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SCIENCE DIRECT
Bioorganic & Medicinal Chemistry
Bioorganic & Medicinal Chemistry 14 (2006) 2446–2449

Chemo-enzymatic synthesis of 1,4-oxazepanyl sugar as potent inhibitor of chitinase

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Received 17 October 2005; revised 10 November 2005; accepted 11 November 2005
Available online 29 November 2005

Abstract—*N*-Acetyl glucosamine **1** is selectively converted into **2** without protection of the other hydroxyl groups by allylation of the anomeric alkoxide in *N*,*N*-dimethylformamide containing lithium bromide. We use cell density cultures to produce the allylated derivative of penta-*N*-acetyl-chitopentaose by using **2** as the initial acceptor for the synthesis of **3** in vivo. Upon periodate oxidation, **3** is transferred to **4**. Compound **4** is quickly subjected to sodium borohydride reduction and NH_3 amination, which afforded the target compound **5**. In **5**-binding chitinase assay, it indicates that the chitinase is obviously inactivated by **5** with $IC_{50} = 4.7$ μmol/L.

1. Introduction

Chitooligosaccharides (COS), which are oligosaccharides made up of β-1,4-linked D-glucosamine residues, have attracted much attention as new biomedical materials. COS have been reported to possess physiological activities such as antitumor activity[1] and elicitor activity for plants.[2] There has been considerable intensive research on the efficient production of COS.[3–5] It is considered that the greatest physiological activities are shown by COS with a degree of polymerization (d.p.) greater than the chitopentaose.[1] In principle, the chemical stability of COS should influence their biological activities. Plant chitinases have been studied extensively in the context of plant–pathogen interactions. In fact, chitinases and other enzymes rapidly cleave COS. The acylated degradation products that are either di-, tri- or tetrameric are only weakly active on their respective hosts.[6] Therefore, improving the stability of the active COS is the key to developing the biomedicines of COS.

The inhibitors of glycosidases have generally been designed to mimic the charge and/or the shape of the oxocarbenium ion-like transition-state or intermediate:[6] incorporating a basic nitrogen center or a positive charge at or adjacent to C-1 (anomeric carbon) into a sugar surrogate is a common practice in designing glycosidase inhibitors.[7,8] The ability of sugars which incorporated a basic nitrogen center to become protonated in biological medium and to form a cation which can interact strongly with an anionic group (carboxylate) at the enzyme active site explains their high affinity for glycosidases.[7]

With the aim of improving the stability of active COS and the study of its protein-binding ability to chitinase, herein we have synthesized 1,4-oxazepanyl sugar **5** (Scheme 1) by the chemo-enzymatic method. It is an analogue of penta-*N*-acetyl-chitopentaose with a nitrogen atom in the nonreducing sugar ring and with allyl group at the reducing end as the aglycone.

2. Results and discussion

2.1. Synthesis of allyl 2-acetamido-2-deoxy-β-D-glucopyranoside (*2*)[9]

N-Acetyl D-glucosamine **1** and LiBr (2 equiv) were suspended in DMF (1 mL/mmol of **1**) and successively treated with sodium hydride (1.3 equiv) and allyl bromide (3 equiv) at room temperature for 3 h. The crude

Keywords: 1,4-Oxazepanyl sugar; Chemo-enzymatic synthesis; Chitinase.
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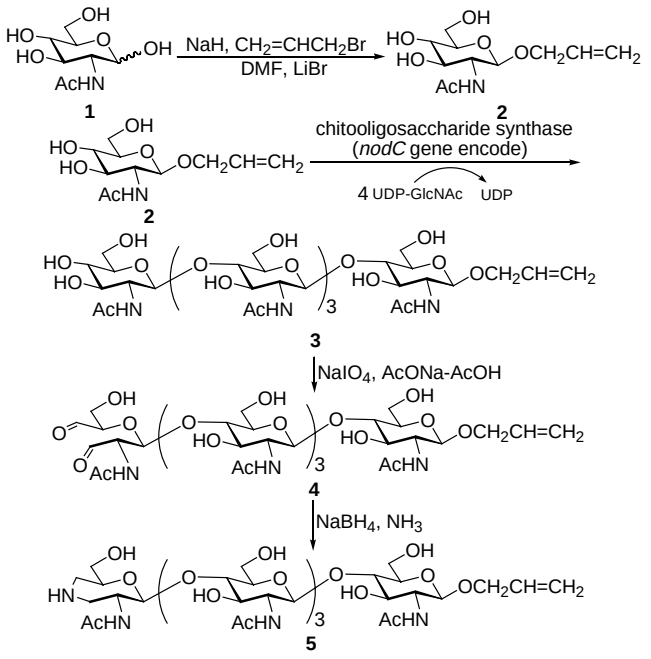
doi:10.1016/j.bmc.2005.11.020



Scheme 1. Synthetic route of **5**.

product directly was purified by flash chromatography (eluant: AcOEt/MeOH 4:1), and the β-allyl glycoside **2** was obtained in 82% yield. This one-step glycoside synthesis compares well with the standard peracetylation, selective anomeric deprotection, *O*-alkylation at C1, and deacetylation steps.[10]

2.2. Synthesis of allyl penta-*N*-acetyl-chitopentaose (3)

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. Cell density cultures were carried out as previously described[11] in 2 or 10 L bioreactors containing an initial culture volume of 1 or 7 L, respectively. Compound **2** was added to culture system. The *Escherichia coli* strain BL21(DE3) containing a plasmid carrying the cloned *nodC* gene from *Mesorhizobium loti* strain E1R[12] was used as the source of NodC protein. The culture time lasted 48 h.

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 P2. And the β-allyl penta-*N*-acetyl-chitopentaose **3** was obtained in 65% yield. Therefore, it indicates that **2** is clearly used as substrates by NodC in vivo. The most obvious explanation for this phenomenon is that the allylated derivative **2** of GlcNAc 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.

2.3. Synthesis of the target compound 5

As shown in Scheme 1, **3** was transferred to **4** upon periodate oxidation. Compound **4** was quickly subjected to sodium borohydride reduction and NH_3 amination, which afforded the target compound **5** in total 65% yield.

2.4. Binding chitinase assay

In the **5**-binding chitinase assay, we found that the chitinase was obviously inactivated by **5** with $IC_{50} = 4.7$ μmol/L, which indicated that introducing a nitrogen atom can improve the binding ability of substrate to chitinase.

3. Experimental

3.1. General methods

Kieselgel $60F_{254}$ (E. Merck) was used for TLC. Compounds were visualized using UV light and charring with 1:4 H_2SO_4/ethanol. Flash chromatography was performed on a column of silica gel (Baker, 0.063–0.200 nm). The size exclusion chromatography was performed on Biogel P2. Optical rotations were determined at 25 °C with a Perkin-Elmer Model 241-Mc automatic polarimeter. ^{13}C and ^{1}H NMR spectra were recorded using a Bruker DPX-300 spectrometer at 75 and 300 MHz, respectively. Mass spectra were recorded with a VG PLATFORM mass spectrometer using the ESI mode. The chitinase from *Serratia marcescens* was purchased from Sigma Company.

3.2. Synthesis of allyl 2-acetamido-2-deoxy-β-D-glucopyranoside (2)

For this experiment, **1** and LiBr (2 equiv) were suspended in DMF (1 mL/mmol of **1**) under argon. This suspension was stirred at room temperature until a homogeneous solution was obtained (3 h), after which sodium hydride (1.3 equiv) and allyl bromide (3 equiv) were added. The crude product directly was purified by flash chromatography (eluant: AcOEt/MeOH 4:1), and the β-allyl glycoside **2** was obtained in 82% yield. $[\alpha]_D$ +70.3 (*c* 1.1, H_2O). ^{13}C NMR (75 MHz, CD_3OD): δ 173.5 (C=O NHAc), 135.0 ($CH_2CH=CH_2$), 117.1 ($CH_2CH=CH_2$), 101.4 (β-C-1), 71.7, 75.6, 77.3, (C-3, C-4, C-5), 70.5 ($CH_2CH=CH_2$), 56.8 (C-2), 62.4 (C-6), 23.0 (CH_3 NHAc). ^{1}H NMR (300 MHz, CD_3OD): δ 5.98–5.79 (m, 1H, $CH_2CH=CH_2$), 5.31–5.04 (m, 2H, $CH_2CH=CH_2$), 4.43 (d, 1H, α-H-1, $J_{1,2}$ 8.0 Hz), 4.39–3.24 (m, 8H, H-2, H-3, H-4, H-5, H-6, $CH_2CH=CH_2$), 1.97 (s, 3H, CH_3 NHAc). ESIMS: *m*/*z* 284.1 $[M+Na]^+$. Anal. Calcd for $C_{11}H_{19}NO_6$: C, 50.57; H, 7.33; N, 5.36. Found: C, 50.85; H, 7.12; N, 5.41.

3.3. Bacterial strains, plasmids, and growth conditions

The *E. coli* strain BL21(DE3) containing a plasmid carrying the cloned *nodC* gene from *M. loti* strain E1R[12] was used as the source of NodC protein. Routine cultures were grown in LB medium.[13] Cell density cultures were carried out as previously described[11] in 2 or 10 L bioreactors containing an initial culture volume of 1 or 7 L, respectively. The culture medium was slightly modified and had the following composition: **2** (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), and KOH (2 g/L). $MgSO_4$ was added from a concentrated solution that was autoclaved separately. Thiamine was sterilized by filtration. 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). Antibiotic ampicillin was used to ensure maintenance of the plasmid and was prepared in concentrated stock solution as described by Sambrook et al.[13] 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 4.8 mL/h/L and kept constant until the end of the culture.

3.4. Purification of compound 3

After being harvested by centrifugation (20 min at 12,000*g*), bacterial cells were resuspended in a volume of distilled water equal to that of the original culture medium and disrupted by boiling for 45 min. After cooling, 10 μL concentrated HCl was added. Cell debris and precipitated proteins were eliminated by centrifugation (30 min at 12,000*g*) and the 1 L 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 1.3 L of 55% (v/v) aqueous ethanol. This last fraction was loaded onto a 100 × 1-cm column of Biogel P2. The flowthrough fraction was recovered and lyophilized. The 1.8 g **3** was obtained in 65% yield. $[\alpha]_D$ +57.8 (*c* 1.1, H_2O). ^{13}C NMR (75 MHz, D_2O): δ 175.57, 175.48 (C=O NHAc), 135.5 ($CH_2CH{=}CH_2$), 117.4 ($CH_2CH{=}CH_2$), 102.44, 102.23 (C-1^{II}, C-1^{III}, C-1^{IV}, C-1^{V}), 102.0 (β-C-1), 80.65, 80.20, 79.95, 76.95, 75.51, 74.49, 73.46, 73.07, 71.04, 70.71, 70.25, 61.55, 61.00, 56.58, 56.08, 54.68 (C-2^{I-V}, C-3^{I-V}, C-4^{I-V}, C-5^{I-V}, C-$6a^{I-V}$, C-$6b^{I-V}$), 70.9 ($CH_2CH{=}CH_2$), 23.11, 22.90 (CH_3 CH_3 NHAc). ^1H NMR (300 MHz, D_2O): δ 6.27 (d, 1H, $J_{1,2}$ 7.9 Hz, α-H-1), 6.00–5.77 (m, 1H, $CH_2CH{=}CH_2$), 5.37–5.04 (m, 2H, $CH_2CH{=}CH_2$), 4.47 (m, 4H, H-1^{II}, H-1^{III}, H-1^{IV}, H-1^{V}), 4.30–3.99 (m, 2H, $CH_2CH{=}CH_2$), 3.90–3.30 (m, 46H), 1.97, 1.95 (s, 15H, CH_3 NHAc). ESIMS: *m*/*z* 1096.4 $[M+Na]^+$. Anal. Calcd for $C_{43}H_{71}N_5O_{26}$: C, 48.09; H, 6.66; N, 6.52. Found: C, 48.10; H, 6.29; N, 6.48.

3.5. Synthesis of the target compound 5

$NaIO_4$ (9 mg) was added to a solution of **3** (40 mg) in 100 mmol/L AcONa–AcOH (pH 5.5, 1.8 mL) and stirred for 2 h. After adjusting the solution pH to 9.0, $NaBH_4$ (5 mg) was added to the reaction solution and further stirred for 2 h. Then 5 mL concentrated NH_3-H_2O was added and stirred for 1h again. All reactions went along at room temperature. The reaction solution was purified by Biogel P2 to afford 26 mg of the target compound **5** in total 65% yield. $[\alpha]_D$ +63.5 (*c* 1.1, H_2O). ^{13}C NMR (75 MHz, D_2O): δ 175.57, 175.46 (C=O NHAc), 135.3 ($CH_2CH{=}CH_2$), 117.4 ($CH_2CH{=}CH_2$), 102.44, 102.20 (C-1^{II}, C-1^{III}, C-1^{IV}, C-1^{V}), 102.1 (β-C-1), 80.60, 80.20, 79.95, 77.01, 75.50, 74.49, 73.43, 73.00, 70.84, 70.56, 70.25, 61.73, 61.00, 56.83, 56.10, 54.68 (C-2^{I-V}, C-3^{I-V}, C-4^{I-V}, C-5^{I-V}, C-$6a^{I-V}$, C-$6b^{I-V}$), 70.6 ($CH_2CH{=}CH_2$), 23.10, 22.87 (CH_3 CH_3 NHAc). ^1H NMR (300 MHz, D_2O): δ 6.26 (d, 1H, $J_{1,2}$ 7.9 Hz, α-H-1), 6.00–5.78 (m, 1H, $CH_2CH{=}CH_2$), 5.37–5.04 (m, 2H, $CH_2CH{=}CH_2$), 4.45 (m, 4H, H-1^{II}, H-1^{III}, H-1^{IV}, H-1^{V}), 4.31–3.99 (m, 2H, $CH_2CH{=}CH_2$), 3.85–3.29 (m, 47H), 1.95, 1.93 (s, 15H, CH_3 NHAc). ESIMS: *m*/*z* 1079.5 $[M+Na]^+$. Anal. Calcd for $C_{43}H_{72}N_6O_{24}$: C, 48.86; H, 6.87; N, 7.95. Found: C, 48.79; H, 6.53; N, 8.00.

3.6. 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 substrate **3** under the condition stated. 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 **5** (20 mmol/L) were incubated at 18 °C in 40 mmol/L sodium acetate buffer at pH 5.0. The residual activity was determined at 100 min intervals.

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