Bioorganic & Medicinal Chemistry 19 (2011) 3054–3059

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Preparation of allosamidin and demethylallosamidin photoaffinity probes and analysis of allosamidin-binding proteins in asthmatic mice

Yosuke Sato [a], Shigeo Suzuki [a], Seiko Muraoka [a], Naoya Kikuchi [a], Naotaka Noda [b], Takafumi Matsumoto [b], Hiromasa Inoue [b,*,†], Hiromichi Nagasawa [a], Shohei Sakuda [a,*]

[a] Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bnkyo-ku, Tokyo 113-8657, Japan
[b] Research Institute for Diseases of the Chest, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

ARTICLE INFO

Article history:
Received 23 February 2011
Revised 7 April 2011
Accepted 8 April 2011
Available online 13 April 2011

Keywords:
Allosamidin
Demethylallosamidin
Photoaffinity probe
Ym1
Asthma
Chitinase-like protein
Chitinase

ABSTRACT

Allosamidins, metabolites of *Streptomyces* with strong inhibitory activities toward family 18 chitinases, show a variety of biological activities in various organisms. We prepared photoaffinity and biotinylated probes of allosamidin and demethylallosamidin, the *N*-demethyl derivative that shows much stronger anti-asthmatic activity than allosamidin. Mild acid hydrolysis of allosamidins afforded mono-amine derivatives, which were amidated to prepare probes with a photoactivatable aryl azide and/or biotin moieties. The derivatives with an *N*-acyl group at C-2 of the D-allosamine residue at the non-reducing end of allosamidins inhibited *Trichoderma* chitinase as strongly as the original compounds. Since the target of allosamidins in asthma is unclear, photoaffinity probes were used to analyze allosamidin-binding proteins in bronchoalveolar lavage (BAL) fluid in IL-13-induced asthmatic mice. Ym1, a chitinase-like protein, was identified as the main allosamidin-binding protein among proteins whose expression was upregulated by IL-13 in BAL fluid. Binding of allosamidins with Ym1 was confirmed by the experiments with photoaffinity probes and recombinant Ym1.

1. Introduction

Allosamidin (**1**), a metabolite of *Streptomyces*, has a unique pseudotrisaccharide structure that mimics chitin (Scheme 1).[1] It inhibits all family 18 chitinases, which hydrolyze chitin and are widely present not only in chitin-containing organisms such as insects and fungi, but also in non-chitin-containing ones such as plants and mammals.[2] Allosamidin has been used to investigate the physiological role of chitinases involved in a variety of organisms. For example, it inhibits cell separation of yeast,[3] transmission of the malaria parasite,[4] and insect molting[5] by inhibiting chitinases.

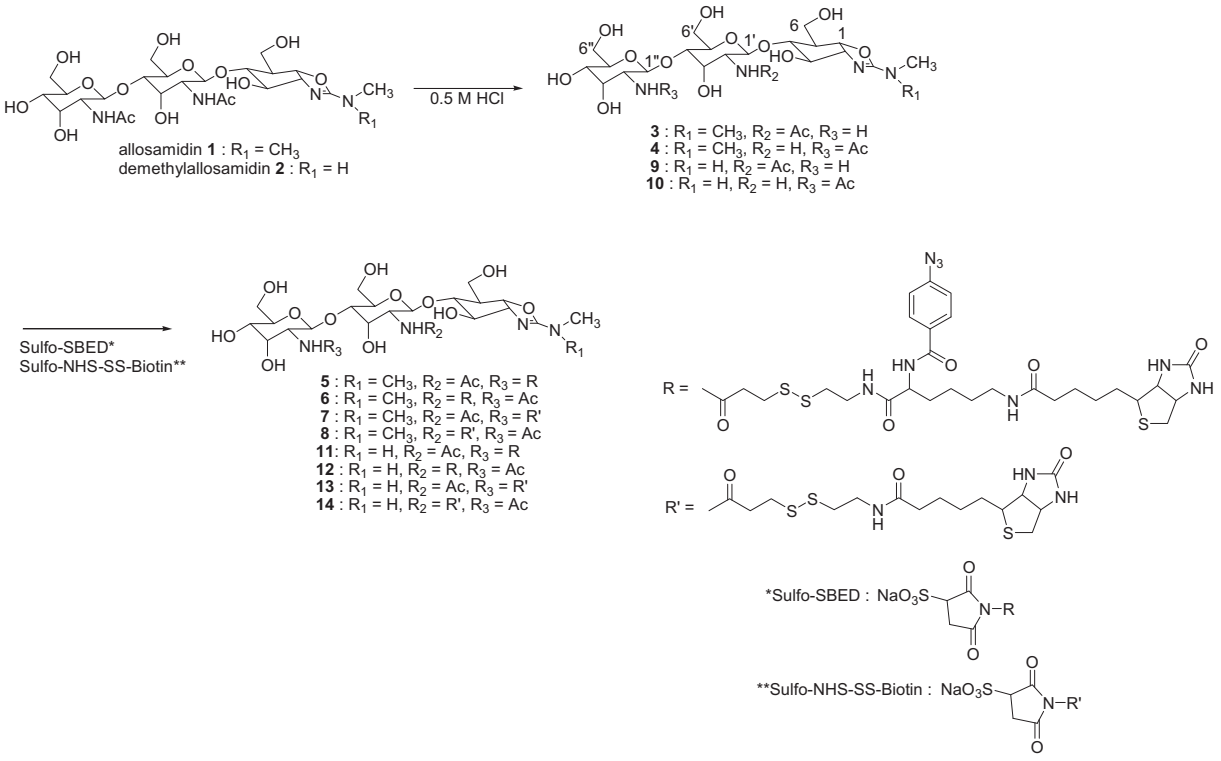
Recently, novel biological or physiological activities of allosamidin in plants, mammals, and allosamidin-producing bacteria have been found. Allosamidin enhanced stress tolerance in *Arabidopsis thaliana*[6] and attenuated asthmatic Th2 inflammation in a mouse model of asthma.[7] It strongly promoted chitinase production and growth in allosamidin-producing *Streptomyces* through a two-component regulatory system.[8–10]

The anti-asthmatic activity of allosamidin was thought to be due to its action on acidic mammalian chitinase (AMCase), since expression of AMCase has been shown to be upregulated in response to allergen exposure or IL-13-induced inflammation in mouse lung[11] and allosamidin strongly inhibits AMCase.[12] However, it has been reported that transgenic mice overexpressing AMCase show no signs of allergic inflammation.[13] Furthermore, we recently showed that demethylallosamidin (**2**), the demethyl derivative of allosamidin, showed stronger anti-asthmatic activity than allosamidin in mouse models and the inhibitory activity of demethylallosamidin toward AMCase was not different from that of allosamidin.[14] Thus, the mechanism of allosamidin causing anti-asthmatic activity remains unclear.

Allosamidin derivatives with a photoaffinity or biotinylated residue are very useful probes to study the biological and physiological activities of allosamidin and its target molecules. We previously synthesized a biotinylated probe from a dialdehyde prepared by sodium periodate oxidation of allosamidin.[15] Although this probe inhibited chitinase just as strongly as allosamidin, it has a somewhat labile imine linkage between the allosamidin residue and the biotin moiety. Therefore, we planned to prepare more stable probes of allosamidin and demethylallosamidin. Demethylallosamidin probes may be equally useful as allosamidin ones

* Corresponding authors. Tel.: +81 3 5841 5133; fax: +81 3 5841 8022 (S.S.); tel.: +81 99 275 6476; fax: +81 99 275 6482 (H.I.).
E-mail addresses: inoue-pulm@umin.net (H. Inoue), asakuda@mail.ecc.u-tokyo.ac.jp (S. Sakuda).
† Present address: Department of Pulmonary Medicine, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan.

0968-0896/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2011.04.012



Scheme 1.

to study the mode of action of allosamidins as anti-asthmatic agents.

In this paper, we describe the preparation of new photoaffinity and biotinylated allosamidin probes which maintain strong chitinase inhibitory activity. Experiments with the photoaffinity probes to detect allosamidin binding proteins in bronchoalveolar lavage (BAL) fluid in a mouse model of asthma are also described.

2. Results and discussion

2.1. Preparation of photoaffinity and biotinylated probes of allosamidins

With respect to the structure-activity relationship of allosamidin, it is known that modification of the *N*-acetyl-D-allosamine residue at the non-reducing end of **1** does not reduce the chitinase inhibitory activity significantly.[16] Therefore, we planned to use deacetylated allosamidin **3** with an amino group at C-2″ as a precursor for the probe. Mild acid hydrolysis of allosamidin with 0.5 M HCl at 80 °C afforded several products, including a disaccharide previously reported.[17] After reacting for 10 h, two monodeacetylated molecules, **3** and **4**, were obtained. A residue with a photoactivatable aryl azide and a biotin moiety was introduced by a coupling with Sulfo-SBED at each amino group of compounds **3** and **4** to afford photoaffinity probes **5** and **6**, respectively (Scheme 1). The coupling reaction was done by mixing the solutions of Sulfo-SBED in dimethyl sulfoxide and **3** or **4** in 1% $NaHCO_3$ with stirring for 30 min at room temperature in the dark. Similarly, biotinylated probes **7** and **8** were prepared from compounds **3** and **4**, respectively, by coupling with Sulfo-NHS-SS-Biotin. Demethylallosamidin probes **11** and **13**, and **12** and **14**, were prepared from deacetylated demethylallosamidins **9** and **10**, respectively, according to similar methods that were used for the preparation of the allosamidin probes.

The inhibitory activities of compounds **1**–**14** toward *Trichoderma* chitinase are summarized in Table 1. Amino compounds **3**, **4**, **9**, and **10** were not as potent as the corresponding acylated compounds. Conversely, the photoaffinity and biotinylated probes **5**, **7**, **11**, and **13** with newly introduced acyl groups at C-2″ maintained strong chitinase inhibitory activities, while the inhibitory activities of probes **6**, **8**, **12**, and **14** with the acyl group at C-2′ were very weak. These probes have stable amide linkage between the allosamidin residue and the photoaffinity or biotin moiety; for example, probe **7** was stable in a solution of 0.05 M HCl or 0.05 M NaOH for 30 min at room temperature.

2.2. Detection and identification of allosamidin-binding proteins in BAL fluids in IL-13-induced asthmatic mice

Next, the photoaffinity probes prepared above were used to detect allosamidin-binding proteins in BAL fluids in a mouse model of asthma. IL-13-induced asthmatic mice were used for the experiments. A solution of the BAL fluid proteins prepared from each of the control and IL-13 instillation mice was incubated with the

Table 1
Inhibitory activities of compounds **1**–**14** toward *Trichoderma* chitinase

Compound	IC_{50} (μM)
1	5.4
2	10.3
3	34.3
4	69.3
5	10.7
6	125.1
7	3.6
8	50.3
9	82.9
10	308.5
11	7.7
12	155.2
13	7.9
14	49.7

allosamidin- or demethylallosamidin-photoaffinity probe (**5** or **11**). The solution was UV-irradiated to cross-link the probe and proteins, and biotinylated proteins were detected by western blotting using HRP–avidin after SDS–PAGE (Fig. 1B). A strong band at 70 kDa was detected in all samples (lanes 1–8 in Fig. 1B) due to non-specific binding of probe **5** or probe **11** with albumin. The band at 45 kDa was observed in the BAL fluid of IL-13-induced mice (lanes 5 and 7 in Fig. 1B) and it was identical to the band of the protein whose expression was clearly upregulated by IL-13 as detected by silver staining (lane 2 in Fig. 1A). The density of the band at 53 kDa detected by western blotting increased in the BAL fluid of IL-13-induced mice (lanes 5 and 7 in Fig. 1B) compared to that of control mice (lanes 1 and 3 in Fig. 1B). The bands at 45 kDa and 53 kDa were disappeared in the experiments with excess amounts of allosamidin or demethylallosamidin (lanes 2, 4, 6 and 8 in Fig. 1B).

N-terminal amino acid sequence analysis of the 45 kDa and 53 kDa proteins afforded the sequence YQLMXYYTSW (X was unidentifiable.) and EDVQETDTSQ, which showed that the predominant proteins involved in the bands at 45 kDa and 53 kDa was Ym1 and/or Ym2[18–20] and α-1 antitrypsin,[21] respectively. Ym1 and Ym2 are congeners with similar molecular size and high amino acid sequence homology. The amino acid sequence determined by the N-terminal analysis is commonly present in both proteins. Therefore, mouse lung cDNA was analyzed by the method of Webb et al.[20] to estimate the component(s) in the 45 kDa band. Lung cDNA were prepared from an asthmatic mouse exposed to IL-13 or ovalbumin and PCR products were prepared from the cDNA using primers whose sequences commonly exist in both cDNA of Ym1 and Ym2. The PCR products were digested with *Sca* I restriction endonuclease. cDNA encoding Ym1 has a *Sca* I site in its sequence, while that encoding Ym2 does not have the site. As shown in Figure 2, the PCR products were digested almost entirely by *Sca* I for both IL-13 and ovalubmin exposures. This suggested that Ym1 was expressed much more abundantly than Ym2 in the lung of an IL-13- or ovalbumin-induced asthmatic mouse and Ym1 is the main allosamidin-binding protein in the 45 kDa band.

Recombinant Ym1 (rYm1) was prepared using an *Escherichia coli* expression system, followed by refolding. The rYm1 was cross-linked with a photoaffinity probe (**5** or **11**), and the binding

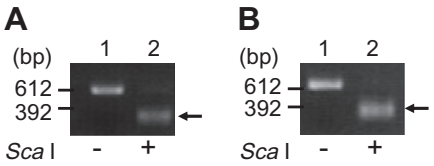


Figure 2. *Sca* I digestion of RT-PCR product. Lung cDNA were prepared from lung tissue of an asthmatic mouse exposed to ovalbumin or IL-13. PCR products were prepared from the cDNA using primers whose sequences commonly existed in both Ym1 and Ym2 cDNA. The PCR products were digested almost entirely by *Sca* I to afford the digest (arrows) in ovalubmin (A) and IL-13 (B) exposure experiments.

of rYm1 with the probes was detected by western blotting (Fig. 3). The density of the band at 45 kDa increased depending on concentrations of the probes in both experiments using **5** (lanes 1–3) and **11** (lanes 5–7), and the band was disappeared in the experiments with excess amounts of allosamidin or demethylallosamidin (lanes 4 and 8), suggesting the specific binding of allosamidins to rYm1. Significant difference was not observed between the density of the band detected by allosamidin-probe **5** and that by demethylallosamidin-probe **11**.

Ym1 and Ym2 belong to chitinase-like proteins that have structures homologous to chitinases but do not have chitinase activity due to the lack of essential acidic amino acid residue(s) commonly present in the active site of family 18 chitinases.[22] Chitinase-like proteins are widely present in a variety of organisms including mammals. However, little is known about their physiological functions at the molecular level. Although they do not show chitinase activity, some of them have been shown to bind to poly and/or oligosaccharides. Binding of Ym1 to chitin,[18] heparin, and glucosamine oligomers has been reported.[23] In addition, it has been shown that binding of chitinase-like proteins YKL-40 and SPG-40 to *N*-acetylglucosamine oligomers with relatively high affinity.[24,25] These carbohydrate-binding properties of chitinase-like proteins may be implicated in their biological activity. Therefore, allosamidins may have the potential to inhibit the function of these proteins by binding to them.

It has been reported that Ym1 shows eosinophil chemotaxis[18] and anti-sense Ym1 RNA suppresses asthmatic responses in a murine model of allergic asthma.[26] Our experiments with the

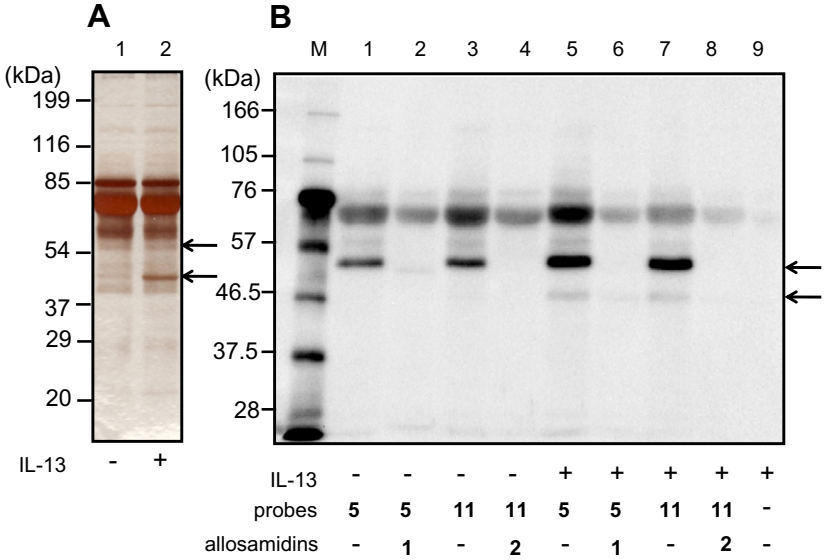


Figure 1. Detection of allosamidin-binding proteins in BAL fluids. Proteins in the BAL fluid of control mice (lane 1 in (A), lanes 1–4 in (B)) and IL-13-induced asthmatic mice (lane 2 in (A), lanes 5–8 in (B)) were analyzed by silver staining (A), and photoaffinity probes (B). BAL fluid was incubated with probe **5** (lanes 3 and 5 in (B)), probe **11** (lanes 4 and 6 in (B)), probe **5** and **1** (lane 2 in (B)), or probe **11** and **2** (lane 4 in (B)). The reaction solution was UV-irradiated to enable cross-linking, and biotinylated proteins were analyzed using streptavidin-HRP after SDS–PAGE. Prestained and biotinylated protein markers were used in (A) and (B), respectively. Arrows indicate 45 kDa and 53 kDa proteins.

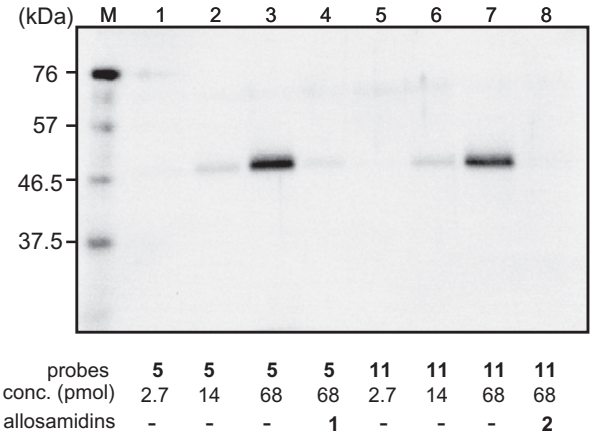


Figure 3. Binding of allosamidin-photoaffinity probes with recombinant Ym1. Recombinant Ym1 (13 pmol) was incubated with probe **5** (lanes 1–3), **1** (68 nmol) and probe **5** (lane 4), probe **11** (lanes 5–7), and **2** (68 nmol) and probe **11** (lane 8). The reaction solution was analyzed using streptavidin–HRP after SDS–PAGE.

photoaffinity probes suggested that Ym1 was the main protein specifically binding to allosamidins whose expression was much upregulated by IL-13. An increase of AMCase activity in BAL fluid after IL-13 treatment has been shown,[14] but AMCase protein was not clearly detected by the probes probably due to its low concentration in the fluids. It is known that the amount of Ym1 expressed in the BAL fluid of allergen-induced asthmatic mice is much higher than that of AMCase.[27] BRP-39, another murine chitinase-like protein, and its human homolog YKL-40 have been strongly suggested to play a critical role in asthma.[28] The photoaffinity and biotinylated allosamidin probes we prepared may be useful to investigate the mode of action of not only chitinases but also chitinase-like proteins.

Alpha-1 antitrypsin is a serine protease inhibitor which protects the alveolar matrix from proteolytic attack.[29] Although its expression is not strongly upregulated by IL-13, there is a possibility that allosamidins might affect an unknown role of α-1 antitrypsin in asthma. Works to investigate the binding affinities of allosamidin and demthylallosamidin to not only Ym1, BRP39 and AMCase but also α-1 antitrypsin are now in progress.

In conclusion, new photoaffinity and biotinylated probes of allosamidin and demethylallosamidin with strong chitinase-inhibitory activity were prepared. Ym1 was identified as the major allosamidin-binding protein whose expression was upregulated by IL-13 in the BAL fluid in a mouse model of asthma by using the photoaffinity probes prepared.

3. Experimental

3.1. Allosamidin and demethylallosamidin

Allosamidin and demethylallosamidin were isolated from the mycelial extracts of *Streptomyces* sp. AJ9463 according to the method described previously.[30]

3.2. Preparation of allosamidin photoaffinity and biotinylated probes

Allosamidin **1** (25 mg) or demethylallosamidin **2** (25 mg) was hydrolyzed with 0.5 M HCl (2.5 mL) at 80 °C for 10 h and the reaction solution was separated using reverse-phase HPLC (column: Capcell-Pak C_{18}, 4.6 mm × 250 mm, Shiseido, Tokyo, Japan; a gradient elution of 0–20% acetonitrile in 10 mM $AcONH_4$–NH_4OH pH 8.9 over 30 min; flow rate: 1.0 mL/min; detection: 215 nm; retention times of **3**, **4**, **9**, and **11**: 20.7 min, 21.6 min, 14.1 min, and 16.3 min) to afford crude **3** (12.0 mg) and crude **4** (3.4 mg) or **9** (2.2 mg) and **11** (2.8 mg). Crude **3** and crude **4** were further purified using cation-exchange HPLC (column: Shodex Asahipak ES502C 7.6 mm × 250 mm, Showa Denko, Tokyo, Japan; an isocratic elution of 10 mM $AcONH_4$–NH_4OH pH 10.1; flow rate: 1.0 mL/min; detection: 220 nm; retention times of **3** and **4**: 6.8 min and 6.0 min) to obtain **3** (1.2 mg) and **4** (0.9 mg). **3**: HRESIMS *m/z* 581.2698 $(M+H)^+$ (calcd for $C_{23}H_{41}N_4O_{13}$, 581.2670); δ_H (D_2O + 0.3% CD_3COOD, 500 MHz, acetone δ_H 2.225 as internal reference): 5.38 (H-1, dd, *J* = 5 Hz and 9 Hz), 5.05 (H-1″, d, *J* = 9 Hz), 4.77 (H-1′, d, *J* = 9 Hz), 4.38 (H-3′, t, *J* = 3 Hz), 4.37 (H-2, dd, *J* = 4 Hz and 9 Hz), 4.28 (H-3″), 4.28 (H-3, t, *J* = 5 Hz), 3.93 (H-5′), 3.90 (H-6′a), 3.88 (H-4), 3.88 (H-4′), 3.86 (H-6″a), 3.84 (H-2′), 3.81 (H-5″), 3.81 (H-6a), 3.74 (H-6″b), 3.72 (H-6′b), 3.71 (H-4″), 3.66 (H-6b), 3.33 (H-2″, dd, *J* = 3 Hz and 9 Hz), 3.08 (N–CH_3, s), 3.06 (N–CH_3, s), 2.53 (H-5), 2.08 (Ac, s). Compound **4**: HRESIMS *m/z* 581.2660 $(M+H)^+$ (calcd for $C_{23}H_{41}N_4O_{13}$, 581.2670); δ_H (D_2O + 0.3% CD_3COOD, 500 MHz, acetone δ_H 2.225 as internal reference): 5.33 (H-1, dd, *J* = 5 Hz and 9 Hz), 5.01 (H-1′, d, *J* = 9 Hz), 4.78 (H-1″, d, *J* = 9 Hz), 4.59 (H-3′, t, *J* = 3 Hz), 4.36 (H-2, dd, *J* = 5 Hz and 9 Hz), 4.32 (H-3, dd, *J* = 5 Hz and 6 Hz), 4.03 (H-3″, t, *J* = 3 Hz), 3.97 (H-4, dd, *J* = 6 Hz and 8 Hz), 3.90 (H-5′), 3.88 (H-2″, dd, *J* = 3 Hz and 9 Hz), 3.86 (H-6a), 3.86 (H-6″a), 3.80 (H-6′a, dd, *J* = 3 Hz and 12 Hz), 3.76 (H-4′), 3.76 (H-6b), 3.75 (H-5″), 3.73 (H-6″b), 3.67 (H-4″, dd, *J* = 3 Hz and 10 Hz), 3.61 (H-6′b, dd, *J* = 6 Hz and 12 Hz), 3.28 (H-2′, dd, *J* = 3 Hz and 9 Hz), 3.08 (N–CH_3, s), 3.06 (N–CH_3, s), 2.64 (H-5), 2.06 (Ac, s). **9**: HRESIMS *m/z* 567.2485 $(M+H)^+$ (calcd for $C_{22}H_{39}N_4O_{13}$, 567.2514); δ_H (D_2O + 0.3% CD_3COOD, 500 MHz, acetone δ_H 2.225 as internal reference): 5.37 (H-1, dd, *J* = 5 Hz and 9 Hz), 5.05 (H-1″, d, *J* = 9 Hz), 4.78 (H-1′, d, *J* = 9 Hz), 4.39 (H-2), 4.39 (H-3′, t, *J* = 3 Hz), 4.30 (H-3), 4.29 (H-3″, t, *J* = 3 Hz), 3.95 (H-5′), 3.93 (H-6′a), 3.90 (H-4′), 3.89 (H-4), 3.86 (H-2′), 3.84 (H-6″a), 3.83 (H-6a), 3.82 (H-5″), 3.75 (H-6″b), 3.75 (H-6′b), 3.71 (H-4″), 3.67 (H-6b), 3.32 (H-2″, dd, *J* = 3 Hz and 9 Hz), 2.91 (N–CH_3, s), 2.55 (H-5), 2.05 (Ac, s). Compound **10**: HRESIMS *m/z* 567.2496 $(M+H)^+$ (calcd for $C_{22}H_{39}N_4O_{13}$, 567.2514); δ_H (D_2O + 0.3% CD_3COOD, 500 MHz, acetone δ_H 2.225 as internal reference): 5.35 (H-1), 5.02 (H-1′, d, *J* = 9 Hz), 4.81 (H-1″, d, *J* = 9 Hz), 4.60 (H-3′, t, *J* = 3 Hz), 4.38 (H-2), 4.38 (H-3), 4.06 (H-3″, t, *J* = 3 Hz), 4.01 (H-4), 3.93 (H-5′), 3.91 (H-2″, dd, *J* = 3 Hz and 9 Hz), 3.91 (H-6a), 3.89 (H-6″a), 3.83 (H-6′a, dd, *J* = 3 Hz and 12 Hz), 3.78 (H-4′), 3.78 (H-5″), 3.77 (H-6b), 3.75 (H-6″b), 3.70 (H-4″, dd, *J* = 3 Hz and 10 Hz), 3.63 (H-6′b, dd, *J* = 6 Hz and 12 Hz), 3.27 (H-2′, dd, *J* = 3 Hz and 9 Hz), 2.93 (N–CH_3, s), 2.68 (H-5), 2.07 (Ac, s).

A solution of 4.8 mg of Sulfo-SBED (Pierce, Rockford, IL) in dimethyl sulfoxide (200 μL) was added to a solution of **3** (0.6 mg), **4** (0.45 mg), **9** (2.0 mg), or **10** (2.0 mg) in 1% $NaHCO_3$(aq) (220 μL), and the mixture was stirred for 30 min at room temperature in the dark. Each reaction mixture was purified by reverse-phase HPLC (column: Capcell-Pak C_{18}, 4.6 mm × 250 mm; a gradient elution of 0–80% acetonitrile in 10 mM $AcONH_4$–NH_4OH pH 8.9 over 20 min; flow rate: 1.0 mL/min; detection: 220 nm; retention times of **5** and **6**: 18.2 min and 18.3 min, or column: Capcell-Pak C_{18}, 10 mm × 250 mm; a gradient elution of 0–70% acetonitrile in 10 mM $AcONH_4$–NH_4OH pH 8.9 over 30 min; flow rate: 4.0 mL/min; detection: 270 nm; retention times of **11** and **12**: 25.2 min and 25.4 min) to obtain **5** (0.8 mg), **6** (0.7 mg), **11** (0.5 mg), and **12** (0.8 mg). **5**: HRESIMS *m/z* 1243.4815 $(M+H)^+$ (calcd for $C_{51}H_{79}N_{12}O_{18}S_3$, 1243.4797). **6**: HRESIMS *m/z* 1243.4847 $(M+H)^+$ (calcd for $C_{51}H_{79}N_{12}O_{18}S_3$, 1243.4797). Compound **11**: HRESIMS *m/z* 1229.4593 $(M+H)^+$ (calcd for $C_{50}H_{77}N_{12}O_{18}S_3$, 1229.4641). Compound **12**: HRESIMS *m/z* 1229.4667 $(M+H)^+$ (calcd for $C_{50}H_{77}N_{12}O_{18}S_3$, 1229.4641).

A solution of 4.0 mg of Sulfo-NHS-SS-Biotin (Pierce) in dimethyl sulfoxide (200 μL) was added to an aqueous solution of 2.0 mg of **3**,

4, **9**, or **10** and the mixture was stirred for 30 min at room temperature in the dark. Each reaction mixture was purified using reverse-phase HPLC (column: Capcell-Pak C_{18}, 10 mm × 250 mm; a gradient elution of 0–70% acetonitrile in 10 mM $AcONH_4$–NH_4OH pH 8.9 over 30 min; flow rate: 4.0 mL/min; detection: 215 nm; retention times of **7**, **8**, **13**, and **14**: 22.8 min, 24.4 min, 21.5 min, and 22.2 min) to obtain **7** (0.6 mg), **8** (0.8 mg), **13** (0.5 mg), and **14** (0.8 mg). Compound **7**: HRESIMS *m/z* 970.3580 $(M+H)^+$ (calcd for $C_{38}H_{64}N_7O_{16}S_3$, 970.3572). Compound **8**: HRESIMS *m/z* 970.3585 $(M+H)^+$ (calcd for $C_{38}H_{64}N_7O_{16}S_3$, 970.3572). Compound **13**: HRESIMS *m/z* 956.3463 $(M+H)^+$ (calcd for $C_{37}H_{62}N_7O_{16}S_3$, 956.3415). **14**: HRESIMS *m/z* 956.3451 $(M+H)^+$ (calcd for $C_{37}H_{62}N_7O_{16}S_3$, 956.3415).

3.3. Chitinase assay

The chitinase assay was performed in 0.1 M citric acid–Na_2HPO_4 buffer (pH 7.0) using 4-methylumbelliferyl-*N*,*N*′,*N*″-triacetyl chitotrioside [4MU-$(GlcNAc)_3$] (Sigma–Aldrich, St. Louis, MO) as a substrate according to the method described previously.[8] *Trichoderma* chitinase (Chitinase T-1) was purchased from Takara Shuzo Co., Ltd, Kyoto, Japan.

3.4. Detection of allosamidin binding proteins in BAL fluids using photoaffinity probes

Eight to 10-wk-old male A/J mice received an intratracheal instillation of a solution containing 0.5 μg of recombinant murine IL-13 (Sigma–Aldrich) or phosphate-buffered saline on days 1, 3, and 5. Mice were given a lethal dose of pentobarbital, and lungs were gently lavaged with 1.0 mL of saline via tracheal cannula to obtain the BAL fluids. A BAL fluid containing 5 μg of proteins (10 μL) was mixed with 6 μL of 0.1 M citric acid–0.2 M Na_2HPO_4 buffer (pH 7.0). After incubation at 37 °C for 1 h, an aqueous solution of the probe (84 μL containing 0.068 nM of **5** or **11**) was added to the solution. In the experiments with excess amounts of allosamidin or demethylallosamidin, 68 nM of allosamidin or demethylallosamidin was added to the solution 30 min before the addition of probe **5** or **11**. The reaction solution was further incubated at 37 °C for 1 h and UV-irradiated (365 nm) at 4 °C for 15 min. Acetone (400 μL) was added to the reaction solution and the mixture was kept at −20 °C overnight to afford a precipitate. After collecting the precipitate by centrifugation (6000*g*, 15 min), the precipitate was dissolved in 25 μL of water and the solution was mixed with 25 μL of electrophoresis sample buffer. The mixture was boiled for 5 min and subjected to 10% SDS–PAGE. After SDS–PAGE, proteins on the gel were transferred to a polyvinylidene difluoride (PVDF) membrane (Clear Blot Membrane-p, ATTO, Tokyo, Japan) by electroelution at 100 mA for 60 min (per 75 × 80 mm membrane) with three transfer buffers (300 mM Tris containing 20% methanol and 0.05% SDS, 25 mM Tris containing 20% methanol and 0.05% SDS, and 25 mM Tris containing 40 mM 6-aminohexanoic acid, 20% methanol and 0.05% SDS), which was probed using HRP–avidin (Pierce).

3.5. Protein sequencing

Acetone was added to the BAL fluid and the mixture was kept at −20 °C overnight to afford a precipitate. After collecting the precipitate by centrifugation (6000*g*, 15 min), the precipitate was subjected to 10% SDS–PAGE according to the above-mentioned method. Proteins on the gel were transferred to a PVDF membrane and the PVDF membrane was stained with 0.2% Coomassie Brilliant Blue (CBB) R-250 in methanol for 5 min and bleached in 50% methanol containing 10% acetic acid for several minutes. The band to be analyzed was cut from the membrane and subjected to N-terminal amino acid sequencing using a protein sequencer (Model 492HT, Applied Biosystems, Foster City, CA), affording the sequences of the 45 kDa and 53 kDa proteins.

3.6. RT-PCR and *Sca* I digestion

Lung tissue was obtained from asthma-induced mice that had received ovalbumin or IL-13. RNA was purified using the ISOGEN reagent (Nippon Gene Co., Ltd, Tokyo, Japan) and reverse-transcribed using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) with oligo(dT) according to the manufacturer's protocol. PCR was performed using the forward primer 5′-AGGCTGCTACTCACTTCCAC-3′ and the reverse primer 5′-CTTGCAACTTGCACTGTGTA-3′ for 25 cycles at an annealing temperature of 70 °C. The PCR product was digested with *Sca* I and analyzed by agarose gel electrophoresis.

3.7. Preparation of recombinant Ym1

A plasmid construct with the reading frame encoding mouse Ym1 was generated using a pET28a vector (Novagen, Darmstadt, Germany), which expressed Ym1 as a His-tag fusion protein under the control of a T7 promoter. PCR was performed using the oligonucleotide primers (sense primer 5′-GCCGGATCCTACCAGCTGATGTGCTACTAT-3′ and anti-sense primer 5′-CGCGAATTCAATAAGGGCCCTTGC-3′) flanking the coding region of mouse Ym1 and murine lung cDNA (BD Biosciences-Clontech, Palo Alto, CA) as the template. The PCR fragment obtained was A-tailed and cloned into the plasmid vector pGEM-T Easy (Promega, Madison, WI). The vector was digested with *BamH* I and *EcoR* I, and the fragment containing sequence encoding Ym1 was ligated to the pET28a vector. The construct was transfected into *E. coli* BL21 (DE3), and the Ym1/His-tag fusion protein was expressed with 1 mM IPTG. The cells containing the expressed recombinant protein in the inclusion body were disrupted by sonication in PBS buffer with Complete protease inhibitors (Roche Molecular Biochemicals, Mannheim, Germany), 1 mM EDTA, and 1% Triton X-100. The suspension was centrifuged at 6000*g* for 10 min at 4 °C and the obtained precipitates were resuspended in 20 mM sodium phosphate buffer (pH 7.8) containing 500 mM NaCl and 6 M guanidine–HCl. After centrifuging the suspension at 6000*g* for 10 min at 4 °C, the supernatant was purified using a Ni-NTA agarose column according to the manufacturer's protocol (Invitrogen). The eluted Ym1/His-tag fusion protein was dialyzed as a refolding reaction against 0.1 M Tris buffer (pH 8.0) containing 0.5 M arginine, 2 mM EDTA, 6 mM oxidized glutathione, and 0.6 mM reduced glutathione at 4 °C for 24 h. The remaining solution in the dialysis membrane was centrifuged at 6000*g* for 10 min at 4 °C, and the supernatant was dialyzed again in PBS buffer at 4 °C for 24 h.

3.8. Binding experiments of photoaffinity probes to recombinant Ym1

A solution of recombinant Ym1 (586 ng) in 16 μL of 0.1 M citric acid–0.2 M Na_2HPO_4 buffer (pH 7.0) was mixed with an aqueous solution of the probe (84 μL containing **5** or **11**). The solution was incubated at 37 °C for 1 h and UV-irradiated (365 nm) at 4 °C for 15 min. Detection of the binding of recombinant Ym1 and the probe in the reaction solution was done according to the same method as described above.

Acknowledgment

This work was supported by grants-in aid for scientific research from the Japan Society for the Promotion of Sciences (Nos.

22658037 and 22390167) and by the National Institute of Biomedical Innovation, Japan.

References and notes

1. Sakuda, S.; Isogai, A.; Matsumoto, S.; Suzuki, A.; Koseki, K. *Tetrahedron Lett.* **1986**, *27*, 2475.
2. Henrissat, B. A. *Biochem. J.* **1991**, *280*, 309.
3. Sakuda, S.; Nishimoto, Y.; Ohi, Y.; Watanabe, M.; Takayama, S.; Isogai, A.; Yamada, Y. *Agric. Biol. Chem.* **1990**, *54*, 1333.
4. Vinetz, J. M.; Dave, S. K.; Specht, C. A.; Brameld, K. A.; Xu, B.; Hayward, R.; Fidock, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 14061.
5. Sakuda, S.; Isogai, A.; Matsumoto, S.; Suzuki, A. *Journal. Antibiot.* **1987**, *40*, 296.
6. Takenaka, Y.; Nakano, S.; Tamoi, M.; Sakuda, S.; Fukamizo, T. *Biosci. Biotechnol. Biochem.* **2009**, *73*, 1066.
7. Zhu, Z.; Zheng, T.; Homer, R. J.; Kim, Y. K.; Chen, N. Y.; Cohn, L.; Hamid, Q.; Elias, J. A. *Science* **2004**, *304*, 1678.
8. Suzuki, S.; Nakanishi, E.; Ohira, T.; Kawachi, R.; Nagasawa, H.; Sakuda, S. *Journal. Antibiot.* **2006**, *59*, 402.
9. Suzuki, S.; Nakanishi, E.; Ohira, T.; Kawachi, R.; Ohnishi, Y.; Horinouchi, S.; Nagasawa, H.; Sakuda, S. *Journal. Antibiot.* **2006**, *59*, 410.
10. Suzuki, S.; Nakanishi, E.; Furihata, K.; Miyamoto, K.; Tsujibo, H.; Watanabe, T.; Ohnishi, Y.; Horinouchi, S.; Nagasawa, H.; Sakuda, S. *Int. Journal. Biol. Macromol.* **2008**, *43*, 13.
11. Homer, R. J.; Zhu, Z.; Cohn, L.; Lee, C. G.; White, W. I.; Chen, S.; Elias, J. A. *Am. Journal. Physiol. Lung Cell Mol. Physiol.* **2006**, *291*, L502.
12. Boot, R. G.; Blommaart, E. F. C.; Swart, E.; van der Vlugt, K. G.; Bijl, N.; Moe, C.; Place, A.; Aerts, J. M. F. *Journal. Biol. Chem.* **2001**, *276*, 6770.
13. Reese, T. A.; Liang, H. E.; Tager, A. M.; Luster, A. D.; van Rooijen, N.; Voehringer, D.; Locksley, R. M. *Nature* **2007**, *447*, 92.
14. Matsumoto, T.; Inoue, H.; Sato, Y.; Kita, Y.; Nakano, T.; Noda, N.; Eguchi-Tsuda, M.; Noriwaki, A.; Kan-o, K.; Matsumoto, K.; Shimizu, T.; Nagasawa, H.; Sakuda, S.; Nakanishi, Y. *Biochem. Biophys. Res. Commun.* **2009**, *390*, 103.
15. Sakuda, S.; Sakurada, M. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2987.
16. Kinoshita, M.; Sakuda, S.; Yamada, Y. *Biosci. Biotechnol. Biochem.* **1993**, *57*, 1699.
17. Sakuda, S.; Isogai, A.; Makita, T.; Matsumoto, S.; Koseki, K.; Kodama, H.; Suzuki, A. *Agric. Biol. Chem.* **1987**, *51*, 3251.
18. Owashi, M.; Arita, H.; Hayai, N. *Journal. Biol. Chem.* **2000**, *275*, 1279.
19. Welch, J. S.; Lozach, E.; Sykes, D. B.; Liddiard, K.; Greaves, D. R.; Glass, C. K. *Journal. Biol. Chem.* **2002**, *277*, 42821.
20. Webb, D. C.; McKenzie, A. N. J.; Foster, P. S. *Journal. Biol. Chem.* **2001**, *276*, 41969.
21. Barbour, K. W.; Goodwin, R. L.; Guillonneau, F.; Wang, Y.; Baumann, H.; Gerger, F. G. *Mol. Biol. Evol.* **2002**, *19*, 718.
22. Sutherland, T. E.; Maizels, R. M.; Allen, J. E. *Clin. Exp. Allergy* **2009**, *39*, 943.
23. Chang, N. C. A.; Hung, S. I.; Hwa, K. Y.; Kato, I.; Chen, J. E. G.; Liu, C. H.; Chang, A. C. *Journal. Biol. Chem.* **2001**, *276*, 17497.
24. Houston, D. R.; Recklies, A. D.; Krupa, J. C.; van Aalten, D. M. F. *Journal. Biol. Chem.* **2003**, *278*, 30206.
25. Kumar, J.; Ethayathulla, A. S.; Srivastava, D. B.; Singh, N.; Sharma, S.; Kaur, P.; Srinivasan, A.; Singh, T. P. *Acta Crystallogr., Sect. D* **2007**, *63*, 437.
26. Iwashita, H.; Morita, S.; Sagiya, Y.; Nakanishi, A. *Am. Journal. Respir. Cell Mol. Biol.* **2006**, *35*, 103.
27. Zhao, J.; Zhu, H.; Wong, C. H.; Leung, K. Y.; Wong, W. S. F. *Proteomics* **2005**, *5*, 2799.
28. Lee, C. G.; Elias, J. A. *Allergy Asthma Immunol. Res.* **2010**, *2*, 20.
29. Campos, M. A.; Wanner, A.; Zhang, G.; Sandhaus, R. A. *Chest* **2005**, *128*, 1179.
30. Zhou, Z.; Sakuda, S.; Yamada, Y. *Journal. Chem. Soc., Perkin Trans. 1* **1992**, 1649.