ORIGINAL ARTICLE

Chitinase inhibitors: extraction of the active framework from natural argifin and use of *in situ* click chemistry

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In situ click chemistry is a target-guided synthesis technique for discovering potent protein ligands by assembling azides and alkynes into triazoles inside the affinity site of a target protein. We report the rapid discovery of a new and potent inhibitor of bacterial chitinases by the use of *in situ* click chemistry. We observed a target-templated formation of a potent triazole inhibitor of the chitinase-catalyzed chitin hydrolysis, through *in situ* click chemistry between a biologically active azide-containing scaffold and structurally unrelated alkyne fragments. Chitinase inhibitors have chemotherapeutic potential as fungicides, pesticides and antiasthmatics. Argifin, which has been isolated and characterized as a cyclopentapeptide natural product by our research group, shows strong inhibitory activity against chitinases. As a result of our efforts at developing a chitinase inhibitor from an azide-bearing argifin fragment and the application of the chitinase template and a library of alkynes, we rapidly obtained a very potent and new 1,5-disubstituted triazole inhibitor against *Serratia marcescens* chitinase (*Sm*Chi) B. The new inhibitor expressed 300-fold increase in the inhibitory activity against *Sm*ChiB compared with that of argifin. To the best of our knowledge, our finding of an enzyme-made 1,5-disubstituted triazole, using *in situ* click chemistry is the second example reported in the literature.

INTRODUCTION

Chitin, the second most abundant polysaccharide in nature, is a constituent of fungal cell walls, the exoskeletons of crustaceans and insects, and the microfilarial sheaths of parasitic nematodes.[1–3] 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 (for a review, see Andersen *et al.*[4]). They also offer significant potential for the treatment of asthma and other diseases in humans.[5] Argifin (**1**) (Figure 1),[6–8] a member of a family of natural cyclic peptides produced by microorganisms, was isolated in our laboratory from the cultured broth of the fungal strain (*Gliocladium* sp. FTD-0668), and was found to be a potent inhibitor of blowfly[6] and *Serratia marcescens* chitinases (*Sm*Chi).[9–12] Recently, argifin complexes with fungal (*Aspergillus fumigatus*), human and bacterial chitinases have been resolved by X-ray crystallography.[11,12] These studies 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 examined to date. The remarkable fidelity of the hydrogen-bonding network between the chitinases and the argifin ligand implicates its critical role in revealing the micro- to nanomolar range of inhibition. In fact, van Aalten and co-workers revealed through 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.[13] Hence, we concluded that the N^{ω}-methylcarbamoyl-L-arginine core represents an ideal anchor to derivatize and elaborate better chitinase inhibitors.
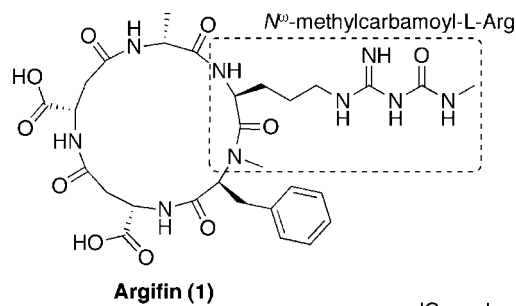
Herein we report our early results of the design and simplification of azide-bearing N^{ω}-methylcarbamoyl-L-arginine substrate as a smaller analog of macrocyclic peptide natural product **1** and the use of target-guided synthesis (TGS) (for reports of TGS, see Rideout,[14] Rideout,[15] Ingelese and Benkovic,[16] Boger *et al.*,[17] Maly *et al.*,[18] Nicolaou *et al*,[19] Greasley *et al.*,[20] Nguyen and Huc,[21] Nicolaou *et al.*,[22] Kehoe *et al.*,[23] Poulin-Kerstein and Dervan,[24] and Hu *et al.*[25]) for the screening of new and more potent chitinase inhibitors, using the 1,3-dipolar cycloaddition[26] between an azide ligand and a library of acetylenes. The *in situ* click chemistry for drug discovery is

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Figure 1 Argifin (**1**) and the IC_{50} values against *Serratia marcescens* chitinases A, B and C_1.

Serratia marcescens chitinase A (*Sm*ChiA)	0.025 ± 0.0024
B (*Sm*ChiB)	6.4 ± 1.0
C_1(*Sm*ChiC$_1$)	>30

dependent on irreversibly reacting reagents that are inert under physiological conditions,[27] as shown earlier by the discovery of highly potent inhibitors of acetylcholine esterase,[28–31] carbonic anhydrase II[32] and HIV-1 protease.[33]

RESULTS AND DISCUSSION

Preparation of the azide-bearing inhibitors

Initially, we investigated the appropriate inhibitors to develop *in situ* click chemistry from the N^ω-methylcarbamoyl-L-arginine scaffold. Although a simple arginine-derived inhibitor **2** discovered by van Aalten and co-workers had been reported independently,[13] it showed low inhibitory activity against *Sm*Chi in contrast to argifin **1**, which was examined by our group (Figure 2). We synthesized the azide-bearing inhibitor **3** as a reactive scaffold for capturing complementary acetylenic reagents to form triazole-linked inhibitors by TGS. Competition assay with 4-methylumberiferyl diacetylchitobiose (4-MU-(GluNAc)$_2$)[34,35] showed that this azide-bearing inhibitor **3** expressed a low inhibitory activity similar to that of the azide-lacking inhibitor **2**, which are in striking contrast to the potency of the natural product **1**. Hence, amide derivatives of azide **3** with amines other than methylamine were made and tested to see whether the binding could be restored to a level that would make azide **3** a sufficiently good anchor at the active site, to be used for the capture of alkyne-bearing candidates through *in situ* triazole formation. Fortunately, the dibenzylamide analog **5** of azide **3** emerged as a potent inhibitor (0.045 and 0.58 μM IC_{50} values against *Sm*ChiA and B, respectively). Interestingly, the IC_{50} value against *Sm*ChiB of **5** was 10-fold stronger than that of parent **1**. The monobenzylamide **4** was also active, but less than **5**. As seen in Figure 2, compounds **2–5** can be ranked by inhibition constants as **5** > **4** > **3** ≈ **2**. We therefore used potent azide analog **5** as a target 'anchor' molecule for *in situ* click chemistry.

Screening for enzyme-templated reaction

The *in situ* click chemistry experiments were carried out in parallel in 96-well microtiter plates to explore the chitinase-accelerated reaction using a mixture of *Sm*Chi A, B and C_1 (Sigma Co., Tokyo, Japan). Utilization of the mixed *Sm*Chi has the advantage of accelerating the identification of new inhibitors against each isozyme of chitinase through a one-off screening. Although a single 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 **5** (100 μM) and 71 structurally diverse alkynes (300 μM) were incubated in the presence
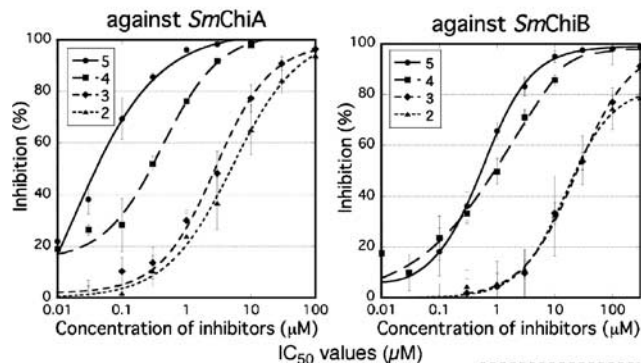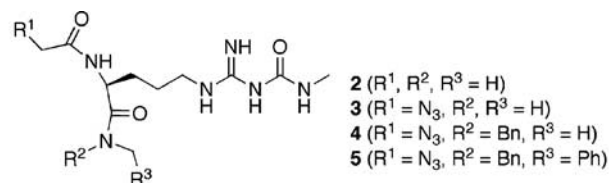

Figure 2 Structures and IC_{50} values of N^ω-methylcarbamoyl-L-arginine-derived inhibitors.

of *Sm*Chi A, B and C_1 (9.6 mUnit ml^{-1}) in 10% methanol containing phosphate buffer solution at pH 7.0 (Scheme 1). The formation of the triazole products was monitored by HPLC and mass spectrometry by 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 **6** (IC_{50} > 30 μM) was sufficiently accelerated in its cycloaddition with azide **5** in the presence of the enzymes to yield a detectable amount of triazole **7** (at this point not distinguished as to whether a *syn*- or *anti*-substituted triazole) in the background with great reproducibility by LCMS-SIR measurement (Figure 3). In effect, the chitinases had performed as a reaction vessel of molecular scale to create its own better inhibitor.

Preparations and inhibitory activities of triazole 7

Subsequently, azide **5** and alkyne **6** were subjected to copper(I)-catalyzed azide–alkyne cycloaddition conditions (CuAAC)[36–38] along with the ruthenium-catalyzed azide-alkyne cycloaddition reaction conditions (RuAAC),[39,40] to prepare pure regioisomers of **7** allowing the identification of the regiochemistry of the triazole formed by the enzymes. As expected, pure 1,4- and 1,5-disubstituted triazole products (*anti*-**7** and *syn*-**7**) were obtained (Scheme 2). Having both pure triazoles in hand, we turned our attention to the identification of the generated triazole analog by TGS and the participating isozymes of the enzyme for this *in situ* click chemistry. For the determination of IC_{50} values against each *Sm*Chi isozyme, *anti*- and *syn*-**7** were assessed in a competition assay with 4-MU-(GluNAc)$_2$. As shown in Figure 4, the inhibitory activities of both regioisomers of **7** against *Sm*Chi A and C_1 were almost the same as those of **5**. On the other hand, *syn*-**7** displayed high inhibitory activity against *Sm*Chi B (IC_{50} value of 0.022 μM), which is approximately 30-fold stronger than that of **5** (approximately 300-fold improved potency as compared with the natural product **1**). These results strongly indicate that *syn*-**7** is most likely formed *in situ* by the *Sm*Chi B isozyme in the enzyme mixture.
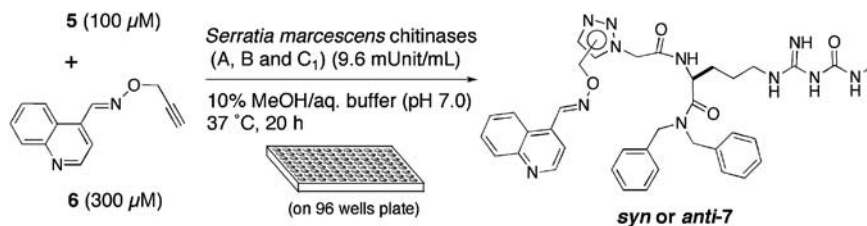
Scheme 1 SmChi templated *in situ* click chemistry protocol and the guided triazole analog.
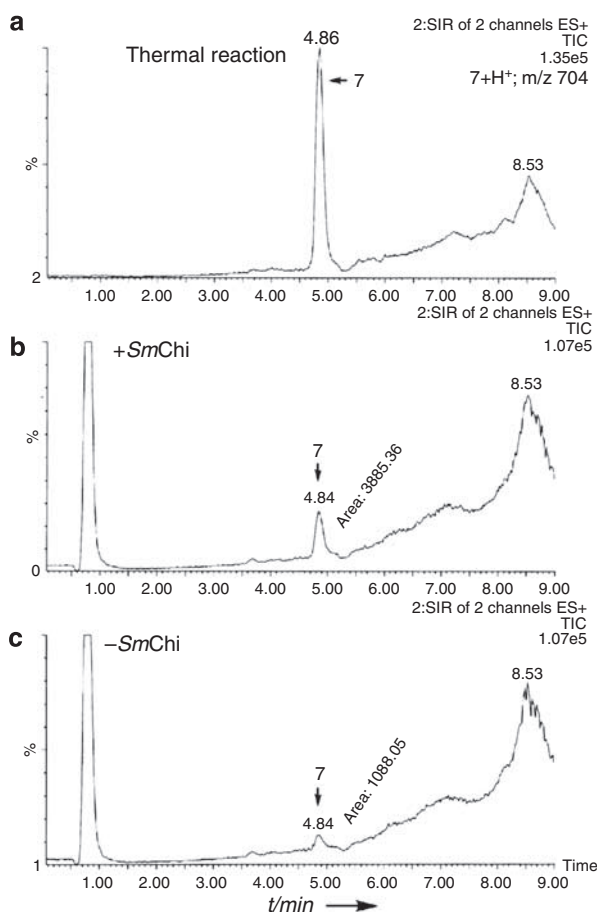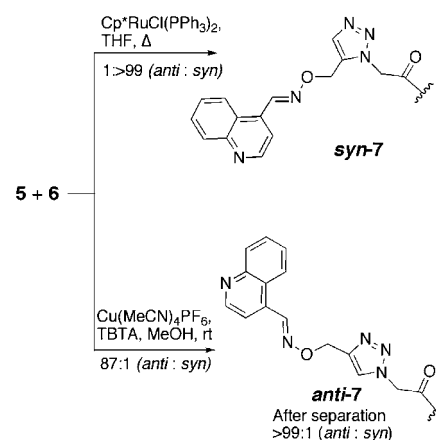

Figure 3 Results of *in situ* click chemistry between **5** and **6**, monitored by LCMS-SIR. (**a**) Authentic sample of **7** from thermal reaction (100 °C, 12 h), apparently single peak (4.9 min) of **7** (*anti*: *syn*=3: 2) was observed; (**b**) reaction (37 °C, 20 h) between **5** (100 μM) and **6** (300 μM) in the presence of SmChi (9.6 mUnit ml⁻¹); (**c**) without SmChi (background reaction).

Scheme 2 Preparation of *anti* and *syn*-**7**. TBTA=*tris*-(benzyltriazolylmethyl) amine, THF=tetrahydrofuran.

	anti-7	*syn*-7
SmChiA	0.050±0.0021	0.061±0.010
SmChiB	1.0 ±0.094	**0.022±0.0015**
SmChiC₁	>30	>30
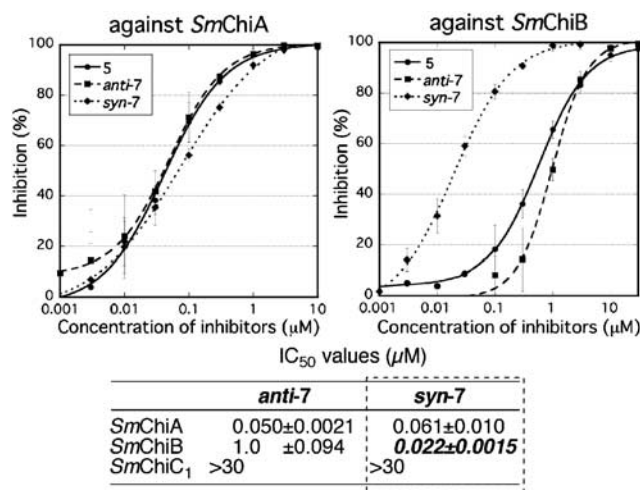
Figure 4 IC$_{50}$ values of *anti* and *syn*-**7** against SmChiA, B and C$_1$.

Acceleration effect of *syn*-triazole formation through SmChiB

Analysis of *syn-anti* selection for the *in situ* screening by LCMS-SIR revealed that a combination of azide **5** and alkyne **6** had led to the accelerated formation of **syn-7** in the presence of pure (His)$_6$-SmChiB in an enzyme-dose-dependent manner (Figure 5). Moreover, no *syn*-triazole formation was observed in the control incubation containing SmChi B and the same azide and alkyne in the presence of a natural product argadin acting as a potent inhibitor (see Supplementary Information for structure)[41] (IC$_{50}$ values against SmChiB, 33 ± 2.8 nM), thereby validating **syn-7** as an *in situ* hit and confirming that its formation required the enzyme active site to be accessible. Interestingly, the regioisomer **anti-7**, which is predominantly formed

under pure thermal Huisgen conditions, is less active against SmChiB than the 'anchor' molecule **5**, which probably presents the –CH$_2$–N$_3$ group in a unique position when **5** and the protein form their complex. At this point, the chitinase–**5** complex is most likely a single entity presenting the azide properly to the 'well-suited' alkyne ligand and correctly binding to the complex so that a *syn*-triazole selectively clicks into its existence. Now the two small molecules have become one, and this enzyme-templated molecule invariably binds more strongly than either component alone. The high kinetic barrier would effectively lower **5** and **6** until they are attracted to each other to form the thermodynamically unfavorable *syn*-isomer.
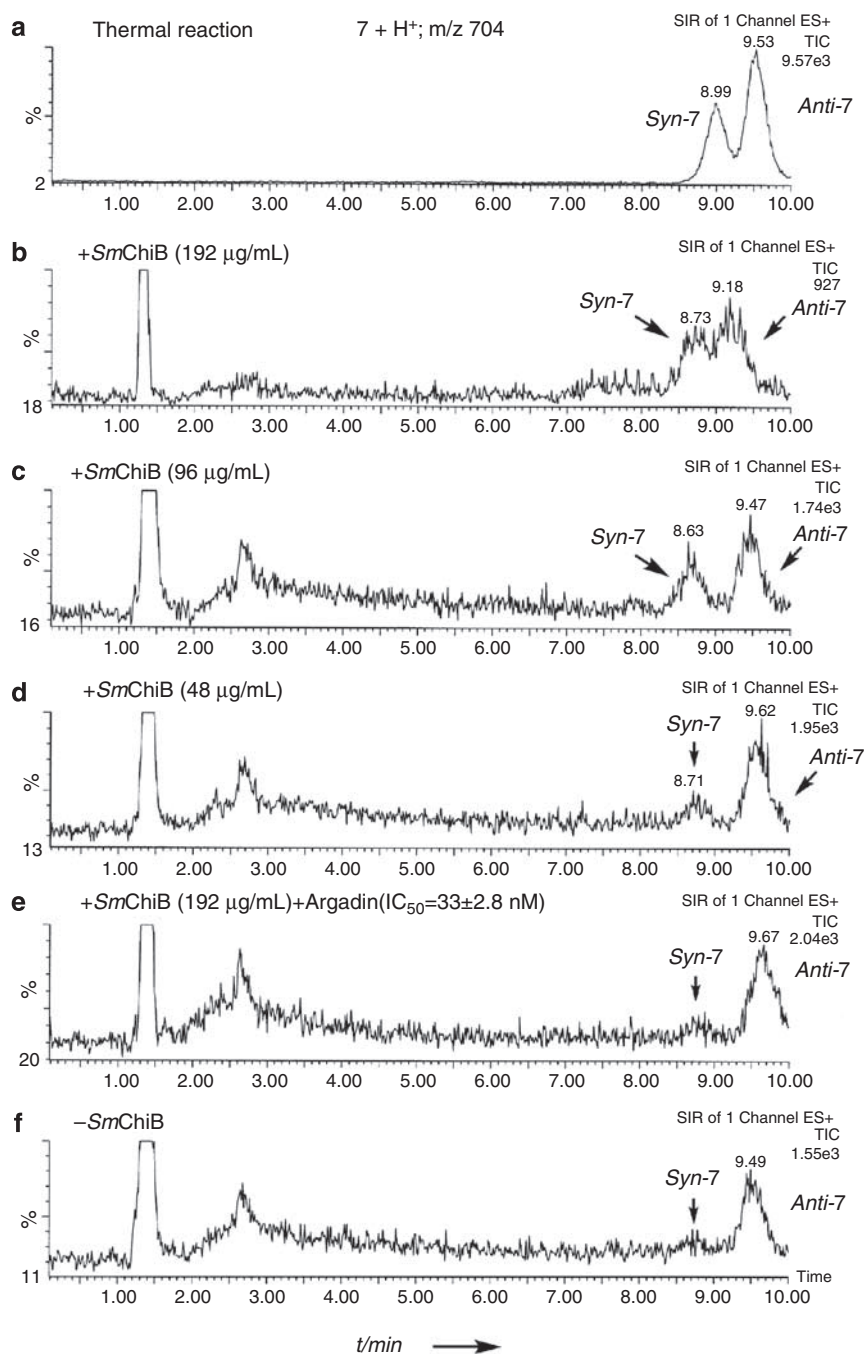
Figure 5 Identification of *syn-anti* selection for *in situ* click chemistry between **5** (100 μM) and **6** (300 μM), monitored by LCMS-SIR under different conditions from Figure 3. (**a**) Authentic sample of **7** from thermal reaction (100 °C, 12 h): *syn*-**7** (9.0 min) and *anti*-**7** (9.5 min) (2:3 ratio); (**b**) reaction with pure (His)$_6$-*Sm*ChiB (192 μg ml^{-1}; 37 °C, 20 h); (**c**) (His)$_6$-*Sm*ChiB (96 μg ml^{-1}; 37 °C, 20 h); (**d**) (His)$_6$-*Sm*ChiB (48 μg ml^{-1}; 37 °C, 20 h); (**e**) (His)$_6$-*Sm*ChiB (192 μg ml^{-1}), argadin (100 μM; 37 °C, 20 h); and (**f**) without enzyme (background reaction).

Conclusion and remarks

We discovered a highly active inhibitor of chitinase through *in situ* click chemistry. The strategy used an azide substituent appended to an active domain excised, as it were, from the more complex natural macrocyclic peptide **1**. The *Sm*Chi, which in this case was specifically *Sm*ChiB, acts 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, would have to be synthesized to reveal the SAR for the affinity of a 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 long and costly analog syntheses. Ongoing studies are focused on optimizing the structure and further evaluating

bioactivities against various types of chitinases, as well as promoting efforts to seek entirely new inhibitors, not only for chitinases but also for thorough application and refinement of the basic *in situ* methodology.

METHODS

Details of the experimental procedures and characterization of the new compounds, list of the acetylenes library, the expression of each *Serratia marcescens* chitinases and IC_{50} determination can be found in the Supplementary Information.

In situ click chemistry experiment using a mixture of *Sm*ChiA, B and C_1

Azide **5** (5 µl; 2.0 mM in MeOH) was diluted in 0.1 M phosphate buffer (pH 7.0) (60 µl). Subsequently, the required alkynes (5 µl; 6.0 mM in MeOH) were added to the azide solution, followed immediately by the addition of a solution of *Sm*Chi A, B and C_1, which was purchased from Sigma Co. (C-7809-1UN) (for the ratio of the *Sm*Chi isozymes, see Supplementary Figure 10 in the Supplementary Information), in 0.1 M phosphate buffer (30 µl; 32 mUnit ml^{-1}) to give final concentrations of 9.6 mUnit ml^{-1} *Sm*Chi, 100 µM azide **5** and 300 µM of each alkyne (Supplementary Figure 7) in 10% MeOH/phosphate buffer (100 µl). After mixing thoroughly, the reaction mixtures were incubated at 37 °C for 20 h, then diluted with 100 µl MeOH to restrain the enzyme activity and injected directly into the LC/UV-MS instrument to perform LCMS-SIR analysis (Column: Senshu Pak Pegasil (Senshu Scientific Co., Tokyo, Japan) ODS 2ø×50 mm; conditions for HPLC: gradient 10% MeCN (0.05% TFA)/H$_2$O (0.1% TFA) to 100% MeCN (0.05% TFA) over 8 min, flow 0.3 ml min^{-1}, detect 200–400 nm, temp 20 °C; MS-SIR: cone voltage 60 V, source temp 110 °C, desolvation temp 350 °C, selected mass 704 [M+H]$^+$). The triazole products were identified by their retention times and molecular weights. Control experiments without chitinases (azide **5** (5 µl; 2.0 mM in MeOH) and alkynes (5 µl; 6.0 mM in MeOH) in 0.1 M phosphate buffer (pH 7.0) (90 µl) at 37 °C for 20 h) were run consecutively.

One unit of chitinases can liberate 1.0 mg of *N*-acetyl-D-glucosamine from chitin per hour at pH 6.0 at in a 2-h assay.

Expression and purification of (His)$_6$-*Sm*ChiB

*Sm*ChiB gene was amplified by PCR and cloned *in frame* to pTrcHis B vector (Invitrogen, Tokyo, Japan) to give pTrcHis*Sm*ChiB. *E. coli* strain DH5α harboring plasmid pTrcHis*Sm*ChiB was harvested and disrupted by the same procedure used for expression of crude *Sm*ChiB. After centrifugation, the supernatant was applied to HisTrap Chelating HP column (Amersham Biosciences, Tokyo, Japan), and the bound proteins were eluted by a stepwise gradient of imidazole in 100 mM phosphate buffer. (His)$_6$-*Sm*ChiB was eluted at a concentration of over 150 mM imidazole and showed a molecular mass of 53 kDa on SDS-PAGE (Supplementary Figure 10). The combined fraction was concentrated and imidazole was removed using HiTrap Desalting column (Amersham BioSciences). The protein concentration (320 µg ml^{-1}) was determined by Bradford method using protein quantification kit-rapid (Dojindo, Kumamoto, Japan) with BSA as a standard protein (0.80 absorbance at 595 nm for diluted protein solution (×10)) (Supplementary Figure 10). The chitinolytic activity of purified (His)$_6$-*Sm*ChiB was assayed by the procedure in the Supplementary Information (Supplementary Figure 10).

In situ formation of syn-7 by *Sm*ChiB template reaction

A chromatographic and analytical method different from *in situ* click screening was used for determining the *syn-anti* selection of the *in situ* click chemistry product **7** by (His)$_6$-*Sm*ChiB. The assignment was accomplished by comparing the retention times of the *in situ* products with authentic samples prepared by the thermal reaction of triazole formation.

Azide **5** (5 µl; 2.0 mM in MeOH) was diluted in 0.1 M phosphate buffer (pH 7.0) (60 µl). Subsequently, the alkyne **6** (5 µl; 6.0 mM in MeOH) was added to the azide solution, followed immediately by the additon of a solution of (His)$_6$-*Sm*ChiB (320 µg ml^{-1}) in 0.1 M phosphate buffer (30 µl) to give a final concentration of 96 µg ml^{-1} (His)$_6$-*Sm*ChiB, 100 µM azide **5** and 300 µM alkyne

6 in 10% MeOH/phosphate buffer (100 µl). After mixing thoroughly, the reaction mixtures were kept at 37 °C for 20 h, then cooled to 20 °C and diluted with 100 µl MeCN to restrain the enzyme activity and injected directly into the LC/UV-MS instrument to perform LCMS-SIR analysis (Column: Develosil (Nomura Chemical Co., Aichi, Japan) C30-UG-5, 2ø×150 mm; conditions for HPLC: isocratic 29% MeCN(0.05% TFA)/H$_2$O(0.1%TFA), flow 0.3 ml min^{-1}, detect 200–400 nm, temp 20 °C; MS-SIR: cone voltage 95 V, source temp 110 °C, desolvation temp 350 °C, selected mass 704 [M+H]$^+$). The triazole products were identified by their retention times and molecular weights. In concurrence, experiments to investigate the dose dependency of the enzyme for production of **syn-7** were carried out under the same condition by using different concentrations of (His)$_6$-*Sm*ChiB (final concentrations 192 and 48 µg ml^{-1} (His)$_6$-*Sm*ChiB, respectively, 100 µM azide **5** and 300 µM of alkyne **6** in 10% MeOH/phosphate buffer). From these comparisons, dose-dependent production of **syn-7** was clearly observed (Figure 5). LCMS-SIR peaks of **syn-** and **anti-7** were confirmed by co-injection of authentic sample **7** (from thermal reaction) and a crude mixture of enzyme template reaction ((His)$_6$-*Sm*ChiB 192 µg ml^{-1}, 100 µM azide and 300 µM alkyne at 37 °C, 20 h) (Supplementary Figure 11). Control experiments in the presence of the known *Sm*ChiB inhibitor Argadin[41] (IC_{50} 33 ± 2.8 nM) (final concentration: 192 µg ml^{-1} *Sm*ChiB, 100 µM argadin, 100 µM azide **5** and 300 µM alkyne **6**) and in the absence of enzyme (100 µM azide **5** and 300 µM alkyne **6**) were run consecutively.

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