>30

Fig. 8. Structures and IC_{50} values of N^{ω}-methylcarbamoyl-L-Arg derived inhibitors.

blocks with the highest affinity. The enforced propinquity 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 monovalent building blocks.

Initially, we investigated appropriate inhibitors to develop *in situ* click chemistry from the N^{ω}-methylcarbonyl-L-Arg scaffold. A simple Arg-derived inhibitor (**45**), discovered by van Aalten and coworkers had been reported independently,[31] it showed low inhibitory activity against *Sm*Chi in contrast to **8**, which was examined by our group (Fig. 8). We consequently synthesized the azide-bearing inhibitor **46** as a reactive scaffold for capturing complementary acetylenic reagents to form triazole-linked inhibitors by TGS. Elucidations of IC_{50} values against each *Sm*Chi 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 dibenzylamide analog **48** of azide **46** emerged as a potent inhibitor (0.045 μM and 0.58 μM IC_{50} values against *Sm*ChiA and B, respectively). Interestingly, the IC_{50} value against *Sm*ChiB 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 *Sm*ChiA, B, and C_1. Utilization of the mixed *Sm*Chi has the advantage of accelerating the identification of novel inhibitors against each isozyme of chitinase through a one-off screening. Although a singular isozyme or multiple isozymes of chitinase may participate in the formation of triazoles under this particular screening condition, the identification of the actual templating isozyme or isozymes can be determined in a follow-up assay using separate isozymes. Consequently, azide **48** (100 μM) and 71 structurally-diverse alkynes (300 μM) (structures are shown in supplementary information of reference 30) were incubated in the presence of *Sm*ChiA, B and C_1 (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



Scheme 5. *Sm*Chi templated *in situ* click chemistry protocol and the guided triazole analog.

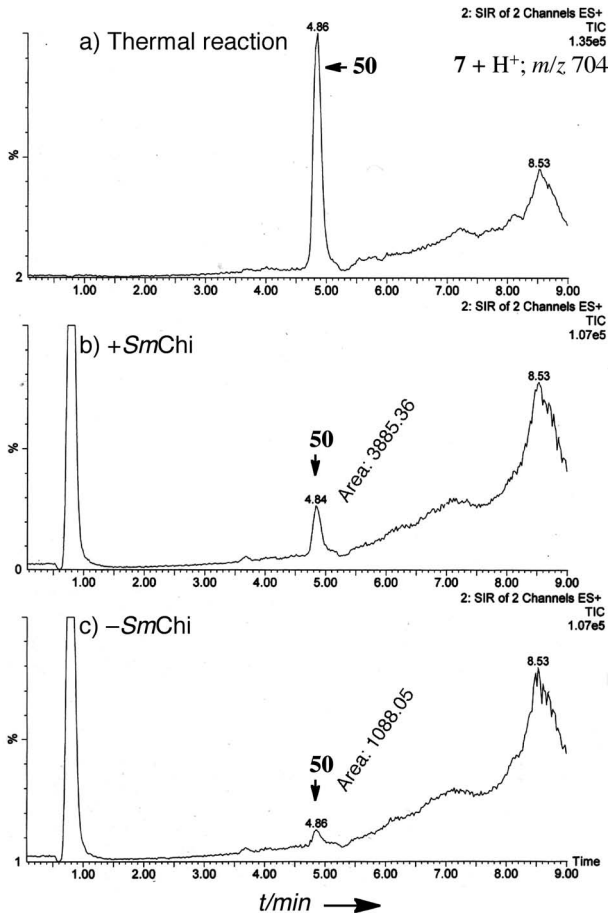
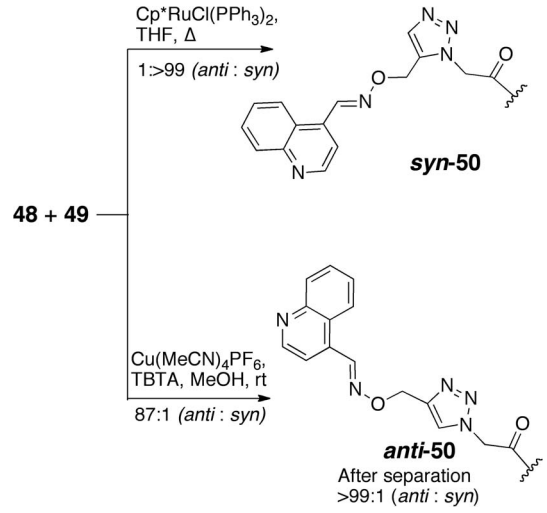


Fig. 9. Results of *in situ* click chemistry between **48** and **49**, monitored by LCMS-SIR. a) Authentic sample of **50** from thermal reaction (100 °C, 12 h), apparently single peak (4.9 min) of **50** (*anti*:*syn* = 3:2) was observed; b) Reaction (37 °C, 20 h) between **48** (100 μM) and **49**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



Scheme 6. Preparation of ***anti*** and ***syn*-50**. TBTA = *tris*-(benzyltriazolylmethyl)amine, THF = tetrahydrofuran.

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** (IC_{50} > 30 μM) had been sufficiently accelerated in its cycloaddition with azide **48** in the presence of the enzymes to yield a detectable amount of triazole **50** (at this point unidentified whether *syn*- or *anti*-substituted triazole) over background with great reproducibility by LCMS-SIR measurement (Fig. 9). In effect, the chitinases had performed as a molecular scale reaction vessel, creating its own better inhibitor.

Subsequently, azide **48** and alkyne **49** were then 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 *Sm*ChiA and C_1 were almost the same as that of **48**. On the other hand, ***syn*-50** displayed high inhibitory activity against *Sm*ChiB (IC_{50} 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 *Sm*ChiB 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 $(His)_6$-*Sm*ChiB in an enzyme-dose dependent manner (Fig. 10). Moreover, no *syn*-triazole formation was observed in the control incubation containing *Sm*ChiB and the same azide and alkyne in the presence of argadin natural product **7** (IC_{50} values against *Sm*ChiB;

Table 3. IC_{50} values of **48**, ***anti*-**, and ***syn*-50** against *Sm*ChiA, B and C_1

	IC_{50} values (μM)		
	48	***anti*-50**	***syn*-50**
*Sm*ChiA	0.045 ± 0.01	0.050 ± 0.002	0.061 ± 0.01
*Sm*ChiB	0.58 ± 0.04	1.0 ± 0.09	**0.022 ± 0.002**
*Sm*ChiC$_1$	>30	>30	>30

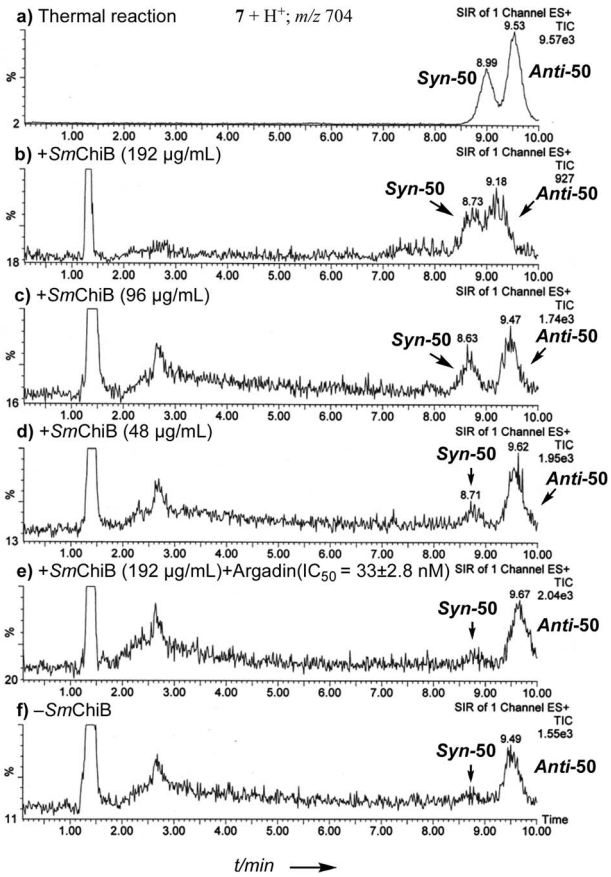


Fig. 10. Identification of *syn*-selectivity for *in situ* click chemistry between **48** (100 μM) and **49** (300 μM), monitored by LCMS-SIR. a) Authentic **50** from thermal reaction: ***syn*-** and ***anti*-50** (2 : 3 ratio); b) Reaction with pure $(His)_6$-*Sm*ChiB (192 μg/mL; 37 °C, 20 h); c) $(His)_6$-*Sm*ChiB (96 μg/mL; 37 °C, 20 h), d) $(His)_6$-*Sm*ChiB (48 μg/mL; 37 °C, 20 h), e) $(His)_6$-*Sm*ChiB (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 *Sm*ChiB 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 *Sm*Chi, which in this case was specifically *Sm*ChiB, 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 *Sm*Chi, 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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Profiles

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