BIOSYNTHETIC STUDIES OF ALLOSAMIDIN

2. ISOLATION OF DIDEMETHYLALLOSAMIDIN, AND CONVERSION EXPERIMENTS OF ^{14}C-LABELED DEMETHYLALLOSAMIDIN, DIDEMETHYLALLOSAMIDIN AND THEIR RELATED COMPOUNDS

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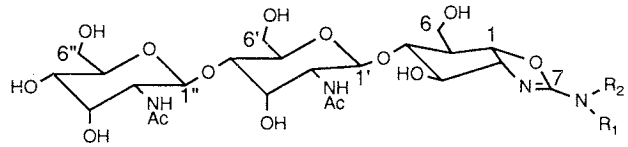
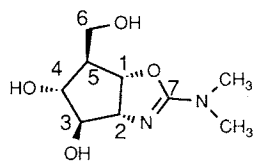
A new allosamidin analog, termed didemethylallosamidin (**3**), was isolated from the mycelia of *Streptomyces* sp. AJ 9463 which is a producer of allosamidin (**1**) and demethylallosamidin (**2**). ^{14}C-Labeled **1**, **2** and **3** as well as their related compounds, **4**, **5**, and **6**, were prepared to investigate the biosynthesis of **1**. Conversion experiments with the labeled allosamidins revealed that **2** was a precursor of **1**, but **3** was not incorporated. This suggests that the first *N*-methyl group is introduced before the cyclization of the aminooxazoline ring during the biosynthesis of **1**. Although none of the compounds **4**, **5**, and **6** were converted to **1**, the production of **1** was inhibited by the addition of **4**.

Allosamidin (**1**) is the first chitinase inhibitor to be isolated from the mycelia of a *Streptomyces* sp.[1] It has a unique trisaccharide structure consisting of two units of *N*-acetyl-D-allosamine and one unit of a novel aminocyclitol derivative, named allosamizoline (**7**).[2~4] *N*-Acetyl-D-allosamine hitherto unknown in nature is a C-3 epimer of *N*-acetyl-D-glucosamine. Allosamizoline has a novel cyclopentanoid structure which is fused with a dimethylaminooxazoline ring. Because of the unique structure and interesting biological activities of **1** against chitin-containing organisms, such as insects and fungi,[1,5~10] its chemistry has recently been developed, especially in synthetic studies.[11~16] Concerning the biosynthesis of **1**, we reported the origin of the carbon and nitrogen atoms of **1** following feeding experiments with ^{13}C- and ^{15}N-labeled precursors.[17]



1 $R_1=R_2=CH_3$ **2** $R_1=CH_3$, $R_2=H$ **3** $R_1=R_2=H$



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Demethylallosamidin (**2**), a demethyl derivative of **1**, was isolated as a minor component from mycelia of an allosamidin-producing microbe.[18] This time, our screening search for a biosynthetic intermediate of **1** revealed the presence of a new allosamidin derivative in the mycelia of *Streptomyces* sp. AJ 9463 which is a high producer of **1** and **2**. Its structure was determined to be a didemethyl derivative of **1** named didemethylallosamidin (**3**). The *N*-methylation steps involved in the biosynthesis of **1** are very important because the number of the methyl groups on the aminooxazoline moiety strongly affects the inhibitory activities of allosamidins depending on the origins of the chitinases.[6,19] As both **2** and **3** are likely intermediates in the *N*-methylation steps, we attempted conversion experiments with ^{14}C-labeled **1**, **2** and **3**, and also with their related compounds **4**, **5** and **6**. In this paper, we describe the isolation and characterization of **3** as well as the conversion experiments with the labeled compounds, which suggest that **2** is a biosynthetic intermediate of **1**, but **3** is not.

Materials and Methods

General Procedure

NMR spectra were recorded on Bruker AM-600 and JEOL JNM-GSX400 spectrometers at 25°C, using dioxane δ_C 67.4 as an external reference for ^{13}C NMR spectra and DHO δ_H 4.8 as internal reference for ^{1}H NMR spectra. Mass spectra were obtained on a JEOL JMS-DX303 spectrometer. [*guanidino*-^{14}C]-L-Arginine was purchased from NEN Research Products. Radioactivity was measured by scintillation counting with a Beckman LS 6000 IC instrument and by a Beckman HPLC system equipped 171-RI detector.

Isolation of Didemethylallosamidin

Streptomyces sp. AJ 9463 was cultured in the medium consisting of glucose 1.5%, peptone 0.2%, meat extract 0.5% and yeast extract 0.1% (pH 7.2) in a 2000-liter jar fermentor. Fermentation was carried out at 28°C for 97 hours under aeration (500 liters/minute) and agitation (245 rpm). The mycelial cake obtained by filtration from the culture broth (1,000 liters) was extracted with 80% aqueous methanol (40 liters). The extract was concentrated and then adsorbed onto a charcoal (7 liters, activated charcoal, Wako pure chemical Ind.) column. After being washed with water (2.1 liters), the column was eluted successively with 10% EtOH (35 liters), 25% EtOH (35 liters) and 50% EtOH (35 liters). The 50% EtOH eluate was concentrated to remove EtOH. After being adjusted to pH 3.5 with acetic acid, the solution was applied to a SP-Sephadex C-25 column (350 ml) pre-equilibrated with 50 mM $AcONH_4$-AcOH (pH 5.0) and the column was eluted with the same buffer. Demethylallosamidin (**2**) was eluted from the column a little later than allosamidin (**1**). The allosamidin fractions were combined, concentrated and adjusted to pH 9.0 with NH_4OH. The white crystalline powder of allosamidin produced was collected by filtration and the mother liquor was further purified by preparative HPLC (column: Capcell Pak C_{18}, 20×250 mm, Shiseido; mobile phase: gradient elution of 0~40% CH_3CN in 10 mM $AcONH_4$-NH_4OH pH 8.9 in 60 minutes; flow rate: 10 ml/minute). The peak having a retention time 14.1 minutes afforded 35 mg of didemethylallosamidin (**3**) as a white powder; **3**: MP >231°C (dec); $[\alpha]_D^{13}$ −29.2° (*c* 0.16, 0.1 M AcOH); HRFAB-MS (glycerol matrix) *m/z* 595.2458 $(M+H)^+$ (Calcd for $C_{23}H_{39}O_{14}N_4$: 595.2462); ^{1}H and ^{13}C NMR (see Table 1). Signals were assigned by analogy with those of **1**[3] as well as ^{1}H-^{1}H COSY and DEPT experiments.

Preparation of ^{14}C-Labeled Compounds

Both [7-^{14}C]-**1** and [7-^{14}C]-**2** were prepared by adding [*guanidino*-^{14}C]-L-arginine to a culture of *Streptomyces* sp. AJ 9463. Culture, administration of a labeled compound, and isolation of **1** and **2** were performed with the procedure as previously described.[17] By the addition of 0.62 MBq of [*guanidino*-^{14}C]-arginine into each 500-ml flask containing 100 ml Bennet medium, 5.6×10^{-2} MBq of [7-^{14}C]-**1** and 4.4×10^{-2} MBq of [7-^{14}C]-**2** were obtained from the broth (100 ml×3).

In order to prepare [7-^{14}C]-**3**, **5** and **6**, 1.7×10^{-2} MBq (0.7 mg) of [7-^{14}C]-**2** was mixed with 4.3 mg

of non-labeled **2** and 3 ml of 28% aqueous ammonia, and the mixture was treated at 121°C for 40 minutes in a sealed tube.[19] The reaction solution was evaporated under reduced pressure and the residue was purified by HPLC (column: Cepcell Pak C_{18}, 4.6×250 mm, Shiseido; mobile phase: gradient elution of 0~40% CH_3CN in 10 mM $AcONH_4$-NH_4OH (pH 8.9) in 30 minutes; flow rate: 1 ml/minute). The peaks having retention times of 4.5, 6.9 and 12.9 minutes afforded 6.4×10^{-4} MBq of **6**, 1.8×10^{-3} MBq of **5** and 2.5×10^{-3} MBq of [7-^{14}C]-**3**. By the same procedure, 1.3×10^{-2} MBq of **4**, which had the retention time of 10.7 minutes on the HPLC, was obtained from the mixture of 4.5×10^{-2} MBq (2.4 mg) of [7-^{14}C]-**1** and 2.6 mg of non-labeled **1**. The characterization of non-labeled **4** was described in a separate paper.[19] FAB-MS spectra of non-labeled **5** and **6** showed $(M+H)^+$ ion at *m/z* 627 and 613, which were smaller than that of non-labeled **4** by 14 and 28 mass units, respectively. ^{1}H NMR spectra of them were almost the same as that of non-labeled **4** except that a signal of one (δ_H 2.73) and no *N*-methyl group was observed in spectra of non-labeled **5** and **6**, respectively, against two *N*-methyl signals in the spectrum of non-labeled **4**.

Conversion Experiments of ^{14}C-Labeled Compounds

The ^{14}C-labeled compound was dissolved in distilled water and the solution was passed through a sterile Millipore filter before addition. The solution was added to each 100-ml flask containing the medium of 40 ml at the 36th hour of cultivation. After 120 hours cultivation, mycelia obtained was extracted with 50 ml of 80% methanol. The radioactivity of this solution was defined as that incorporated into the cell. The extract was chromatographed on a charcoal column (5 ml). After being washed with water (15 ml), the column was eluted successively with 10% EtOH (15 ml) and 50% EtOH (50 ml). The 50% EtOH fraction was concentrated and dissolved in 200 μl of 0.1 N acetic acid. A half of this solution was directly analyzed by HPLC under the same conditions as ^{14}C-labeled compounds were prepared. In the conversion experiments, 1.1×10^{-2} MBq (0.6 mg) of [7-^{14}C]-**1**, 2.7×10^{-2} MBq (1.0 mg) of [7-^{14}C]-**2**, 2.45×10^{-3} MBq (1.1 mg) of [7-^{14}C]-**3**, 1.0×10^{-2} MBq (1.5 mg) of **4**, 1.8×10^{-3} MBq (0.8 mg) of **5** and 6.4×10^{-4} MBq (0.3 mg) of **6** was fed to the culture, and 58, 54, 49, 34, 39 and 78% of the radioactivity was incorporated into the cell, respectively. The resulting chromatograms obtained by the conversion experiments were shown in Fig. 2.

Results and Discussion

Isolation and Characterization of Didemethylallosamidin

Didemethylallosamidin (**3**) was isolated from the mycelial extracts of *Streptomyces* sp. AJ 9463. The isolation procedure of **3** was almost the same as that of **1** because **3** was found in the mother liquor of **1**. After crystallizing **1**, the mother liquor was further purified by reverse phase HPLC under basic conditions to afford **3**. The yields of **1**, **2** and **3** were typically 7.0, 3.5 and 0.04 mg/liter, respectively.

The FAB-MS spectrum of **3** showed a $(M+H)^+$ ion at *m/z* 595 and its molecular formula was determined as $C_{23}H_{38}N_4O_{14}$ by its high resolution

Table 1. ^{13}C and ^{1}H NMR assignments of **3**[a].

Carbon No.	C	H
1	87.9 d	5.40 dd (5, 9)
2	65.0 d	4.40 dd (4, 9)
3	80.9 d	4.30 dd (4, 5)
4	85.6 d	3.96 dd (5, 7)
5	52.1 d	2.59 m
6	60.3 t	3.70 dd (7, 12), 3.81 dd (5, 12)
7	163.1 s	
1′	100.1 d	4.81 d (9)
2′	53.3 d	3.89 dd (3, 9)
3′	69.8 d	4.37 t (3)
4′	77.6 d	3.76 dd (3, 10)
5′	73.4 d	3.92 ddd (2, 7, 10)
6′	61.8 t	3.65 dd (7, 12), 3.83 dd (2, 12)
1″	101.3 d	4.83 d (9)
2″	53.6 d	3.91 dd (3, 9)
3″	70.9 d	4.08 t (3)
4″	67.2 d	3.71 dd (3, 10)
5″	74.4 d	3.78 ddd (2, 5, 10)
6″	61.8 t	3.75 dd (5, 12), 3.89 dd (2, 12)
NAc (C=O)	174.6 s	
	174.7 s	
(CH_3)	22.8 q	2.07 s
	22.8 q	2.10 s

[a] Measured in D_2O+0.3% CD_3COOD at 25°C; chemical shifts are given in ppm, coupling constants in Hz are given in parentheses.

spectrum. The molecular weight of **3** was 28 mass units (C_2H_4) smaller than that of **1**. The ^1H NMR and ^{13}C NMR spectra of **3** were quite similar to those of **1**, except that the *N*-dimethyl signals at δ_H 3.14 and δ_C 38.4 in the spectra of **1** were not observed in those of **3** (Table 1). These data indicated that didemethylallosamidin had the structure of **3**. It shows weaker chitinase inhibitory activities than **1** and are described in a separate paper in detail.[19]

Preparation of ^{14}C-Labeled Compounds for Conversion Experiments

As the quaternary carbon atom of the aminooxazoline moiety of **1** originated from the guanidino group of L-arginine,[17] [7-^{14}C]-**1** and **2** were prepared by feeding experiments with [*guanidino*-^{14}C]-L-arginine. The amount of [7-^{14}C]-**3** obtained by this method was too small for conversion experiments, so adequate amounts of **3** were chemically prepared as follows.

It was known that the reaction of **1** or **2** with ammonia water afforded **3** at high temperature in a sealed tube, and that in the reaction, **2** gave a higher yield of **3** than **1**.[19] Therefore, [7-^{14}C]-**2** obtained above was treated with ammonia water to give [7-^{14}C]-**3**. Compounds **5** and **6** were also formed in this reaction as by-products. Compound **6** was thought to be produced *via* [7-^{14}C]-**3** once formed during the reaction. Compound **4** was obtained from [7-^{14}C]-**1** similarly. These reactions are summarized in Fig. 1.

Conversion Experiments of ^{14}C-Labeled Compounds

Conversion experiments of the ^{14}C-labeled compounds were performed with *in vivo* systems using *Streptomyces* sp. AJ 9463.[17] Cultivation was carried out in a 100-ml Erlenmeyer flask, and labeled compounds were added to the culture at 36 hours of cultivation. After 120 hours cultivation, mycelia were extracted with aqueous methanol and the extract was chromatographed on charcoal. The fraction eluted with 50% ethanol was concentrated and directly analyzed by HPLC.

Conversion experiments with [7-^{14}C]-**1**, -**2** and -**3** were carried out first. In all cases, much radioactivity was incorporated into the cells. Fig. 2 showed the resulting HPLC patterns. In the case of the experiment with [7-^{14}C]-**2**, 75% of the radioactivity in the cells was associated with the peak of **1** (Fig. 2b), indicating

Fig. 1. Preparation of [7-^{14}C]-**3**, and compounds **4**, **5** and **6**.

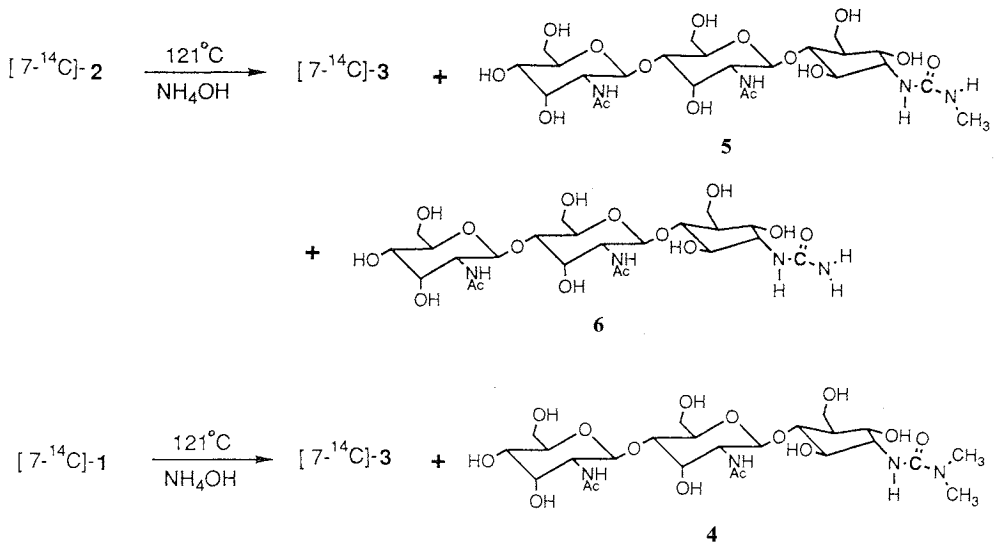
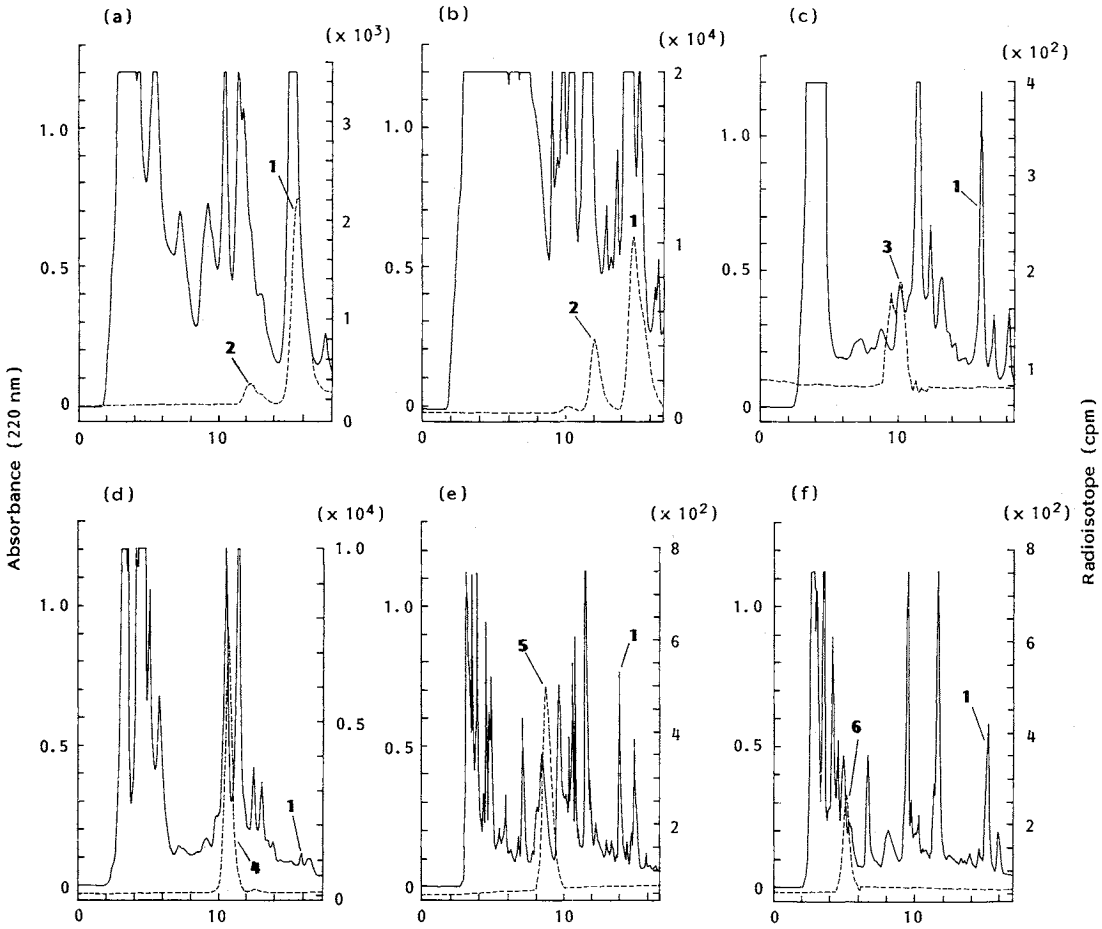


Fig. 2. Conversion experiments of ^{14}C-labeled compounds.

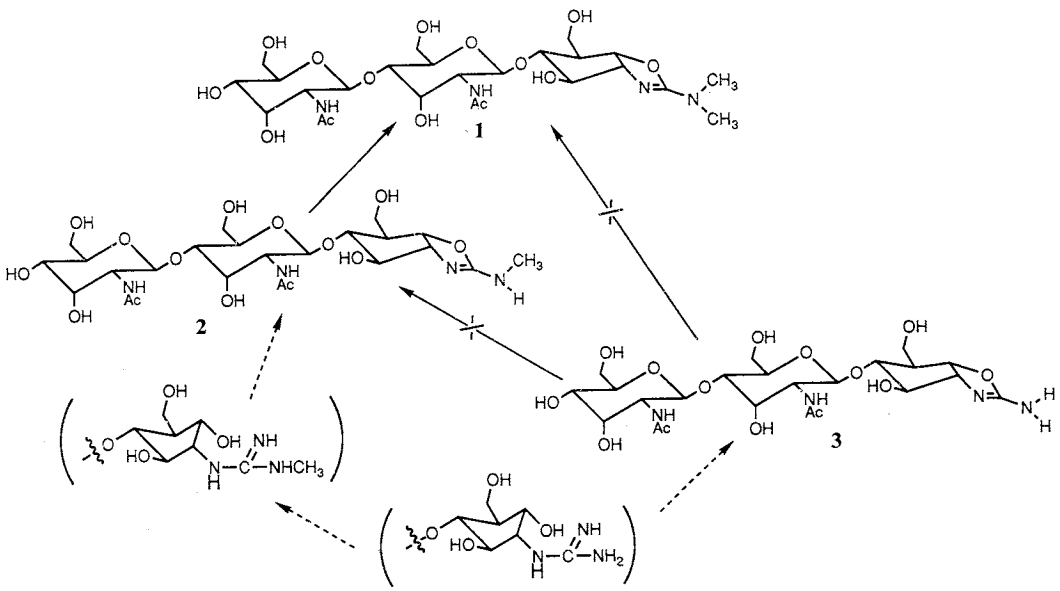


HPLC patterns resulted from the conversion experiments of [7-^{14}C]-**1** (a), [7-^{14}C]-**2** (b), [7-^{14}C]-**3** (c), **4** (d), **5** (e), and **6** (f). Solid line: absorbance at 220 nm; dotted line: radioactivity. HPLC conditions and samples: described in Materials and Methods. UV and RI detectors were connected in this order.

that **2** was efficiently converted to **1**. On the other hand, when [7-^{14}C]-**1** was fed, more than 90% of the radioactivity in the cells was retained in the peak of **1** (Fig. 2a). Unexpectedly, conversion of [7-^{14}C]-**3** to **1** or **2** was not observed at all (Fig. 2c). These results strongly indicated that **2** was a biosynthetic intermediate of **1**, but **3** was not.

Conversion experiments with compounds **4**, **5** and **6** were next carried out. A compound having a ureido group at C-2 was presumed to be a candidate for a precursor just before the formation of the oxazoline ring. However none of them were converted to **1**, **2** or **3** in spite of high incorporation of radioactivity into the cells being detected in all cases (Fig. 2d, 2e and 2f). Interestingly, it was observed that the production of **1** was inhibited by the addition of **4** (Fig. 2d) whose concentration was 36 μg/ml in the experiment. This finding was confirmed by the addition of non-labeled **4** to the cultures at a concentration of 10 or 100 μg/ml. As a result, the amount of **1** produced was reduced by 71% and 93%, respectively. This inhibitory activity of **4** suggested that a compound structurally similar to **4** might be present as a biosynthetic precursor of **1**. It has not been verified whether **5** or **6** are also inhibitors of the

Fig. 3. Plausible biosynthetic pathway of **1**, **2** and **3**.



production of **1**, but it seems that both of them have a similar inhibitory activity because the concentration of **5** and **6** used in the conversion experiments was 20 and 7 μg/ml, respectively, and each of the peak of **1** in Fig. 2e and 2f became smaller than that in Fig. 2c which roughly corresponded to the amount of **1** usually produced.

The results obtained here are summarized in Fig. 3. By the conversion experiment from **2** to **1**, the second *N*-methylation was proved to be the final step of the biosynthesis of **1**. Since **3** was not converted to **1**, the first *N*-methyl group of **1** may be introduced before the cyclization to the aminooxazoline ring. Considering the fact that compound **4** inhibited the production of **1**, for example, it is assumed that an intermediate having a guanidino group at C-2 could accept a methyl group and then cyclize leading to **2**. In the case of the biosynthesis of **3**, an oxazoline ring would be formed before the methylation as shown in Fig. 3.

Acknowledgments

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