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Argifin; efficient solid phase total synthesis and evalution of analogues of acyclic peptide

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ABSTRACT

An effective solid phase synthesis of Argifin, providing subsequent access to effective synthesis of analogues, was developed in 13% overall yield, as well as elucidating structure–activity relationships. The novel acyclic peptide **18b**, prepared from a synthetic intermediate of Argifin, was found to be 70 times more potent as an inhibitor of *Serratia marcescens* chitinases B than Argifin itself

1. Introduction

Chitinases hydrolyze β-(1,4)-linked *N*-acetylglucosamine (Chitin), which is one of the most abundant polysaccharides in nature.[1] Since chitin is a major structural component of fungi and insects,[2] with 18 chitinases playing important physiological roles in these organisms, chitinase inhibitors are of considerable interest as potential agents against fungi,[3] insects,[4] and malaria transmission.[5] They also offer significant potential for treatment of asthma and other diseases in humans.[6] Despite their attractive potential for medicinal usage, no practical use has been developed to date.

During screening for chitinase inhibitors, a new cyclic pentapeptide Argifin (**1**) was isolated from the cultured broth of *Gliocladium* sp. FTD-0668 by our group, and found to be a potent inhibitor of *Serratia marcescens* chitinases (*Sm*Chi) with IC_{50} values of 0.025 and 6.4 μM against *Sm*ChiA and B, respectively (Fig. 1).[7,8] The structure of **1** was elucidated by amino acid analysis and detailed 1D and 2D-NMR experiments. Additionally, three-dimensional structures of **1**, in complex with *Sm*ChiB, were resolved by X-ray crystallography[9], resulting in detailed visualization of the interaction of **1** with *Sm*ChiB. More importantly, there are at least four concentrated hydrogen bond interactions between the N^{ω}-methylcarbamoyl-L-arginine moiety and the motif of the hydrolytic pocket of the chitinases, critically contributing to expression of nanomolar to micromolar range inhibitions.[9]

Design and development of practical and efficient strategies for Argifin synthesis has been an important objective since the original source, *Gliocladium* sp. FTD-0668, no longer produces this cyclic-peptide. Therefore, a method for rapid and diverse synthetic route of **1** is required the supply to the biological tests as well as the SAR studies of Argifin. Herein, we report our efficient solid phase total synthesis of **1**, which also facilitates rapid synthesis of analogues, using 2-chlorotrityl chloride resin along with HPLC purification. Furthermore, during the total synthesis, we found that the acyclic peptide, possessing a much-simplified structure, exhibited 70-fold more potent inhibitory activity than that of **1** against *Sm*ChiB. Our

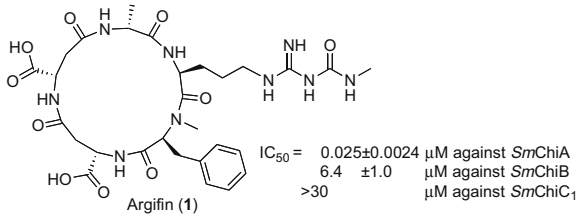

Figure 1. Structure and IC_{50} values of Argifin (**1**).[8]

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work also facilitated elucidation of structure-activity relationships (SAR) of the linear peptides.

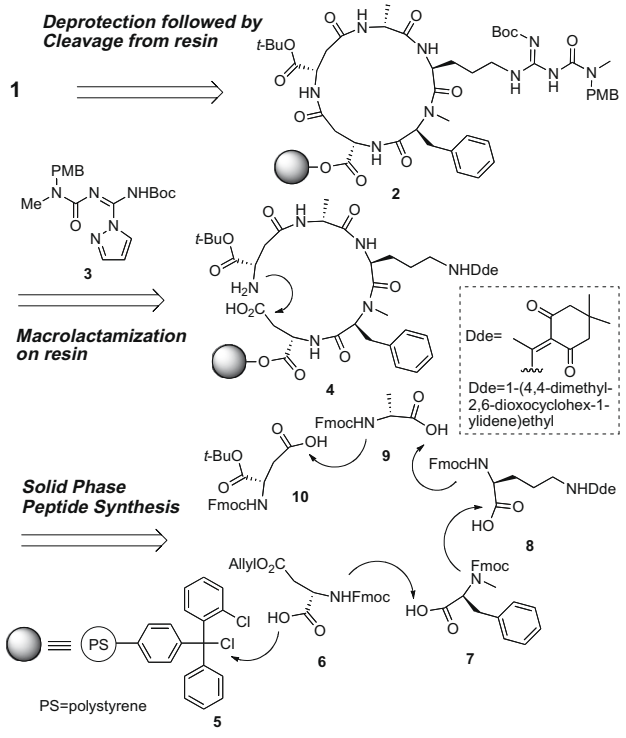
2. Results and discussion

2.1. Chemistry

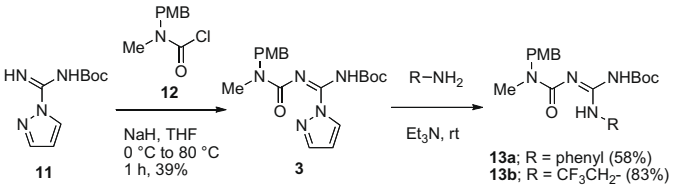
The first total synthesis of **1** was developed by I. M. Eggleston and co-workers in 2005, utilizing solid phase peptide synthesis.[10] Their strategy, however, included liquid phase reaction sequences for introduction of the N^{ω}-methylcarbamoyl group during final stages of total synthesis, with two-time HPLC separation due to its hydrophilicity, indicating that an efficient strategy was still required, especially to ensure rapid synthesis of analogues. Our retrosynthetic analysis is outlined in Scheme 1. To avoid complications of the liquid phase reactions, we envisaged the complete solid phase total synthesis for all reaction sequences, except for final cleavage step from the resin.

Additionally,1-*H*-pyrazole-1-[*N*-(*tert*-butoxycarbonyl)-*N*'-(*N*-*p*-methoxybenzylcarbamoyl-*N*-methyl)]carboxamidine (**3**) was designed to simply introduce the N^{ω}-methylcarbamoylguanidino onto the NH_2 group of Orn for solid phase synthesis.

From our synthetic perspective, **1** could be prepared from fully-functional compound (**2**) with deprotection and cleavage from the resin under acidic conditions. Resin-cyclic peptide (**2**) could be synthesized using macrolactamization on resin and effective introduction of the N^{ω}-methylcarbamoylguanidino group, together with **3** starting from the acyclic compound (**4**). Then, the linear peptide composed blocks Fmoc-Asp(OAllyl)-OH (**6**), Fmoc-*N*-Me-Phe-OH (**7**), Fmoc-Orn(Dde)-OH (**8**) [Dde; 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl][11], Fmoc-D-Ala-OH (**9**), and Fmoc-Asp-O*t*-Bu (**10**) can be assembled by solid phase synthesis, using a 2-chlorotrityl link that is tolerant to reaction conditions in Fmoc-based solid phase synthesis and stable under cleavage conditions.



Scheme 1. Synthetic plan of **1**.



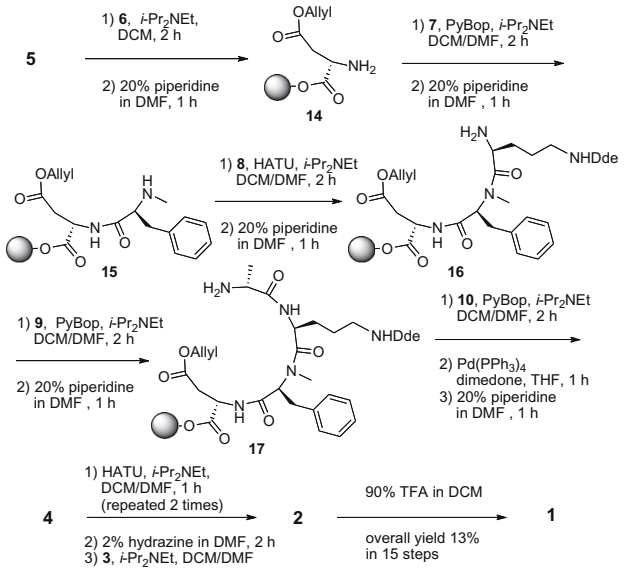
Scheme 2. Synthesis of **13a–b** as a model study.

Initially, synthesis of the N^{ω}-methylcarbamoyl group on resin is required for solid phase total synthesis of **1**.

To address this issue, we constructed the N^{ω}-methylcarbamoyl group in liquid phase as a model study (Scheme 2). Treatment of 1-*H*-pyrazole-1-[*N*-(*tert*-butoxycarbonyl)]carboxamidine (**11**)[12] with *N*-methyl-*N*-(4-methoxybenzyl)carbamoyl chloride (**12**)[13] in the presence of NaH in THF afforded the corresponding urea compound (**3**) in 39% yield. The guanidine formation involved *N*-methylcarbamoyl group (**13a–b**) being effectively introduced using aniline and trifluoroethylamine at room temperature in 58% and 83% yields, respectively (Scheme 2), suggesting that this method can not only be utilized for synthesis of **1**, but also synthesis of similar natural products containing the N^{ω}-methylcarbamoyl group, such as Banyasin A.[14]

In possession of **3**, the carboxylic acid at α position in **6** was loaded onto 2-chlorotrityl resin **5** in the presence of *i*-Pr_2NEt in DCM, resulting in 98% loading yield (1.4 mmol/g), calculated from the weight of **6** after cleavage with 20% TFA in DCM. The Fmoc group was removed with 20% piperidine in DMF, and subsequent condensation with **7** using PyBop (benzotriazol-1-yloxytriopyrrolidinophosphonium hexa-fluorophosphite), *i*-Pr_2NEt provided dipeptide (**15**). After subsequent removal of the Fmoc group (20% piperidine in DMF), the use of HATU (*N*-[(dimethylamino)(3*H*-1,2,3-triazolo(4,5-*b*)pyridin-3-yloxy)methylene]-*N*-methylmethanaminium hexafluorophosphate) as the best coupling reagent with **8** afforded the tripeptide (**16**) with complete consumption of the resulting **15**. When PyBop or PyBrop (Bromo-tris-pyrrolidino-phosphonium hexafluorophosphate) as condensation reagents was used, mixture of **15** and **16** were observed in LC–UV–MS, after cleavage with 20% TFA in DCM. With **16** in hand, Fmoc deprotection (20% piperidine in DMF) and coupling with **9** (PyBop, *i*-Pr_2NEt) afforded the tetrapeptide. The use of the same coupling procedure with **10** and PyBop provided the linear pentapeptide, without aspartimide formation, after cleavage with 20% TFA in DCM. After deprotection of the Allyl ($Pd(PPh_3)_4$/dimedone in THF) and Fmoc (20% piperidine in DMF) groups, macrolactamization of **4** with PyBop in two cycles on resin furnished the corresponding cyclic compound without oligomerization. To complete total synthesis, after Dde deprotection (2% hydrazine in DMF), N^{ω}-methylcarbamoylguanidino formation with **3**, followed by deprotection and cleavage with 90% TFA in DCM from the resin, furnished **1** in overall 13% yield after HPLC purification (Scheme 3). Spectral data and activities of synthetic **1** were found to be identical with those of the natural **1**.

Significantly, we found that an acyclic intermediate exhibited better activity than the parent **1** against *Sm*ChiB, suggesting a cyclic conformation might not be necessary to exhibit inhibition of chitinase activity. The most important functional group to exhibit activity appears to be the N^{ω}-methylcarbamoyl guanidine group, based on Argifin-*Sm*ChiB X-ray study.[9] A similar result from the van Aalten group has been recently reported using *Aspergillus fumigatus* chitinase B_1 (*Af*$ChiB_1$) as the enzyme for their assay.[15] Most of their analogues, however, do not express inhibitory activity greater than that of **1** against *Af*$ChiB_1$.

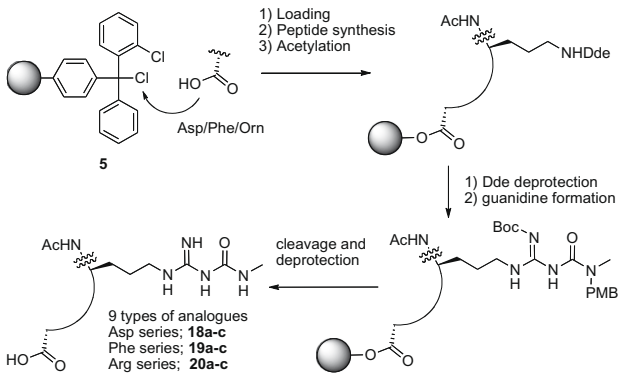


Scheme 3. Solid phase total synthesis of **1**.

We began our investigation of analogues by looking into nine acyclic derivatives bearing the N^{ω}-methylcarbamoylguanidino group to elucidate the SAR against each *Sm*Chi isozyme. For synthesis of acyclic peptides, we utilized the solid phase peptide synthesis strategy using 2-chlorotrityl chloride resin. From the synthetic perspective, elaboration of the acyclic analogues is outlined in Scheme 4. The carboxylic acids at each amino acid (**6**/**7**/**8**) were loaded onto **5** in the presence of *i*-Pr_2NEt in DCM, followed by elaboration of appropriate amino acids (**7**/**8**/**9**/**10**) and acetylation of 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 Boc and PMB group under TFA conditions, readily furnished the nine acyclic analogues (**18**–**20a**–**c**) in 21–78% yields (see Table 1).

2.2. In vitro evaluation

For determination of IC_{50} values against each *Sm*Chi isozyme, nine acyclic compounds were subjected to a competition assay with 4-methylumberiferyldiacetyl-chitobiose[8,16] (Table 1). Interestingly, Arg-(*N*-Me-Phe)-Asp (**18a**) and D-Ala-Arg-(*N*-Me-Phe)-Asp (**18b**) exhibited approximately 50–70-fold more potent activity against *Sm*ChiB (with 0.13 μM and 0.091 μM of IC_{50} values, respectively) than that of parent **1**, suggesting that the D-Ala moiety is not a crucial function to express competent inhibitory



Scheme 4. General procedure of 9 types of linear analogues.

activity on *Sm*ChiB except for Arg-series **20a**–**c** (Table 1). In contrast, possessing the D-Ala moiety increases activity in terms of *Sm*ChiA. Furthermore, the addition of Asp moiety next to D-Ala enfeebles both activities. These correlations for all-series were clearly demonstrated (sigmoidal graphs for Asp-series are provided in Table 1). On the other hand, lack of the Asp unit next to *N*-Me-Phe (in **19a**–**c** and **20a**–**c**) decreases activity of both *Sm*ChiA and B, suggesting the Asp(OAllyl) plays a key role in the activity. Likewise, the *N*-methyl-Phe moiety is also an important amino acid, indicated by the finding that the Arg series display weak activity for both *Sm*ChiA and B. Unfortunately, all of the acyclic compounds, including **1**, do not exhibit activity against *Sm*ChiC_1.

3. Conclusion

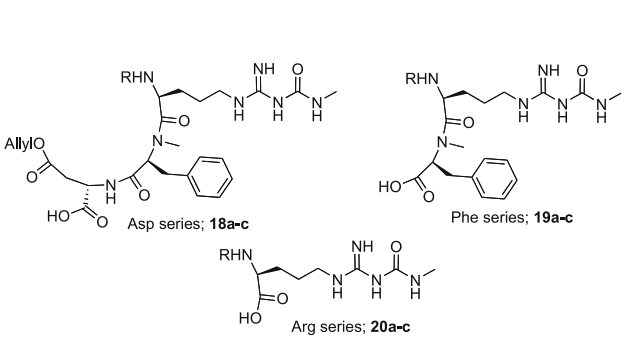
In summary, we have developed the efficient solid phase total synthesis of **1**, involving macrolactamization on resin as well as construction of the N^{ω}-methylcarbamoyl group, with single HPLC purification, allowing us to speedily generate a variety of analogues. It is notable that the novel acyclic peptide **18b** exhibits 70-fold more potent activity against *Sm*ChiB than that of **1**, 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, which has potent inhibitory activity, derived from the natural product. Further studies for acyclic[8] and cyclic analogues are continuing in our laboratory.

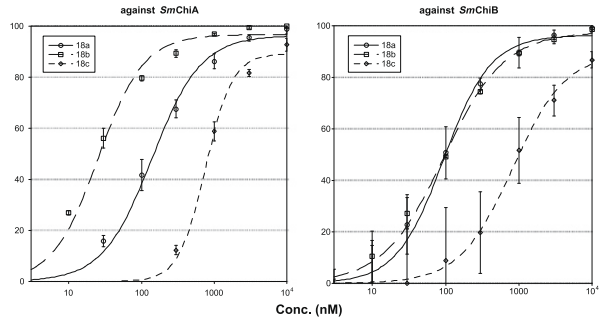
4. Experimental

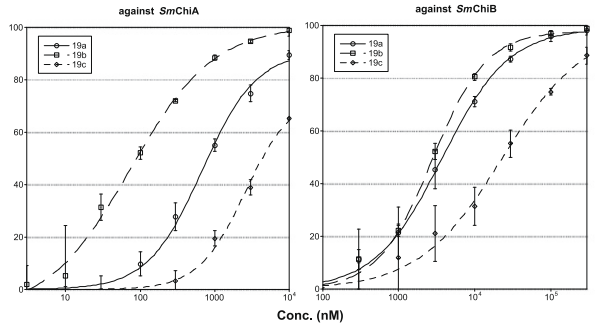
4.1. General

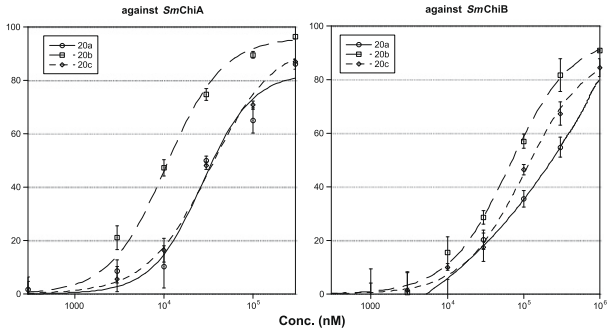
Fmoc-Asp(OAllyl)-OH (**6**), Fmoc-*N*-Me-Phe-OH (**7**), Fmoc-D-Ala-OH (**9**), and Fmoc-Asp-O*t*-Bu (**10**) were purchased from Watanabe Chemical Industries, LTD. 2-Chlorotritylchlorie resin (**5**) was purchased from NovaBiochem. Dry THF, toluene, and CH_2Cl_2 were purchased from Kanto Chemical Co. Fmoc-Orn(Dde)-OH (**8**) was prepared according to similar procedures. Precoated silica gel plates with a fluorescent indicater (Merck 60 F254) were used for analytical and preparative thin layer chromatography. Flash column chromatography was carried out with Kanto Chemical silica gel (Kanto Chemical Co., Inc., Silica Gel 60 N, spherical neutral, 0.040–0.050 mm, Cat.-No. 37563-84). ^{1}H NMR spectra were recorded at 270 MHz or 300 MHz or 400 MHz and ^{13}C NMR spectra were recorded at 67.5 MHz or 75 MHz or 100 MHz on JEOL JNM-EX270 (270 MHz) or Varian VXR-300 (300 MHz) or Varian XL-400 (400 MHz) or Varian UNITY-400 (400 MHz). The chemical shifts are expressed in ppm downfield from internal solvent peaks CH_3OH (3.31, 4.84 ppm, ^{1}H NMR), pyridine (8.71 (br), 7.55 (br), 7.19 (br) ppm, ^{1}H NMR), CD_3OD (49.0 ppm, ^{13}C NMR), pyridine-d_5 (123.5, 135.5, 149.2 ppm, ^{13}C NMR), D_2O (the end of both fields; 0, 200 ppm, ^{13}C NMR) and *J* values are given in hertz. The coupling patterns are expressed by s (singlet), d (doublet), dd (double doublet), t (triplet), m (multiplet) or br (broad). The All infrared spectra were measured on a Horiba FT-210 spectrometer. High- and low-resolution mass spectra were measured on a JEOL JMS-DX300 and JEOL JMS-AX505 HA spectrometer. Liquid chromatographic analysis was conducted on a Hitachi ELITE LaChrom with Senshu Pak-PEGASIL ODS-II (4.6φ × 250 mm). Optical rotations were measured by JASCO DIP-370 polarimeter. Melting points were measured on a Yanagimoto Micro Apparatus. Fluoroscence for measurments of chitinases inhibitory activities was measured by fluorometer on a Labsystems Fluoroscan II. LC/UV-MS was performed on a Waters 2795 Separation Module with Alliance® HT and micromass ZQ (Column; Senshu Pak-PEGASIL ODS

Table 1
IC_{50} results for 9 types of linear analogues









	R	IC_{50} (μM)			Yields[c] (%)
		*Sm*ChiA[b]	*Sm*ChiB[b]	*Sm*ChiC[b]	
18a	Ac	0.14 ± 0.0057	0.13 ± 0.053	>30	78
18b	Ac-D-Ala	*0.025 ± 0.0015*	*0.091 ± 0.0038*	>30	44
18c	Ac-Asp- D-Ala	1.1 ± 0.061	1.0 ± 0.19	>30	20
19a[a]	Ac	0.75 ± 0.072	3.7 ± 0.67	>30	22
19b	Ac-D-Ala	0.090 ± 0.014	2.7 ± 0.63	>30	40
19c	Ac-Asp- D-Ala	8.4 ± 1.0	30 ± 6.8	>30	30
20a	Ac	31 ± 1.2	260 ± 55	>30	58
20b	Ac-D-Ala	6.6 ± 0.64	64 ± 12	>30	35
20c	Ac-Asp- D-Ala	32 ± 7.6	110 ± 17	>30	21
1	Argifin	0.025 ± 0.0024	6.4 ± 1.0	>30	13

[a] Ref. 15.
[b] The preparation of *Sm*ChiA, B, and C_1; see Ref. 8.
[c] Isolated yields after HPLC purification.

2φ × 50 mm: Condition of HPLC; gradient 10% MeCN(0.05% TFA)/H_2O (0.1% TFA) to 100% MeCN(0.05% TFA) over 8 min, flow 0.3 mL/min, detect 200–400 nm, temp 20 °C). LC–UV was performed on a ELITE LaChrom (Column; Senshu Pak PEGASIL ODS 20φ × 250 mm with a flow rate of 8 mL/min. Mobile phase A was 0.05% TFA in MeCN , mobile phase B was 0.05% TFA in H_2O).

Gradient 1 was *T* = 0 min, *A* = 15% ; *T* = 30 min, *A* = 98%. Gradient 2 was *T* = 0 min, *A* = 20%; *T* = 30 min, *A* = 98%. Gradient 3 was *T* = 0 min, *A* = 25%; *T* = 30 min, *A* = 98%. Gradient 4 was *T* = 0 min, *A* = 0%; *T* = 5 min, *A* = 5% ; *T* = 30 min, *A* = 98%.

4.2. Abbreviations

Ac = Acetyl, Boc = *tert*-butyloxycarbonyl, DCM = dichloromethane, Dde = 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl, DIPEA = *N*,*N*-diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, HATU; *N*-[(dimethylamino)(3*H*-1,2,3-triazolo(4,5-*b*)pyridin-3-yloxy) methylene-]-*N*-methylmethanaminium hexafluorophosphate, Orn = ornithin, PMB = *p*-methoxybenzyl, PyBop; benzotriazol-1-yloxytriopyrrolidinophosphonium hexafluorophosphite. TFA = trifluoroacetic acid, THF = tetrahydrofuran.

4.3. *N*-(*p*-Methoxybenzyl)-*N*-methylamidoyl chloride 12

Although **12** has been reported by the Yoakim group (but there is no spectra data for **12**), we have synthesized **12** by using our reaction sequence.

To a solution of *p-methoxybenzaldehyde* (30.00 mL, 248.0 mmol) in MeOH (83.0 mL) was added 40% $MeNH_2$ in water (17.30 mL) at room temperature. After being stirred at room temperature for 1 h, the mixture was added $NaBH_4$ (4.69 g, 124 mmol) at 0 °C and then was stirred at 0 °C for 1 h. After the reaction was quenched with 3 N HCl aq the solvent was removed in vacuo. The residue was washed with $CHCl_3$ (300 mL × 1) and the water layer was basified with 3 M NaOH aq (100 mL) at 0 °C to approximately pH 10. The resulting water layer was extracted with CH_2Cl_2 (200 mL × 3), dried over Na_2SO_4, and concentrated in vacuo to produce the crude *N-(p-methoxybenzyl)-N-methylamine*, which is used without further purification. To a solution of the crude *N-(p-methoxybenzyl)-N-methylamine* in THF (330 mL) was carefully added DIPEA (34.5 mL, 0.198 mmol), triphosgene (17.18 g, 0.058 mmol) at 0 °C. After being stirred for 1 h, the mixture was diluted with EtOAc (300 mL), quenched with satd $NaHCO_3$ aq

(100 mL) and separated. The mixture was washed with sat. $NaHCO_3$ aq (100 mL × 1), satd NaCl aq (100 mL × 1), dried over Na_2SO_4, and concentrated in vacuo. Flash column chromatography (hexane/EtOAc = 10:1) afforded **12** (19.5 g, 0.032 mol) in 55% yield as a colorless oil.

R_f = 0.70 (silica gel, hexane/EtOAc = 1:1), IR (KBr) ν cm^{-1}: 2945 (m), 1736 (s), 1512 (m), 1248 (s), 1182 (m), 1070 (s), ^1H NMR (270 MHz, $CDCl_3$) *The mixture of rotamers was observed.* δ: 7.20 (m, 2H, Ph), 6.90 (m, 2H, Ph), [4.65 , 4.51 (s × 2, 2H, –$PhCH_2$–), (rotamer)], 3.81 (s, 3H, O–CH_3), [3.04, 2.97 (s × 2, 3H, –*N*–CH_3), (rotamer)], ^{13}C NMR (67.5 MHz, $CDCl_3$) *The mixture of rotamers was observed.* δ: 159.4, [150.1, 149.1 (rotamer)], [129.5, 128.6, 127.3, 127.0 (rotamer)], [114.2, 114.1 (rotamer)], [55.3, 53.7 (rotamer)], 55.2, [37.5, 36.0 (rotamer)], HR-MS (FAB, PEG200 + 400): calcd for $C_{10}H_{12}O_2NCl$: 213.0557 [M+H], found *m/z*: 213.0556[M+H]$^+$.

4.4. 1-*H*-Pyrazole-1-(*N*-(*tert*-butoxycarbonyl)-*N*′-(*N*-methyl-*N*-*p*-methoxybenzylcarbamoyl)-carboxamidine 3

To a solution of **11** (6.40 g, 30.4 mmol) in THF (304 mL) was added NaH (2.0 g, 45.6 mmol) at 0 °C. After being stirred at 0 °C for 10 min, the reaction was allowed to warm up to room temperature and was added a solution of **12** (19.5 g, 91.3 mmol) in THF (102 mL) before heated up to reflux. The mixture was stirred at reflux for 1 h and cooled to room temperature. After the mixture was diluted with EtOAc (200 mL), quenched with satd aq NH_4Cl (100 mL) and separated, the organic layer was washed with H_2O (100 mL × 1), dried over Na_2SO_4 and concentrated to yield the crude product. Flash column chromatography (hexane/EtOAc = 10:1) afforded **3** (4.6 g, 11.9 mmol) in 39% yield as a colorless oil.

R_f = 0.41 (silica gel, hexane/EtOAc = 1:2), IR (KBr) ν cm^{-1}: 2941 (w), 2359 (w), 1761 (s), 1641 (s), 1504 (s), 1242 (s), 1153 (s), ^1H NMR (270 MHz, $CDCl_3$) *The mixture of rotamers was observed.* δ: 8.24 (d, *J* = 18.2 Hz, 1H, pyrazole), 7.65 (d, *J* = 1.3 Hz, 1H, pyrazole), 7.32 (m, 2H, Ph), 6.87 (m, 2H, Ph), 6.44 (s, 1H, pyrazole), 4.56 (s, 2H, *p*-OCH_3PhCH_2–), 3.83 (s, 3H, *p*-OCH_3PhCH_2–), 2.88 (s, 3H, *N*-CH_3), 1.50 (s, 9H, O$(CH_3)_3$), ^{13}C NMR (67.5 MHz, $CDCl_3$) *The mixture of rotamers was observed.* δ: 160.5 (amidine), [158.8, 158.7 CH_3O-C_6H_4-, (rotamer)], 149.3 (–H*N*–C(=O)O-^{t}Bu), [142.5, 142.4 pyrazole, (rotamer)], [140.6, 139.7 –CH_3*N*–C(=O)*N*–, (rotamer)], [129.6, 128.8, 128.7 Ph, (rotamer)], 129.6 (pyrazole), [113.8, 113.6, Ph (rotamer)], 109.3 (pyrazole), [82.8, 82.7 –OC$(CH_3)_3$, (rotamer)], 55.1, (–OCH_3), [53.0, 50.6 –Ph–CH_2–*N*(Me)–, (rotamer)], [34.5, 32.8 –*N*–CH_3, (rotamer)], [27.9, 27.8 –OC$(CH_3)_3$, (rotamer)], HR-MS (FAB, NBA): calcd for $C_{19}H_{26}O_4N_5$: 388.1985 [M+H], found *m/z*: 388.1995 [M+H]$^+$.

4.5. Fmoc-Orn(Dde)-OH (8)

To a solution of Fmoc-Orn-OH hydrochloride (6.0 g, 0.015 mol) in DCM–MeOH (7:3, 300 mL, 0.05 M) was added DIPEA (10.7 mL, 0.060 mol), 4,4-dimethyl-2,6-dioxocyclohexeylidene (5.6 g, 0.030 mol) at room temperature. After being stirred at room temperature for 48 h, the solvent was removed in vacuo. The residue was diluted with EtOAc (300 mL), washed with 1 N aq HCl (300 mL), satd aq NaCl (200 mL), dried over Na_2SO_4, and concentrated. Recrystallization from EtOAc and hexane provided **8** (6.0 g, 0.0116 mol) in 75% yield as a colorless solid.

R_f = 0.35 (silica gel, CH_3Cl–MeOH = 4:1), IR (KBr) ν cm^{-1}: 3415, 2958, 1574, 1464, 1450, 1267, 1149, 1034, 741, $[\alpha]_D^{27} = 4.32$ (*c* 1.0, CH_3Cl), mp = 150–152 °C, ^1H NMR (270 MHz, pyridine-d_5): 13.8 (br s, 1H, –COOH), 8.93 (d, *J* = 8.6 Hz, 1H, aromatic), 7.83 (d, *J* = 7.6 Hz, 2H, aromatic), 7.70 (dd, *J* = 7.3, 3.3 Hz, 2H, aromatic), 7.39 (m, 2H, aromatic), 7.26 (m, 2H, aromatic), 4.88 (m, 1H, –$COOCH_2$CH–9-fluorenyl), 4.68 (m, 2H, –$COOCH_2$-9-fluorenyl), 4.34 (t, *J* = 6.6 Hz, 2H), 2.59 (s, 3H, Me of Dde group), 2.47 (s, 4H, CH_2 of Dde group), 2.29 (m, 1H, β position of Orn), 2.10 (m, 1H, β position of Orn), 1.90 (m, 2H, δ position of Orn), 0.95 (s, 6H, dimethyl of Dde group), ^{13}C NMR (67.5 MHz, pyridine-d_5): 197.2 (x2), 175.2, 173.3, 157.4, 144.7, 144.4, 141.6 (×2), 128.0 (×2), 127.4 (×2), 125.6 (×2), 120.4 (×2), 108.0, 66.5, 54.7, 53.1 (×2), 47.8, 43.0, 30.0 (×2), 28.2 (×2), 26.1, 17.6, HR-MS (FAB, NBA): calcd for $C_{30}H_{35}O_6N_2$: 519.2495 [M+H], found *m/z*: 519.2487 [M+H]$^+$.

4.6. *N*-*tert*-Butoxycarbonyl-*N*′-(*N*-methyl-*N*-*p*-methoxybenzyl)carbamoyl-*N*″-phenylguanidine 13a

To a solution of **3** (100 mg, 0.26 mmol) in acetonitrile (2.6 mL) was added triethylamine (109 μL, 0.78 mmol), aniline (36 μL, 0.39 mmol) at room temperature. The mixture was stirred at room temperature for 40 h. The solution was diluted with EtOAc (5 mL), and was successively washed with sat. aq. NH_4Cl (3 mL × 1), H_2O (3 mL × 1), and brine (5 mL × 1), dried over Na_2SO_4, and concentrated. The resulting colorless oil was purified by flash column chromatography (hexane/EtOAc = 80:1–50:1) to afford **13a** (59.8 mg, 0.15 mmol) in 58% yield as a colorless oil.

R_f = 0.39 (silica gel, hexane/EtOAc = 4:1), IR (KBr) ν cm^{-1}: 3286 (w), 3151 (w), 2979 (w), 1712 (s), 1639 (s), 1512 (s), 1452 (s), 1412 (s), 1246 (s), 1153 (s), ^1H NMR (270 MHz, $CDCl_3$) *The mixture of rotamers was observed.* δ: 12.6 (s, 1H, –*N*H–, rotamer), [10.2, 10.1 (s, 1H, –*N*H–, rotamer)], 7.63 (d, *J* = 7.58 Hz, 1H, Ph), 7.53 (d, *J* = 7.58 Hz, 1H, Ph), 7.43 (t, *J* = 7.26 Hz, 1H, Ph), 7.28-7.00 (complex m, 4H, Ph), 6.86 (dd, *J* = 8.9, 2.3 Hz, 2H, Ph), [4.67, 4.53 (s ×2, 2H, *p*-OCH_3PhCH_2–, rotamer)], 3.79 (s, 3H, –OCH_3), [3.04, 2.92 (s ×2, 3H, –*N*–CH_3–, rotamer)], 1.53 (s, 9H, –$COO(CH_3)_3$), ^{13}C NMR (67.5 MHz, $CDCl_3$) *The mixture of rotamers was observed.* δ: 164.1, 158.6, 153.5, 151.6, [137.4, 137.3 (C × 1, Phe, rotamer)], [130.5, 130.1 (C × 1, aromatic, rotamer)], [128.8, 128.5 (C × 2, aromatic),], 124.1, (Ph), 122.0 (C × 2, Ph), 113.8 (C × 2, aromatic), 82.8 (–OC$(CH_3)_3$), 55.2 (–OCH_3), [52.3, 50.5, (–Ph–CH_2–N(Me)–, rotamer)], [34.9, 33.2, (–(O=C)N-CH_3, rotamer)], 28.0 (C × 3, –OC$(CH_3)_3$), HR-MS (FAB, PEG600+NaI): calcd for $C_{22}H_{29}O_4N_4$: 413.2189 [M+H], found *m/z* 413.2196 [M+H]$^+$.

4.7. *N*-*tert*-Butoxycarbonyl-*N*′-(1,1,1-trisfluoroethyl)-*N*″-(*N*-methyl-*N*-*p*-methoxybenzyl)-carbamoylguanidine 13b

To a solution of **3** (70 mg, 0.18 mmol) in THF/MeOH (1.8 mL/1.0 mL) was added triethylamine (75 μL, 0.54 mmol), trifluoroethylamine (73 μL, 0.27 mmol). The mixture was stirred at room temperature for 25 h. The solution was diluted with EtOAc (2 mL), and was successively washed with sat. aq. NH_4Cl (2 mL × 1), H_2O (5 mL × 1), brine (5 mL × 1), dried over Na_2SO_4, and concentrated in vacuo. The resulting colorless oil was purified by flash column chromatography (hexane/Et OAc = 80:1–50:1) to provide **13b** (63.2 mg, 0.15 mmol) in 83% yield as a colorless oil.

R_f = 0.47 (silica gel, Hexane/EtOAc = 3:1), IR (KBr) ν cm^{-1}: 3330 (w), 2983 (w), 1718 (s), 1637 (s), 1513 (s), 1457 (s), 1417 (s), 1246 (s), 1149 (s), ^1H NMR (270 MHz, $CDCl_3$) *The mixture of rotamers was observed.* δ: 12.4, 12.4 (s, 1H, –*N*H–, rotamer), 8.47, 8.46 (s, 1H, –*N*H–, rotamer), 7.16 (t, *J* = 8.91, 2H, Ph), 6.84 (dd, *J* = 8.57, 2.31 Hz, 2H, Ph), [4.67, 4.49 (s × 2, 2H, *p*-OCH_3PhCH_2–, rotamer)], 4.02 (m, 2H, CH_2CF_3), 3.78 (s, 3H, OCH_3), [3.00, 2.86 (s × 2, 3H, –*N*–CH_3–, rotamer)], 1.48 (s, 9H, –$COO(CH_3)_3$), ^{13}C NMR (67.5 MHz, $CDCl_3$) *The mixture of rotamers was observed.* δ: 163.6, 158.7, 154.4, 153.3, [130.4, 128.5 (rotamer, aromatic)], 128.8 (C × 2, aromatic), 124.0 (–CH_2CF_3, J_{CF} = 276.5 Hz), 113.8 (C × 2, aromatic), 83.0 (–OC$(CH_3)_3$), 55.2 (–OCH_3), [52.4, 50.4, (–Ph–CH_2–N(Me)–, rotamer)], 41.6 (–CH_2CF_3, J_{CF} = 34.6 Hz), [34.7, 32.9, (–*N*–CH_3, rotamer)], 28.0 (C × 3, –OC$(CH_3)_3$), HR-MS (FAB, PEG600+NaI): calcd for $C_{18}H_{26}O_4N_4F_3$: 419.1906 [M+H], found *m/z* 419.1905 [M+H]$^+$.

4.8. Solid phase total synthesis of argifin (1)

The solid phase total synthesis of Argifin (**1**) was carried out in a MicroKan microreactor initially filled with ca. 30 mg of 2-chlorotrityl resin **5** (1.4 mmol/g, purchased from NovaBioChem) and radio frequency chip with a view to an application of a combinatorial synthesis of an Argifin library in near future.

4.8.1. General procedure for loading of Fmoc-Asp(OAllyl)-OH 6 on to 2-chlorotrityl chloride resin 5

The MicroKan microreactor, containing 2-chlorotrityl resin **5**, was placed into a 20 mL screw vial, swollen in DCM (1.5 mL) for 1 h, and filtered. The MicroKan microreactor was treated with a cocktail of *N*-diisopropylethylamine (43.9 μL, 2.52 mmol), Fmoc-Asp(OAllyl)-OH (**6**) (49.8 mg, 0.126 mmol) in DCM (1.5 mL). The mixture was vigorously agitated at room temperature. After being agitated for 2 h, the reaction was quenched with MeOH (0.05 mL) to cap remaining reactive sites and agitated for additional 15 min. the MicroKans were filtered, washed with DMF (5 mL × 4), DCM (5 mL × 4), and dried in vacuo.

4.8.2. General procedure for deprotection of Fmoc group

The MicroKan microreactor was placed into a 20 mL screw vial and swollen in DCM (1.5 mL) for 1 h, and filtered. The MicroKan microreactor was sequentially treated with a solution of 20% piperidine in DMF (1.5 mL). The mixture was vigorously agitated at room temperature. After being agitated for 1 h, the reaction was filtered, washed with DMF (5 mL × 4), DCM (5 mL × 4), and dried in vacuo.

4.8.3. General procedure for peptide coupling

The MicroKan microreactor was placed into a 20 mL screw vial and swollen in DMF (1.5 mL) for 1 h, and filtered. The MicroKan microreactor was treated with a cocktail of each amino acids (3.0 equiv, Fmoc-*N*-Me-Phe-OH (**7**), Fmoc-Ala-OH (**9**), Fmoc-Asp-Ot-Bu (**10**)), PyBop (3.0 equiv), *N*-diisopropylethylamine (6.0 equiv) in DCM/DMF (4/1, 1.5 mL), except in the case of coupling to dipeptide **15** when Fmoc-Orn(Dde)-OH 8 (3.0 equiv), HATU (3.0 equiv), and *N*-diisopropylethylamine (6.0 equiv) in DCM/DMF (4/1, 1.5 mL) were used. The mixture was vigorously agitated at room temperature. After being agitated for 2 h, the reaction was filtered, washed with DMF (5 mL × 4), DCM (5 mL × 4), and dried in vacuo.

4.8.4. General procedure for deprotection of allyl group

The MicroKan microreactor was placed into a 20 mL screw vial and swollen in THF (1.5 mL) for 1 h under N_2 atmosphere, and drained THF by a syringe. To the MicroKan microreactor was sequentially added a solution of $Pd(PPh_3)_4$ (1.5 equiv), dimedone (10 equiv) in THF (1.5 mL) through a syringe under N_2 atmosphere after this solution was stirred and activated at room temperature for 1 h under N_2 atmosphere. The mixture was vigorously agitated at room temperature. After being agitated for 1 h, the reaction was filtered, washed with THF (5 mL × 4), DMF (5 mL × 4), DCM (5 mL × 4), and dried in vacuo.

4.8.5. Cyclization

The MicroKan microreactor was placed into a 20 mL screw vial and swollen in DCM (1.5 mL) for 1 h, and filtered. The MicroKan microreactor was treated with a cocktail of HATU (2 equiv), DIPEA (4 equiv), in DCM/DMF (4/1, 1.5 mL). The mixture was vigorously agitated at room temperature. After being agitated for 2 h, the reaction was filtered, washed with DMF (5 mL × 4), DCM (5 mL × 4), and dried in vacuo. The same procedure was repeated in this case.

4.8.6. General procedure for deprotection of Dde group

The MicroKan microreactor was placed into a 20 mL screw vial and swollen in DMF (1.5 mL) for 1 h, and filtered. The MicroKan microreactor was sequentially treated with a solution of 2% hydrazine monohydrate in DMF (1.5 mL). The mixture was vigorously agitated at room temperature. After being agitated for 1 h, the reaction was filtered, washed with DMF (5 mL × 4), DCM (5 mL × 4), and dried in vacuo.

4.8.7. General procedure for guanidination

The MicroKan microreactor was placed into a 20 mL screw vial and swollen in DCM (1.5 mL) for 1 h, and filtered. The MicroKan microreactor was sequentially treated with a 1.0 M solution of **3** in DCM (126 μL) and *N*-diisopropylethylamine (43.9 μL, 252 μmol) in DCM/DMF (4/1, 1.5 mL). The mixture was vigorously agitated at room temperature. After being agitated for 2 h, the reaction was filtered, washed with DMF (5 mL × 4), DCM (5 mL × 4), and dried in vacuo.

4.8.8. Cleavage from the resin

The MicroKan microreactor was placed into a 20 mL screw vial and swollen in DCM (1.5 mL) for 1 h, and filtered. The MicroKan microreactor was sequentially treated with a cocktail of 90% TFA/DCM (1.5 mL). The mixture was vigorously agitated at room temperature. After being agitated for 3 h, the reaction was filtered, and dried in vacuo to provide the crude Argifin (24 mg, 84.5 %). The use of HPLC purification (15% MeCN/H_2O) furnished **1** (3.8 mg, 13.4 %) as a colorless solid.

4.8.9. Synthetic argifin

IR (KBr) ν cm^{-1}: 3370, 3280, 3080, 2940, 1720, 1645, 1600, 1550, 1500, 1450, 1400, 1260, $[\alpha]_D^{20} = -51.2$ (*c* 1.0, H_2O), ^1H NMR (400 MHz, D_2O + 0.4% TFA) δ: 7.36 (dd, *J* = 7.0, 7.0 Hz, 2H, ε1, ε2 position of MePhe), 7.29 (dd, *J* = 7.0, 7.0 Hz, 1H, ζ position of MePhe), 7.23 (d, *J* = 7.0 Hz, 2H, δ1, δ2 position of MePhe), 5.13 (dd, *J* = 11.0, 3.0 Hz, 1H, α position of MePhe), 4.81 (m, 1H, α position of Asp2), 4.56 (dd, *J* = 12.0, 2.5 Hz, 1H, α position of Asp1), 4.29 (dd, *J* = 11.5, 2.5 Hz, 1H, α position of Arg), 4.18 (q, *J* = 7.0 Hz, 1H, α position of Ala), 3.17 (dd, *J* = 14.0, 3.0 Hz, 1H, β position of MePhe), 3.08 (dd, *J* = 16.5, 2.0 Hz, 1H, β position of Asp1), 3.05 (m, 1H, β position of MePhe), 3.01 (m, 2H, δ position of Arg), 2.88 (s, 3H, *N*Me of MePhe), 2.88 (m, 2H, β position of Asp1), 2.80 (m, 2H, β position of Asp2), 2.75 (s, 3H, *N*Me of Arg), 2.52 (dd, *J* = 13.5, 13.5 Hz, 2H, β position of Asp2), 1.40 (m, 1H, γ position of Arg), 1.31 (d, *J* = 7.0 Hz, 3H, β position of Ala), 1.15 (m, 1H, γ position of Arg), 1.13 (m, 1H, β position of Arg), −0.30 (m, 1H, β position of Arg), ^{13}C NMR (100 MHz, D_2O+0.4% TFA) δ: 175.4 (–NHCO– of Ala), 175.2 (–NHCO– of Asp1), 174.4 (–NHCO– of Arg), 174.3 (–NHCO– of Asp2), 171.5 (γ position of Asp2), 171.4 (γ position of Asp1), 170.3 (–NHCO– of MePhe), 155.3 (MeNHCONH–), 153.7 (MeNHCONHC(=NH)NH–), 137.5 (γ position of MePhe), 129.8 (×2, δ1, δ2 position of MePhe), 129.2 (×2, ε1, ε2 position of MePhe), 127.4 (ζ position of MePhe), 62.3 (α position of MePhe), 50.6 (α position of Asp1), 50.0 (α position of Asp2), 49.7 (α position of Ala), 48.8 (α position of Arg), 40.8 (δ position of Arg), 37.9 (β position of Asp2), 35.1 (β position of Asp1), 33.4 (β position of MePhe), 30.0 (NMe of MePhe), 26.7 (β position of Arg), 26.1 (MeNHCO-), 24.1 (γ position of Arg), 16.9 (β position of Ala), HR-MS (FAB, thioglycerol + glycerol, PEG600+NaI) calcd for $C_{29}H_{42}O_{10}N_9$: 676.3055 [M+H], found *m*/*z*: 676.3064 $[M+H]^+$.

4.9. *N*-Ac-Arg{N^{ω}-(*N*-methylcarbanoyl)}-*N*-methyl-Phe-Asp(OAllyl)-OH 18a

IR (KBr) ν cm^{-1}: 3370, 3280, 3080, 3012, 1727, 1641, 1581, 1540, 1500, 1415, 1243, $[\alpha]_D^{27} = -54.1$ (*c* 1.0, MeOH), ^1H NMR (300 MHz, CD_3OD) *The mixture of rotamers was observed.* δ: 7.32 (dd, *J* = 7.0, 7.0 Hz, 2H, ε1, ε2 position of MePhe), 7.26–7.17 (com-

plex m, 3H, ζ position of MePhe, δ1, δ2 position of MePhe), 5.91 (complex m, 1H, β position of OAllyl), 5.31 (dd, *J* = 17.0, 5.0 Hz, 1H, γ position of OAllyl), 5.21 (dd, *J* = 10.0, 1.0 Hz, 1H, γ position of OAllyl) 5.06 (dd, *J* = 12.0, 3.0 Hz, 1H, α position of MePhe), 4.80 (m, 2H, of α position of Asp2), 4.57 (dd, *J* = 5.0, 5.0 Hz, 1H, α position of OAllyl), 4.30 (dd, *J* = 7.0, 2.0 Hz, 1H, α position of Arg), 3.21 (dd, *J* = 14.0, 2.0 Hz, 1H, β position of MePhe), 3.10–2.90 (complex m, 3H, β position of MePhe, δ position of Arg), 2.92 (m, 2H, β position of Asp2), 2.86 (t, 3H, NMe of Arg), 2.78 (s, 1.5H, NMe of MePhe, rotamer), 2.76 (s, 1.5H, NMe of MePhe, rotamer), 1.92 (s, 2H, Me of Ac, rotamer), 1.89 (s, 1H, Me of Ac, rotamer), 1.61(m, 1H, γ position of Arg), 1.28 (m, 1H, γ position of Arg), 1.16 (d, 2H, β position of Arg), ^{13}C NMR (75 MHz, CD_3OD) *The mixture of rotamers was observed.* δ: 174.5, 174.2, 173.5, 171.7, 171.2, 155.9, 155.8, 139.4, 133.4, 130.8 (x2), 130.0 (x2), 129.6, 118.7, 66.6, 63.6, 50.4, 49.1, 42.0, 36.7, 35.1, 30.3, 30.0, 28.9, 26.6, 22.2, HR-MS (FAB, thioglycerol + glycerol, PEG600+NaI) calcd for $C_{27}H_{40}O_8N_7$: 590.2957 [M+H], found *m*/*z*: 590.2938 $[M+H]^+$.

4.10. *N*-Ac-D-Ala-Arg{N^{ω}-(*N*-methylcarbanoyl)}-*N*-methyl-Phe-Asp(OAllyl)-OH 18b

IR (KBr) *ν* cm^{-1}: 3415, 3300, 3080, 2940, 1725, 1675, 1600, 1538, 1486, 1450, 1400, 1200, $[\alpha]_D^{27} = -59.1$ (*c* 1.0, MeOH), ^{1}H NMR (300 MHz, CD_3OD) *The mixture of rotamers was observed.* δ: 7.33 (dd, *J* = 7.0, 7.0 Hz, 2H, ε1, ε2 position of MePhe), 7.26–7.17 (complex m, 3H, ζ position of MePhe, δ1, δ2 position of MePhe), 5.91 (complex m, 1H, β position of OAllyl), 5.30 (dd, *J* = 17.0, 11.0 Hz, 1H, γ position of OAllyl), 5.22 (dd, *J* = 11.0, 7.0 Hz, 1H, γ position of OAllyl), 5.11 (dd, *J* = 12.0, 4.0 Hz, 1H, α position of MePhe), 4.78 (m, 1H, α position of Asp2), 4.57 (dd, *J* = 11.0 Hz, 6.0 Hz, 2H, of α position of OAllyl), 4.28 (m, 2H, α position of Ala, α position of Arg), 3.21 (dd, *J* = 14.0, 3.0 Hz, 1H, β position of MePhe), 3.10–2.92 (complex m, 3H, β position of MePhe, δ position of Arg), 2.69 (s, 2H, NMe of MePhe), 2.67 (s, 1H, NMe of MePhe, rotamer), 2.88 (s, 3H, NMe of Arg), 2.83 (m, 2H, β position of Asp2), 1.89 (s, 1H, Me of Ac, rotamer), 1.87 (s, 2H, Me of Ac, rotamer), 1.52 (m, 1H, γ position of Arg), 1.20 (d, *J* = 6.7 Hz, 3H, β position of Ala), 1.16 (m, 1H, γ position of Arg), 1.03 (m, 2H, β position of Arg), ^{13}C NMR (75 MHz, CD_3OD) *The mixture of rotamers was observed.* δ: 175.2, 174.2, 173.5, 172.1, 171.7, 171.1, 156.0, 155.8, 139.2, 133.4, 130.8 (×2), 129.6 (×2), 129.6, 118.8, 66.7, 63.5, 49.8, 49.1, 48.6, 42.0, 36.8, 35.2, 30.3, 29.8, 28.9, 26.6, 22.6, 18.0, HR-MS (FAB, thioglycerol + glycerol, PEG600+NaI), calcd for $C_{30}H_{45}O_9N_8$: 661.3345 [M+H], found *m*/*z*: 661.3310 $[M+H]^+$.

4.11. *N*-Ac-Asp-D-Ala-Arg{N^{ω}-(*N*-methylcarbanoyl)}-*N*-methyl-Phe-Asp(OAllyl)-OH 18c

IR (KBr) *ν* cm^{-1}: 3423, 3300, 3000, 2900, 1725, 1670, 1600, 1536, 1500, 1434, 1396, 1200, $[\alpha]_D^{27} = -28.1$ (*c* 1.0, MeOH), ^{1}H NMR (300 MHz, CD_3OD) *The mixture of rotamers was observed.* δ: 7.32 (dd, *J* = 7.0, 7.0 Hz, 2H, ε1, ε2 position of MePhe), 7.23–7.17 (complex m, 3H, ζ position of MePhe, δ1, δ2 position of MePhe), 5.91 (complex m, 1H, β position of OAllyl), 5.31 (dd, *J* = 17.0, 7.0 Hz, 1H, γ position of OAllyl), 5.21 (dd, *J* = 10.0, 2.0 Hz, 1H, γ position of OAllyl), 5.08 (dd, *J* = 11.0, 3.0 Hz, 1H, α position of MePhe), 4.86 (m, 1H, α position of Asp2), 4.73 (dd, *J* = 11.0, 5.0 Hz, 1H, α position of Asp1), 4.58 (dd, *J* = 6.0 Hz, 6.0 Hz, 2H, of α position of OAllyl), 4.36 (m, 2H, α position of Ala, α position of Arg), 3.22 (dd, *J* = 14.0, 3.0 Hz, 1H, β position of MePhe), 3.04 (dd, *J* = 13.0, 4.0 Hz, 2H, β position of Asp1), 3.01–2.97 (complex m, 3H, β position of MePhe, δ position of Arg), 2.92 (m, 2H, β position of Asp2), 2.85 (s, 3H, NMe of MePhe,), 2.77 (s, 1.5H, NMe of Arg, rotamer), 2.76 (s, 1.5H, NMe of Arg, rotamer), 1.99 (s, 3H, Me of Ac), 1.62 (m, 1H, γ position of Arg), 1.32 (d, *J* = 6.7 Hz, 3H, β position of Ala), 1.29 (m, 1H, γ position of Arg), 1.18 (m, 2H, β position of Arg), ^{13}C NMR (75 MHz, CD_3OD) *The mixture of rotamers was observed.* δ: 175.2, 174.7, 174.6, 173.5, 172.1, 171.9, 171.7, 171.2, 156.2, 155.9, 139.4, 133.4, 130.8 (x2), 130.0 (x2), 129.6, 118.7, 66.7, 63.9, 50.8, 50.3, 48.9, 48.6, 41.8, 38.7, 36.7, 35.0, 30.3, 29.7, 28.5, 26.6, 22.5, 17.9, HR-MS (FAB, thioglycerol + glycerol, PEG600+NaI) calcd for $C_{34}H_{50}O_{12}N_9$: 776.3579 [M+H], found *m*/*z*: 776.3579 $[M+H]^+$.

4.12. *N*-Ac-Arg{N^{ω}-(*N*-methylcarbanoyl)}-*N*-methyl-Phe-OH 19a

IR (KBr) *ν* cm^{-1}: 3379, 3197, 3095, 3025, 1683, 1639, 1562, 1544, 1494, 1423, 1251, $[\alpha]_D^{26} = -61.9$ (c 1.0, MeOH), ^{1}H NMR (300 MHz, CD_3OD) *The mixture of rotamers was observed.* δ: 7.33 (dd, *J* = 7.0, 7.0 Hz, 2H, ε1, ε2 position of MePhe), 7.28–7.18 (complex m, 3H, ζ position of MePhe, δ1, δ2 position of MePhe), 5.02 (dd, *J* = 12.0, 3.0 Hz, 1H, α position of MePhe), 4.30 (dd, *J* = 7.0, 7.0 Hz, 1H, α position of Arg), 3.24 (dd, *J* = 14.0, 7.0 Hz, 1H, β position of MePhe), 3.10–3.00 (complex m, 3H, β position of MePhe, δ position of Arg), 2.91 (s, 3H, NMe of MePhe), 2.78 (s, 1.5H, NMe of Arg, rotamer), 2.76 (s, 1.5H, NMe of Arg, rotamer), 1.90 (s, 1H, Me of Ac, rotamer), 1.89 (s, 2H, Me of Ac, rotamer), 1.64 (m, 1H, γ position of Arg), 1.28 (m, 1H, γ position of Arg), 1.18 (m, 2H, β position of Arg), ^{13}C NMR (75 MHz, CD_3OD) *The mixture of rotamers was observed.* δ: 173.6, 173.4, 172.7, 156.0, 155.8, 139.1, 130.5 (×2), 130.0 (×2), 129.5, 62.8, 49.1, 41.9, 35.2, 30.8, 29.9, 29.7, 26.5, 22.3, HR-MS (FAB, thioglycerol + glycerol, PEG600 + NaI), calcd for $C_{20}H_{31}O_5N_6$: 435.2356 [M+H], found *m*/*z*: 435.2346 $[M+H]^+$.

4.13. *N*-Ac-D-Ala-Arg{N^{ω}-(*N*-methylcarbanoyl)}-*N*-methyl-Phe-OH 19b

IR (KBr) *ν* cm^{-1}: 3328, 3100, 3000, 2900, 1677, 1641, 1560, 1540, 1442, 1417, 1376, 1200, $[\alpha]_D^{26} = -31.4$ (*c* 1.0, MeOH), ^{1}H NMR (300 MHz, CD_3OD) *The mixture of rotamers was observed.* δ: 7.34 (dd, *J* = 7.0, 7.0 Hz, 2H, ε1, ε2 position of MePhe), 7.30–7.18 (complex m, 3H, ζ position of MePhe, δ1, δ2 position of MePhe), 5.15 (dd, *J* = 11.0, 5.0 Hz, 1H, α position of MePhe), 4.24 (m, 2H, α position of Ala, α position of Arg), 3.11 (dd, *J* = 12.0, 4.0 Hz, 1H, β position of MePhe), 3.17–3.00 (complex m, 3H, β position of MePhe, δ position of Arg), 2.96 (s, 3H, NMe of MePhe), 2.80 (s, 1H, NMe of Arg, rotamer), 2.77 (s, 2H, NMe of Arg, rotamer), 2.00 (s, 2H, Me of Ac, rotamer), 1.97 (s, 1H, Me of Ac, rotamer), 1.61 (m, 1H, γ position of Arg), 1.30 (d, *J* = 4.0 Hz, 3H, β position of Ala), 1.23 (m, 1H, γ position of Arg), 1.09 (m, 2H, β position of Arg), ^{13}C NMR (75 MHz, CD_3OD) *The mixture of rotamers was observed.* δ: 174.8, 173.8, 173.3, 173.2, 156.0, 155.8, 138.9, 130.6 (×2), 130.0 (×2), 129.5, 64.5, 48.9, 48.6, 41.9, 35.3, 30.8, 29.8, 28.8, 26.6, 22.4, 18.0, HR-MS (FAB, thioglycerol + glycerol, PEG600 + NaI), calcd for $C_{23}H_{36}O_6N_7$: 506.2727 [M+H], found *m*/*z*: 506.2716$[M+H]^+$.

4.14. *N*-Ac-Asp-D-Ala-Arg{N^{ω}-(*N*-methylcarbanoyl)}-*N*-methyl-Phe-OH 19c

IR (KBr) *ν* cm^{-1}: 3415, 3100, 3000, 2900, 1720, 1676, 1583, 1539, 1486, 1435, 1400, 1201, $[\alpha]_D^{27} = -38.1$ (*c* 1.0, MeOH), ^{1}H NMR (300 MHz, CD_3OD) *The mixture of rotamers was observed.* δ: 7.32 (dd, *J* = 7.0, 7.0 Hz, 2H, ε1, ε2 position of MePhe), 7.26–7.18 (complex m, 3H, ζ position of MePhe, δ1, δ2 position of MePhe), 5.04 (dd, *J* = 11.0, 3.0 Hz, 1H, α position of MePhe), 4.78 (dd, *J* = 10.0, 6.0 Hz, 1H, α position of Asp1), 4.25 (m, 2H, α position of Ala, α position of Arg), 3.24 (dd, *J* = 12.0, 6.0 Hz, 1H, β position of MePhe), 3.12 (dd, *J* = 12.0, 6.0 Hz, 2H, β position of Asp1), 3.08–2.99 (complex m, 3H, β position of MePhe, δ position of Arg),

2.96 (s, 3H, NMe of MePhe,), 2.79 (s, 1.5H, NMe of Arg, rotamer), 2.76 (s, 1.5H, NMe of Arg, rotamer), 1.98 (s, 1H, Me of Ac, rotamer), 1.97 (s, 2H, Me of Ac, rotamer), 1.66 (m, 1H, γ position of Arg), 1.29 (d, *J* = 7.0 Hz, 3H, β position of Ala), 1.35 (m, 1H, γ position of Arg), 1.00 (m, 2H, β position of Arg), ^{13}C NMR (75 MHz, CD_3OD) *The mixture of rotamers was observed.* δ: 174.4, 174.3, 173.8, 173.4, 172.5, 172.1, 156.0, 155.9, 138.8, 130.6 (×2), 130.1 (×2), 130.0, 63.1, 50.8, 49.7, 49.2, 41.9, 38.5, 35.1, 30.2, 29.9, 29.7, 26.6, 22.6, 18.1, HR-MS (FAB, thioglycerol + glycerol, PEG600 + NaI), calcd for $C_{27}H_{41}O_9N_8$: 621.2997 [M+H], found *m*/*z*: 621.3008 $[M+H]^+$.

4.15. *N*-Ac-Arg{N^{ω}-(*N*-methylcarbanoyl)}-OH 20a

IR (KBr) *ν* cm^{-1}: 3316, 3116, 2921, 2850, 1685, 1640, 1560, 1545, 1508, 1425, 1253, 1205, $[\alpha]_D^{24} = 2.06$ (*c* 1.0, MeOH), ^{1}H NMR (300 MHz, CD_3OD) δ: 4.39 (s, 1H, α position of Arg), 3.73–3.00 (m, 2H, δ position of Arg), 2.77 (s, 3H, NMe of Arg), 2.01 (s, 1H, Me of Ac), 1.73 (m, 1H, γ position of Arg), 1.33 (m, 1H, γ position of Arg), 1.14 (m, 2H, β position of Arg), ^{13}C NMR (75 MHz, CD_3OD) δ: 176.3, 173.4, 156.1, 155.8, 48.9, 41.8, 30.1, 26.5, 25.8, 22.4, HR-MS (FAB, thioglycerol + glycerol, PEG600 + NaI), calcd for $C_{10}H_{20}O_4N_5$: 274.1545 [M+H], found *m*/*z*: 274.1515 $[M+H]^+$.

4.16. *N*-Ac-D-Ala-Arg{N^{ω}-(*N*-methylcarbanoyl)}-OH 20b

IR (KBr) *ν* cm^{-1}: 3369, 3083, 2948, 1675, 1650, 1560, 1546, 1461, 1425, 1379, 1203, $[\alpha]_D^{23} = 11.2$ (*c* 1.0, MeOH), ^{1}H NMR (300 MHz, CD_3OD) *The mixture of rotamers was partly observed.* δ: 4.43 (dd, *J* = 9.0, 5.0 Hz, 1H, α position of Arg), 4.33 (dd, *J* = 14.0, 7.0 Hz, 1H, α position of Ala), 3.16 (t, *J* = 7.0 Hz, 2H, δ position of Arg), 2.76 (s, 3H, NMe of Arg), 1.98 (s, 3H, Me of Ac), 1.73 (m, 1H, γ position of Arg), 1.35 (d, *J* = 7.0 Hz, 3H, β position of Ala), 1.31 (m, 1H, γ position of Arg), 1.13 (m, 2H, β position of Arg), ^{13}C NMR (75 MHz, CD_3OD) *The mixture of rotamers was partly observed.* δ: 174.6, 174.2, 173.4, 156.0, 155.8, 48.9, 48.3, 41.8, 29.7, 29.3, 26.5, 22.4), 17.9. HR-MS (FAB, thioglycerol + glycerol, PEG600 + NaI), calcd for $C_{13}H_{25}O_5N_6$: 345.1917 [M+H], found *m*/*z*: 345.1886$[M+H]^+$.

4.17. *N*-Ac-Asp-D-Ala-Arg{N^{ω}-(*N*-methylcarbanoyl)}-OH 20c

IR (KBr) *ν* cm^{-1}: 3359, 3087, 2958, 1716, 1652, 1558, 1550, 1455, 1434, 1392, 1201, $[\alpha]_D^{24} = 5.66$ (*c* 1.0, MeOH), ^{1}H NMR (300 MHz, CD_3OD) *The mixture of rotamers was observed.* δ: 4.79 (dd, *J* = 12.0, 7.0 Hz, 1H, α position of Asp1), 4.38 (m, 2H, α position of Ala, α position of Arg), 3.17 (dd, *J* = 7.0, 7.0 Hz, 2H, β position of Asp1), 3.10–2.82 (m, 2H, δ position of Arg), 2.77 (s, 3H, NMe of Arg), 1.97 (s, 3H, Me of Ac), 1.70 (m, 1H, γ position of Arg), 1.36 (d, *J* = 6.0 Hz, 3H, β position of Ala), 1.14 (complex m, 3H, γ position of Arg, β position of Arg), ^{13}C NMR (75 MHz, CD_3OD) *The mixture of rotamers was observed.* δ: 174.9, 174.7, 173.8, 173.5, 172.1, 156.0, 155.9, 50.6, 49.7, 48.6, 41.8, 30.0, 29.6, 26.7, 22.5, 18.2, HR-MS (FAB, thioglycerol + glycerol, PEG600 + NaI), calcd for $C_{17}H_{30}O_8N_7$: 460.2140 [M+H], found *m*/*z*: 460.2156 $[M+H]^+$.

4.18. Procedures for IC_{50} measurements against each SmChiA, B, and C_1

Ten microliters of 0.1 M phosphate buffer (pH 7.0), 10 μL of each inhibitors in MeOH, 30 μL of diluted crude chitinase solution (*Sm*ChiA-x10 dilution; *Sm*ChiB-x40 dilution; *Sm*ChiC_1-x4 dilution with 0.1 M phosphate buffer pH 7.0) (see the procedures for the preparation of *Sm*ChiA, B, and C_1), and 50 μL of 80 μM 4-methylumberiferyl-β-D-*N,N'*-diacethylchitobiose [4-MU-$(GluNAc)_2$, Sigma] in 0.1 M phosphate buffer (pH 7.0) were placed in each well of a microplate, and incubated with 10μL of inhibitors in MeOH for 5 min. Fluorescence (excitation at 355 nm, emission at 460 nm) was measured at intervals of 60 seconds by fluorometer (Fluoroscan II, Labsystems), and the rate of 4-methylumbelliferone production was corrected by calibrating the quenching ratio of each inhibitors using the mixture of the inhibitors and 4-methylumbelliferone (4MU).

IC_{50} values were determined from dose-response sigmoidal curves, which were calculated by using KaleidaGraph® (Synergy Software Inc., USA) with the experimental data.

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