ISOLATION AND CHARACTERIZATION OF NEW ALLOSAMIDINS

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Three of new allosamidins, termed glucoallosamidins A (**5**), B (**6**) and methyl-*N*-demethylallosamidin (**4**), were isolated as yeast chitinase inhibitors from the mycelium of *Streptomyces* sp. SA-684.

Chitin is an ideal target for designing an antifungal agent because of its importance in their cell wall and limited occurrence in nature[1,2]. Among chitin metabolite enzymes, chitin synthase is thought to be essential for fungal growth since its inhibitors like polyoxins or nikkomycins cause growth arrest[2]. On the other hand, chitinase activity is also detected during their growth and it has been speculated that fungal chitinase plays an important role in a hyphal growth and in yeast cell separation at its budding stage[2,3]. Recently, during the course of our screeing for yeast chitinase inhibitors, we found that demethylallosamidin (**2**)[4], which was isolated as a minor component from the mycelium of the allosamidin (**1**)[5~8] producing microbe, strongly inhibits the chitinase of *Saccharomyces cerevisiae* and affects its cell division[9]. This time, our further screening for yeast chitinase inhibitors using a chitinase of *Candida albicans* revealed the presence of three new allosamidin derivatives in a mycelial extract of *Streptomyces* sp. SA-684. In this paper, we report the isolation, characterization and biological activity against fungal chitinases of these new allosamidins, termed glucoallosamidins A (**5**), B (**6**) and methyl-*N*-demethylallosamidin (**4**).

Materials and Methods

General Procedure

NMR spectra were recorded on Bruker AM-600 and Jeol JNM-GSX-400 spectrometers (Faculty of Engineering, Osaka University), using dioxane δ_C 67.4 as an external reference for ^{13}C NMR spectra, and DHO δ_H 4.8 and TMS δ_H 0.0 as internal reference for ^1H NMR spectra at 300 K. Mass spectra were obtained on a Jeol JMS DX-303 spectrometer, CD spectra were recorded on a Jasco J-600 spectropolarimeter and optical rotation values were measured on a Jasco DIP-181 polarimeter. IR spectra were recorded on Jeol JIR-AQS20M spectrometer.

Preparation of Fungal Chitinases

The chitinase from *C. albicans* was prepared following the method of Dickinson *et al.*[10] with slight modifications. In brief, *C. albicans* ATCC 10231 was cultured in a 100-liter jar fermenter containing 50-liter Sabouraud medium for 15 hours at 37°C under aeration (50 liters/minute) and agitation (140 rpm). The cells harvested by centrifugation (250 g wet weight) were disrupted with a Dyno-mill disintegrator (Shinmaru Enterprises Co.) in 1,100 ml of a buffer (50 mM Bis-tris, 0.25 M sucrose, 1 mM EDTA-Na_2, pH 6.5). The cell lysates were centrifuged (9,000 × *g*, 20 minutes), and the supernatant was centrifuged (145,000 × *g*,

1 hour) again. The high speed supernatant was concentrated by ultrafiltration (UP-20, Toyo Roshi) to one-fifth of the volume. The concentrate was stored at 4°C and used in the chitinase assay as the enzyme solution.

The chitinase of *S. cerevisiae* was prepared from cells of baker's yeast (Kaneka Yeast, Kanegafuchi Chemical Ind.) by the method of CABIB *et al.*[11]. Chitinase T-1 from *Trichoderma* sp. AF6-T8 was purchased from Takara Shuzo Co., Ltd.

Chitinase Assay

In the assay of *Saccharomyces* and *Trichoderma* chitinase, 0.15 M sodium citrate (pH 3.0) and 0.1 M citric acid - Na_2HPO_4 (pH 5.2) buffer was used, respectively.

The chitinase assay was performed in the reaction mixture containing the enzyme solution 90 μl and ^{3}H-chitin[12] suspension 10 μl (12 μg chitin in the same buffer as the enzyme was dissolved). An inhibitor or a test sample for screening was previously lyophilized in the reaction tube (1.5 ml Eppendorf tube). After incubation for 2 hours at 37°C and addition of 0.2 ml of 10% TCA to stop the reaction, the reaction mixture was filtered through a glass fiber filter (Whatman GF/B). The filter was washed with 0.2 ml of water and the radioactivity of the combined filtrate was measured. The chitinase activity is given as the value calculated by subtraction of the radioactivity of enzyme minus control.

Production and Isolation of Allosamidins

Streptomyces sp. SA-684 was cultured in a medium consisting of soluble starch 2.0%, glucose 0.5%, malt extract 0.5%, Bacto-peptone 0.5%, yeast extract 0.5% and Bacto-soytone 0.5% in a 300-liter jar fermenter. Fermentation was carried out at 27°C for 66 hours under aeration (38 liters/minute) and agitation (200 rpm).

The mycelial cake (3.6 kg wet weight), harvested from the culture broth (150 liters) by centrifugation, was extracted with MeOH (15 liters) and 80% MeOH (8 liters) successively. The extracts were combined and concentrated to an aqueous solution (500 ml) under reduced pressure. After being diluted 12-fold with water, the solution was applied to a charcoal (Charcoal, Activated, Wako Pure Chemical Ind., Ltd.) column (5.5 × 49 cm). The active fractions, eluted with 50% EtOH (5 liters) and 50% EtOH (pH 3.5 adjusted by AcOH, 5 liters), were combined and concentrated under reduced pressure to remove EtOH and AcOH. After being diluted to 5 liters with water and adjusted to pH 3.5, the solution was applied to SP-Sephadex C-25 column (2 × 54 cm) pre-equilibrated with 50 mM $AcONH_4$ - AcOH (pH 5.0), which was eluted with the same buffer. The active fractions were finally purified by two steps of reverse phase HPLC (column: Capcell-Pak C_{18}, 20 × 250 mm, Shiseido; mobile phase: gradient elution of 0 ~ 50% CH_3CN in 10 mM $AcONH_4$ - NH_4OH pH 8.9 in 30 and 90 minutes for first and second step, respectively; flow rate: 5 ml/minute). Six active peaks were detected at Rt of 20.3, 21.3, 22.2, 23.8, 24.5 and 25.4 minutes (Fig. 2) and each fraction finally afforded 6.6 mg of **2**, 28.3 mg of **6**, 4.1 mg of **4**, 24.1 mg of **1**, 51.2 mg of **5** and 181.2 mg of **3**. Glucoallosamidins A (**5**), B (**6**) and methyl-*N*-demethylallosamidin (**4**) were obtained as white powders; **5**: MP 222 ~ 233°C (dec); IR ν_{max} (KBr) cm^{-1} 3450, 3300, 1660, 1620, 1560; $[\alpha]_D^{13}$ −8.5° (*c* 0.51, 0.1 M AcOH); HRFAB-MS *m/z* 637.2944 $(M+H)^+$ (Calcd for $C_{26}H_{45}N_4O_{14}$: 637.2932); ^{1}H and ^{13}C NMR (see Tables 1 and 2); **6**: MP 212 ~ 230°C (dec); $[\alpha]_D^{14}$ −14.8° (*c* 0.53, 0.1 M AcOH); HRFAB-MS *m/z* 623.2781 $(M+H)^+$ (Calcd for $C_{25}H_{43}N_4O_{14}$: 623.2776); ^{13}C NMR (see Table 1) and **4**: HRFAB-MS *m/z* 623.2781 $(M+H)^+$ (Calcd for $C_{25}H_{43}N_4O_{14}$: 623.2776); ^{13}C NMR (see Table 1).

Mild Acid Hydrolysis of 5

12.6 mg of **5** was dissolved in 3.0 ml of 0.5 N HCl, and the solution was heated at 70°C for 6.5 hours. After being lyophilized, the resulting powder was purified by HPLC using the same conditions as those of the second step HPLC in the isolation procedure of allosamidins. The peak at Rt of 32.8 minutes afforded 2.84 mg of pseudodisaccharide (**7**), FAB-MS *m/z* 420 $(M+H)^+$ (glycerol matrix); ^{1}H NMR (see Table 2).

Preparation of Benzoyl Derivatives of Acid Hydrolysate of 5

5 (3 mg) was hydrolyzed in a sealed tube with 4 N HCl (2 ml) at 100°C for 4 hours. The hydrolysate was *N*-acetylated with acetic anhydride and passed through a Dowex-50 column (H^+ form) to separate

allosamizoline, which was adsorbed on the column and eluted with 1 N HCl. The eluted fraction was treated with 5% HCl-MeOH for conversion to methylglycosides and then perbenzoylated with benzoyl cyanide and tri-*n*-butylamine in CH_3CN. The reaction mixture was purified by HPLC (column: Capcell-Pak C_{18}, 10×250 mm; mobile phase: 30 minutes gradient elution of 0~100% CH_3CN in water) to give methyl 6-*O*-methyl-3,5-*O*-dibenzoyl-2-acetamido-2-deoxyallofuranoside (170 μg), CI-MS *m/z* 458 $(M+H)^+$; ^1H NMR (600 MHz, $CDCl_3$) δ 7.0~8.2 (15H), 5.86 (1H, d, J=9 Hz, NH), 5.79 (1H, dd, $J_{3,4}$=3 Hz and $J_{3,2}$=8 Hz, 3-H), 5.54 (1H, dt, $J_{5,4}$=3 Hz, $J_{5,6a}$=6 Hz and $J_{5,6b}$=6 Hz, 5-H), 4.95 (1H, d, $J_{1,2}$=5 Hz, 1-H), 4.77 (1H, ddd, 2-H), 4.54 (1H, t, 4-H), 3.77 (1H, dd, 6-H_a), 3.73 (1H, dd, 6-H_b), 3.46 (3H, s, O-CH_3), 3.34 (3H, s, O-CH_3), 1.91 (3H, s, Ac); CD (CH_3CN): $\Delta\varepsilon_{242}$= +0.5, $\Delta\varepsilon_{235}$=0, $\Delta\varepsilon_{225}$= −1.7, and methyl 3,4,6-*O*-tribenzoyl-2-acetamido-2-deoxy-α-glucopyranoside (160 μg), CI-MS *m/z* 548 $(M+H)^+$; ^1H NMR (600 MHz, $CDCl_3$) δ 7.0~8.0 (15H), 5.92 (1H, d, J=10 Hz, NH), 5.68 (1H, t, $J_{3,2}$ or $J_{4,5}$=10 Hz and $J_{3,4}$=10 Hz, 3-H or 4-H), 5.65 (1H, t, $J_{3,2}$ or $J_{4,5}$=10 Hz, 3-H or 4-H), 4.86 (1H, d, $J_{1,2}$=4 Hz, 1-H), 4.59 (1H, ddd, 2-H), 4.58 (1H, dd, $J_{6a,5}$=4 Hz, $J_{6a,6b}$=13 Hz, 6-H_a), 4.43 (1H, dd, $J_{6b,5}$=5 Hz, 6-H_b), 4.30 (1H, ddd, $J_{5,4}$=10 Hz, 5-H), 3.49 (3H, s, O-CH_3), 1.57 (3H, s, Ac); CD (CH_3CN): $\Delta\varepsilon_{240}$= −14.3, $\Delta\varepsilon_{229}$=0, $\Delta\varepsilon_{223}$= +5.4. 3,4,6-*O*-Tribenzoyl-allosamizoline (360 μg) was obtained from the allosamizoline fraction, FAB-MS *m/z* 530 $(M+H)^+$ (diethanolamine matrix); CD (CH_3CN): $\Delta\varepsilon_{238}$=

Fig. 1. Isolation procedure of new allosamidins produced by *Streptomyces* sp. SA-684.

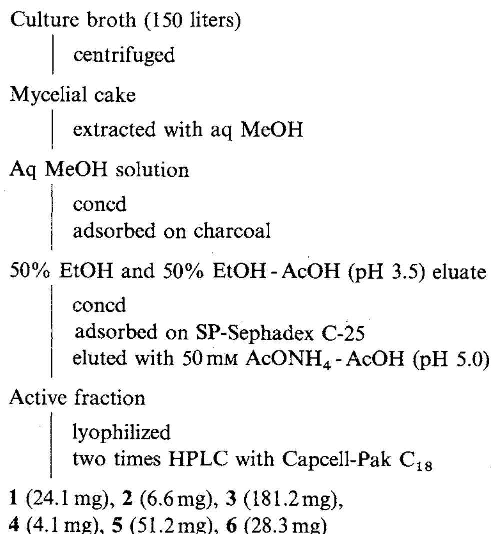
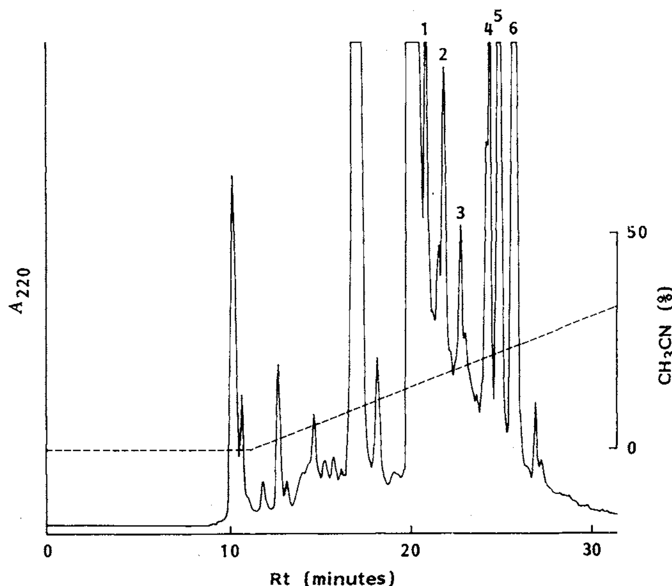


Fig. 2. Purification of allosamidins by HPLC.

Peaks: 1, **2**; 2, **6**; 3, **4**; 4, **1**; 5, **5**; 6, **3**.



HPLC conditions: Column; Capcell-Pak C_{18} (20×250 mm), mobile phase; 30 minutes gradient of 0~50% CH_3CN in 10 mM $AcONH_4$-NH_4OH (pH 8.9), flow rate; 5.0 ml/minute, detector; UV 220 nm, sample; active fractions from SP-Sephadex C-25 column.

-4.8, $\Delta\varepsilon_{231}=0$, $\Delta\varepsilon_{223}=+4.3$.

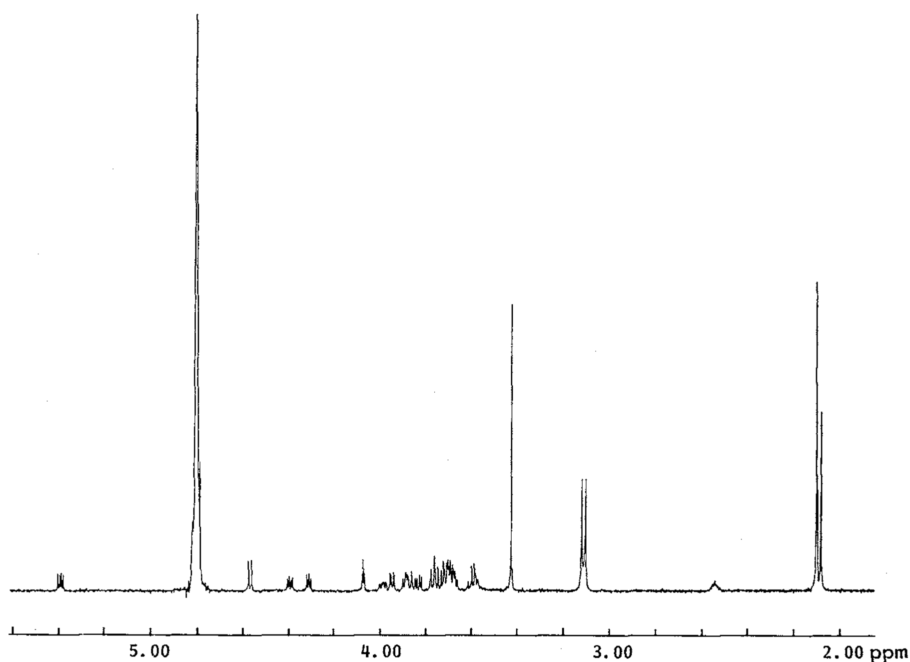
The spectral data of methyl 6-*O*-methyl-3,5-*O*-dibenzoyl-2-acetamido-2-deoxyallofuranoside and 3,4,6-*O*-tribenzoylallosamizoline derived from methylallosamidin, and methyl 3,4,6-*O*-tribenzoyl-2-acetamido-2-deoxy-α-glucopyranoside from authentic *N*-acetyl-D-glucosamine in the same manner were identical with those from **5**.

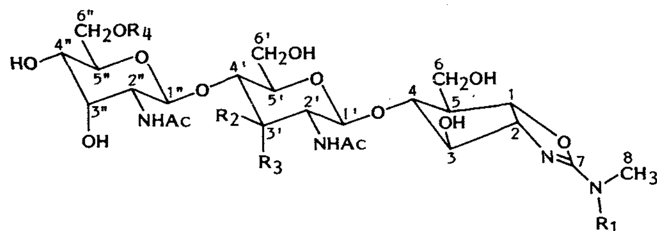
Results

Production and Isolation of New Allosamidins

The length of cultivation time and lower aeration conditions were very important in the production of allosamidins by *Streptomyces* sp. SA-684. The isolation procedure of new allosamidins (summarized in Fig. 1) was very similar to that of allosamidin[5], but the newly used reverse phase HPLC on a basic

Fig. 3. ^{1}H NMR spectrum of **5** (D_2O+CD_3COOD, 600 MHz).



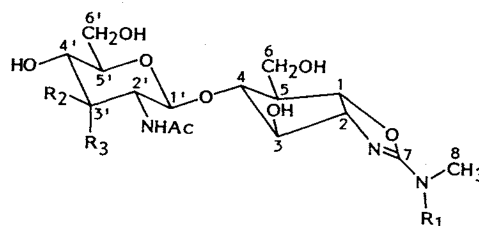


	R_1	R_2	R_3	R_4
Allosamidin (**1**)	CH_3	H	OH	H
Demethylallosamidin (**2**)	H	H	OH	H
Methylallosamidin (**3**)	CH_3	H	OH	CH_3
Methyl-*N*-demethylallosamidin (**4**)	H	H	OH	CH_3
Glucoallosamidin A (**5**)	CH_3	OH	H	CH_3
Glucoallosamidin B (**6**)	H	OH	H	CH_3

condition[4)] was very effective in separating each component (Fig. 2). Methylallosamidin (**3**)[6)] was obtained as a main product and the yield of glucoallosamidin A (**5**), glucoallosamidin B (**6**), allosamidin (**1**), demethylallosamidin (**2**) and methyl-*N*-demethylallosamidin (**4**) were successively smaller.

Structural Elucidation of New Allosamidins

The FAB-MS spectrum of **5** showed a $(M+H)^+$ ion at *m/z* 637, which was the same mass number as that of **3**. The ^1H NMR spectrum of **5** (Fig. 3) was similar to that of **3**, but one of the signals assigned to the 3-H equatorial protons of two *N*-acetylallosamine moiety in **3** was shifted to the complicated region of axial protons in the spectrum of **5**. Analyses of ^1H-^1H and ^{13}C-^1H COSY, and *J*-resolved 2D-NMR spectra of **5** strongly indicated that **5** was the C-3′ epimer of **3**, namely that **5** consisted of 1 mol each of 6-*O*-methyl-*N*-acetylallosamine, *N*-acetylglucosamine and an aminocyclitol derivative termed allosamizoline. To confirm the sequence of these three moieties, **5** was hydrolyzed under mild



	R_1	R_2	R_3
7	CH_3	OH	H
8	CH_3	H	OH

Table 1. ^{13}C NMR assignments of **1**, **2**, **3**, **4**, **5** and **6**[a].

C No.	**1**[b]	**2**[c]	**3**[b]	**4**[h]	**5**[h]	**6**[h]
C-1	87.6	87.7	87.4	87.6	87.1	87.4
C-2	65.4	65.0	64.8	65.0	64.7	64.9
C-3	81.4	80.9	80.9	80.8	81.0	80.7
C-4	85.9	85.6	85.6	85.5	85.5	85.5
C-5	52.4	52.3	52.1	52.3	51.9	52.2
C-6	60.2	60.1	59.7	60.0	59.7	60.0
C-7	161.5	162.4	161.0	162.4	161.2	162.4
C-8	38.4	29.1	38.0	29.0	38.2	29.0
C-9	38.4		38.0		38.0	
C-1′	100.7	100.1	100.3	99.9	102.4	101.9
C-2′	53.4	53.2	53.1[d]	53.2[e]	55.6	55.5
C-3′	69.8	69.7	69.5	69.7	73.1	73.2
C-4′	77.7	77.5	77.4	77.5	80.9	80.9
C-5′	73.4	73.2	73.0[f]	73.2[g]	75.0	75.0
C-6′	61.8	61.6	61.4	61.5	60.9	60.9
C-1″	101.4	101.2	101.1	101.2	100.6	100.6
C-2″	53.7	53.5	53.3[d]	53.4[e]	53.3	53.3
C-3″	70.9	70.7	70.5	70.6	70.6	70.6
C-4″	67.3	67.0	67.1	67.1	67.3	67.3
C-5″	74.4	74.2	72.6[f]	72.7[g]	72.9	72.9
C-6″	61.8	61.6	71.9	72.0	72.1	72.1
NAc(CO)	174.8,	174.6,	174.1,	174.6,	175.1,	175.0,
	174.8	174.4	174.1	174.3	174.5	174.5
$NAc(CH_3)$	22.9,	22.7,	23.0,	22.6,	22.8,	22.8,
	22.9	22.7	22.5	22.6	22.6	22.6
$O-CH_3$			59.2	59.3	59.3	59.3

[a] Chemical shifts are in ppm.
[b] Cited from ref 6.
[c] Cited from ref 4.
[d~g] May be interchanged.
[h] D_2O+CD_3COOD, 600 MHz.

Table 2. ^{1}H NMR assignments of **5** and **7**[a].

H No.	5	7	H No.	5	7
1-H	5.39 dd (5, 9)	5.36 dd (5, 9)	5′-H	3.57 ddd (2, 6, 10)	3.49 ddd (2, 7, 9)
2-H	4.39 dd (4, 9)	4.37 dd (4, 9)	6′-H	3.69 dd (6, 11),	3.72 dd (7, 12),
3-H	4.31 dd (4, 6)	4.29 dd (4, 6)		3.88 dd (2, 12)	3.95 dd (2, 12)
4-H	3.89 dd (6, 8)	3.86 dd (6, 8)	1″-H	4.79 d (9)	
5-H	2.54 m	2.52 m	2″-H	3.95 dd (3, 9)	
6-H	3.70 dd (7, 11),	3.67 dd (7, 11),	3″-H	4.08 dd (3, 3)	
	3.84 dd (5, 11)	3.81 dd (4, 11)	4″-H	3.72 dd (3, 10)	
8-H	3.13 s	3.09 s	5″-H	3.99 ddd (2, 6, 10)	
9-H	3.11 s	3.08 s	6″-H	3.68 dd (6, 11),	
1′-H	4.57 d (8)	4.54 d (8)		3.77 dd (2, 11)	
2′-H	3.77 dd (8, 10)	3.70 dd (8, 10)	NAc	2.08 s, 2.10 s	2.05 s
3′-H	3.72 dd (7, 10)	3.55 dd (9, 10)	OCH_3	3.43 s	
4′-H	3.61 dd (7, 10)	3.42 dd (9, 9)			

[a] Chemical shifts are in ppm ($D_2O + CD_3COOD$, 600 MHz). Coupling constants (J = Hz) are given in parentheses.

Table 3. The inhibitory activity of allosamidins on fungal chitinases.

Chitinase	IC_{50} (μg/ml)[a]							
	1	2	3	4	5	6	7	8
Candida albicans ATCC 10231	6.2	0.7	8.8	0.6	3.4	0.8	1.3	5.7
Saccharomyces cerevisiae	33.8	0.3	37.2	0.4	31.3	0.5	>200	>200
Trichoderma sp. AF6-T8	0.8	0.8	1.2	1.3	0.8	1.6	> 50	> 50

[a] All values are means of triplicate experiments.

conditions (0.5 N HCl, 70°C, 6.5 hours) to give a pseudodisaccharide (**7**) that had one mol each of *N*-acetylglucosamine and allosamizoline. Thus the structure of glucoallosamidin A was determined as **5**. By converting to bezoyl derivatives after acid hydrolysis of **5** and measuring their CD spectra as described in Materials and Methods, it was confirmed that glucosamine moiety of **5** has D configuration and other two parts have the same configurations as those of **3**. The assignments of carbons and protons in the NMR spectra of **5** are summarized in Tables 1 and 2.

The FAB-MS spectrum of **6** showed a $(M+H)^+$ ion at m/z 623, which was 14 mass units smaller than that of **5**. The NMR spectra of **6** were very similar to those of **5** and indicated only one *N*-methyl signal at δ_H 2.93 and δ_C 29.0, while two *N*-methyl signals were observed at δ_H 3.13 and 3.11, and δ_C 38.2 and 38.0 in the spectra of **5** (Table 1). These data indicated that there are the same relation between **5** and **6** as between **1** and **2**. Thus the structure of glucoallosamidin B was determined as **6**. In the same way, the structure of methyl-*N*-demethylallosamidin was assigned as **4**.

Fungal Chitinase Inhibitory Activity of Allosamidins

Chitinase inhibitory activity of **1**~**6**, which are all of hitherto known allosamidins, were tested against three fungal chitinases from *S. cerevisiae*, *C. albicans* and *Trichoderma* sp. The resulting IC_{50} values are listed in Table 3. Allosamidins having a *N*-monomethyl group, **2**, **4** and **6**, show much stronger activity against two yeast chitinases than **1**, **3** and **5** that have a *N*-dimethyl group, but against *Trichoderma* chitinase all allosamidins show about the same activity. It seems that the stereochemistry of C-3′ or the presence of *O*-methyl group at C-6″ affects the activity a little depending on the origins of the chitinases. The activity of two pseudodisaccharide, **7** and **8**[6] derived from **5** and **1**, respectively, were also tested. It

is very interesting that they showed strong inhibitory activity against *Candida* chitinase, while showed no activity against the other two chitinases under the tested conditions. Biological activity of allosamidins *in vivo* against fungi will be reported elsewhere.

Discussion

Allosamidin (**1**) consists of two characteristic moieties, *N*-acetylallosamine and allosamizoline. In this study, we first isolated a new type of allosamidin in which one *N*-acetylallosamine linked to the allosamizoline moiety is replaced by *N*-acetylglucosamine. Glucoallosamidins A (**5**) and B (**6**) showed about the same inhibitory activity against fungal chitinases as methylallosamidin (**3**) and methyl-*N*-demethylallosamidin (**4**), respectively, indicating the stereochemistry of C-3′ is not essential for the activity. The pseudodisaccharide, **7** and **8** inhibit only *Candida* chitinase as far as tested at this time, but it was reported that **8** inhibited the insect chitinase from *Bombyx mori* as strongly as **1**[4]. These activities of **7** and **8** suggest that analogs of the pseudodisaccharide consisting of certain aminosugars and an allosamizoline, or its derivatives, should be possible inhibitors against many kinds of chitinases.

References

1) CABIB, E.: The synthesis and degradation of chitin. Adv. Enzymol. 59: 59~101, 1987
2) GOODAY, G. W.: Chitin metabolism as a target for antifungal and antiparasitic drugs and agrochemicals. Prog. Ind. Microbiol. 27: 139~150, 1989
3) ELANGO, N.; J. U. CORREA & E. CABIB: Secretory character of yeast chitinase. J. Biol. Chem. 257: 1398~1400, 1982
4) ISOGAI, A.; M. SATO, S. SAKUDA, J. NAKAYAMA & A. SUZUKI: Structure of demethylallosamidin as an insect chitinase inhibitor. Agric. Biol. Chem. 53: 2825~2826, 1989
5) SAKUDA, S.; A. ISOGAI, S. MATSUMOTO & A. SUZUKI: Search for microbial insect growth regulators. II. Allosamidin, a novel insect chitinase inhibitor. J. Antibiotics 40: 296~300, 1987
6) SAKUDA, S.; A. ISOGAI, T. MAKITA, S. MATSUMOTO, K. KOSEKI, H. KODAMA & A. SUZUKI: Structures of allosamidins, novel insect chitinase inhibitors, produced by actinomycetes. Agric. Biol. Chem. 51: 3251~3259, 1987
7) SAKUDA, S.; A. ISOGAI, S. MATSUMOTO, A. SUZUKI, K. KOSEKI, H. KODAMA & Y. YAMADA: Absolute configuration of allosamizoline, an aminocyclitol derivative of the chitinase inhibitor allosamidin. Agric. Biol. Chem. 52: 1615~1617, 1988
8) KOGA, D.; A. ISOGAI, S. SAKUDA, S. MATSUMOTO, A. SUZUKI, S. KIMURA & A. IDE: Specific inhibition of *Bombyx mori* chitinase by allosamidin. Agric. Biol. Chem. 51: 471~476, 1987
9) SAKUDA, S.; Y. NISHIMOTO, M. OHI, M. WATANABE, S. TAKAYAMA, A. ISOGAI & Y. YAMADA: Effects of demethylallosamidin, a potent yeast chitinase inhibitor, on the cell division of yeast. Agric. Biol. Chem. 54: 1333~1335, 1990
10) DICKINSON, K.; V. KEER, C. A. HICHICOCK, & D. J. ADAMS: Chitinase activity from *Candida albicans* and its inhibition by allosamidin. J. Gen. Microbiol. 135: 1417~1421, 1989
11) CORREA, J. U.; N. ELANGO, I. POLACHECK & E. CABIB: Endochitinase, a mannan-associated enzyme from *Saccharomyces cerevisiae*. J. Biol. Chem. 257: 1392~1397, 1982
12) MOLANO, J.; A. DURAN & E. CABIB: A rapid and sensitive assay for chitinase using tritiated chitin. Anal. Biochem. 83: 648~656, 1977