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A new fermentation process allows large-scale production of tetra-*N*-acetyl-chitotetraosyl allosamizoline

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Abstract
A new compound **2**, possessing a tetra-*N*-acetyl-chitotetraosyl moiety as a constituent, was synthesized by bacterial fermentation, which used allosamizoline **1** as the initial acceptor. A **2**-binding chitinase assay, indicated that the chitinase was inactivated by **2** with $IC_{50} = 0.03\ \mu g/mL$.

Keywords: *Tetra*-N-*acetyl-chitotetraosyl allosamizoline, fermentation, allosamidin, chitinase, inhibition*

Introduction

Chitooligosaccharides (COS) have been attracting a keen interest in their utilization because they have been reported to possess physiological activities such as antitumor activity [1] and elicitor activity for plants [2]. It is considered that the greatest physiological activities are shown by COS with a degree of polymerization (d.p.) greater than the chitopentaose [1]. However, chitinases and other enzymes rapidly cleave COS. Therefore, improving the stability of the active COS is the key to developing a useful biomedicine.

Allosamidin, a *Streptomyces* metabolite, is an inhibitor of family 18 chitinases and has a pseudo-trisaccharide structure consisting of two units of *N*-acetyl-D-allosamine and one unit of an aminocyclitol derivative, allosamizoline **1** [3]. The allosamidin was synthesized mainly by chemical methods [4–6].

The use of whole cells for the biotransformation of organic substrates is a good technique, which has a number of benefits over conventional, reagent-based methods. With the aim of improving the stability of active COS, we describe here the successful synthesis of a new compound **2** (Scheme 1) by bacterial fermentation. Compound 2 is an analogue of tetra-*N*-acetyl-chitotetraose with one unit of an allosamizoline **1** at the reducing end as the aglycone.

Materials and methods

General methods

Kieselgel $60F_{254}$ (Merck) was used for TLC. Compounds were visualized using UV light and charring with 1:4 H_2SO_4–ethanol. Flash column chromatography was performed on a column of silica gel (Baker, 0.063–0.200 nm). The size exclusion chromatography was performed on Biogel P4. Optical rotations were determined at 25°C with a Perkin–Elmer Model 241-Mc automatic polarimeter. ^1H NMR spectra were recorded using a Bruker DPX-300 spectrometer at 300 MHz, respectively. Mass spectra were recorded with a VG PLATFORM mass spectrometer using the ESI mode. The chitinase from *Serratia marcescens* and allosamidin were purchased from Sigma.

Bacterial strains, plasmids and growth conditions

Cell density cultures were carried out in a 10L batch containing an initial culture volume of 4L.

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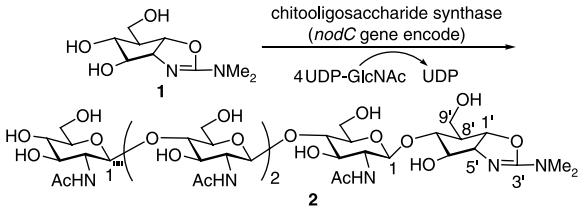

Scheme 1. Chemo-enzymatic synthesis of tetra-*N*-acetyl-chitotetraosyl allosamizoline **2**.

The *Escherichia coli* strain BL21(DE3) containing a plasmid carrying the cloned *nodC* gene from *M. loti* strain E1R [7] was used as the source of NodC protein. Routine cultures were grown in LB medium. Cell density cultures were carried out as previously described [8–10]. The culture medium was slightly modified and had the following composition: **1** [11] (1 g/L), glycerol (15 g/L), $NH_4H_2PO_4$ (7 g/L), KH_2PO_4 (7 g/L), $MgSO_4{\cdot}7H_2O$ (1 g/L), thiamine·HCl (4.5 mg/L), trace mineral solution (7.5 mL/L), citric acid (0.5 g/L), KOH (2 g/L). $MgSO_4$ was added from a concentrated solution that was autoclaved separately, thiamine was sterilized by filtration and the trace mineral stock solution contained: nitrilotriacetate (70 mmol/L, pH 6.5), ferric citrate (7.5 g/L), $MnCl_2{\cdot}4H_2O$ (1.3 g/L), $CoCl_2{\cdot}6H_2O$ (0.21 g/L), $CuCl_2{\cdot}2H_2O$ (0.13 g/L), H_3BO_3 (0.25 g/L), $ZnSO_4{\cdot}7H_2O$ (1.2 g/L) and $Na_2MoO_4{\cdot}2H_2O$ (0.15 g/L). For the cultivation of strain BL21(DE3), the medium was supplemented with leucine (1 g/L). The antibiotic ampicillin was used to ensure maintenance of the plasmid and was prepared in concentrated stock solution. Its final concentration was 50 mg/L for ampicillin. Unless otherwise indicated, the feeding solution contained: glycerol (450 g/L), $MgSO_4{\cdot}7H_2O$ (12 g/L) and trace mineral solution (25 mL/L).

Cell density cultures were inoculated at 2% (v/v) with a culture grown in LB medium. Throughout the cultivation, the dissolved oxygen was maintained at 20% of air saturation by manually increasing the air flow rate and automatically adjusting the stirrer speed, the pH was regulated at 6.8 by automatic addition of aqueous NH_3 (15% w/v) and the temperature was maintained at 34°C. After consumption of the initial glycerol, indicated by a sudden increase in the dissolved oxygen level, the feeding was started with an initial flow rate of 9 mL/h/L. After 5 h of cultivation, the feeding rate was lowered to 4.8mL/h/L and kept constant until the end of the culture.

Purification of compound 2

Bacterial cells from the above culture medium were eliminated by centrifugation (20 min, 12000 × *g*), resuspended in a volume of distilled water equal to that of the original culture medium and disrupted by boiling for 45 min. After cooling, 10μL concentrated HCl was added. Cell debris and precipitated proteins were eliminated by centrifugation (30 min, 12000 × *g*) and the 1L supernatant was mixed with an equal quantity (125 g) of activated charcoal (Norit) and Celite. The slurry was filtered on Whatman no. 4 paper and washed thoroughly with distilled water to remove the salts. The adsorbed oligosaccharides were eluted with 1.3L of 55% (v/v) aq EtOH. After the EtOH soln was concentrated, it was loaded onto a 95 × 4.5-cm column of Biogel P4. The flowthrough fraction (60 mL) was recovered and freeze dried. Compound **2** (1.80 g, 43%) was recovered as a solid material: $[\alpha]_D$ − 14.2 (*c* 0.42, 0.1M AcOH). ^{1}H NMR (300 MHz, D_2O): δ 5.30 (dd, 1H, H-1′), 4.85 (m, 4H, H-1, H-1″, H-1″, H-1″), 4.53 (dd, 1H, H-5′), 4.50 (dd, 1H, H-6′), 4.45–4.14 (m, 4H, H-3, H-3″, H-3″, H-3″), 4.05–3.70 (m, 23H), 3.17, 3.14 (s, 6H, NMe_2), 2.40 (m, 1H, H-8′), 2.14, 2.10 (each s, 12H, CH_3 NHAc). ESIMS: *m/z* 1051.0 $[M + Na]^+$. Anal. Calcd for $C_{41}H_{68}N_6O_{24}$: C, 47.86; H, 6.66; N, 8.17. Found: C, 48.00; H, 6.39; N, 8.01%.

Assay for chitinase activity

The chitinase activity was measured by determining the amount of reducing sugar equivalents released on incubation of the enzyme with the corresponding substrates under the condition stated [12–13]. Assays were terminated by heating to 100°C, and the release of reducing sugars was shown to be linear with respect to time and amount of enzyme used. The chitinase (40 μg/mL), BSA (0.8 mg/mL), glycerol 10% (v/v), and **2** (or allosamidin, 20 mmol/L) were incubated at 18°C in 40 mmol/L sodium acetate buffer at pH5.0. The residual activity was determined at 100 min intervals.

Results and discussion

*Biosynthesis of tetra-*N*-acetyl-chitotetraosyl allosamizoline (2)*

Bacterial fermentation was carried out in 10L bioreactors containing an initial culture volume of 4L. Compound **1** was added to the culture system. The *E. coli* strain BL21(DE3) containing a plasmid carrying the cloned *nodC* gene from *M. loti* strain E1R [7] was used as the source of NodC protein. The culture time lasted 48 hours.

After centrifugation of the culture broth, chitooligosaccharide was recovered exclusively in the pellet containing the bacterial cells. After disruption of the cells by boiling, cell debris was removed by centrifugation and the chitooligosaccharide was purified by activated charcoal adsorption and aq ethanol (55%, v/v) elution. The crude product was further purified by size exclusion chromatography on

Biogel P4. The ^{1}H NMR and MS data confirmed the identification of the obtained sample as the new compound **2**. The yield was 43%. Compound **1** can enter the cells because the hydrophobic methyl contained has affinity for them. It also indicates that **1** is clearly used as a substrate by NodC in vivo. The most obvious explanation for this phenomenon is that **1** does not influence the binding affinity of NodC for the oligosaccharide intermediate and therefore leads to an elongation with additional GlcNAc units. In our system, the exclusive formation of pentamer is probably due to the fact that the synthesis was carried out in growing *E. coli* cells in which the physiological pool of UDP-GlcNAc is maintained at a high level.

Binding chitinase assay

An X-ray structure of allosamidin bound to hevamine, a plant chitinase/lysozyme, confirms that the *N*-acetyl-D-allosamine units in the inhibitor [14] occupy the same subsites as the *N*-acetyl-D-glucosamine units in the natural substrate [15]. This begs the question as to why nature has employed the rare sugar *N*-acetyl-D-allosamine as a mimic for *N*-acetyl-D-glucosamine as occurs in allosamidin. Although the C-3 hydroxyl group of the terminal nonreducing unit of allosamidin can possibly hydrogen bond to Asn45 helping to enhance binding, the unique configuration of the sugar units in this inhibitor may well serve to modify its selectivity profile (compared to the glucoallosamidins, for example) or, more intriguingly, play a role in lessening the likelihood of the inhibitor being recognized as a substrate by *N*-acetylglucosaminidases and degraded. In the **2**-binding chitinase assay, we found that the chitinase was inactivated by **2** with $IC_{50} = 0.03\ \mu g/mL$ (allosamidin with $IC_{50} = 0.01\ \mu g/mL$), which indicated that introducing one unit of an allosamizoline **1** can improve the binding ability of chitotetraose to fungal chitinase.

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