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Note

Enzymatic synthesis and characterization of 6-*O*-β-D-xylopyranosyl-2-acetamido-2-deoxy-D-glucopyranose, a structural analog of primeverose

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Abstract

The synthesis of the disaccharide 6-*O*-β-D-xylopyranosyl-2-acetamido-2-deoxy-D-glucopyranose (*N*-acetylprimeverosamine), structurally related to the natural disaccharide 6-*O*-β-D-xylopyranosyl-D-glucopyranose (primeverose), was obtained via a transglycosylation reaction catalyzed by a crude preparation of β-D-xylosidase from *Aspergillus niger*, using *p*-nitrophenyl β-D-xylopyranoside as the donor and 2-acetamido-2-deoxy-D-glucopyranose as the acceptor. The yield of the reaction was 36% on a molar basis with respect to the donor. The chemical identity of the product was assessed by HPLC, ionspray mass spectrometry and NMR spectroscopy.

Keywords: Transglycosylation; *Aspergillus niger* β-D-xylosidase; Primeverose; *N*-Acetyl-primeverosamine

In the last decade the use of glycosidases has become a common tool for the synthesis of oligosaccharides [1–4], including those of biological relevance. The latter goal was achieved either with the help of chemical [5] or glycosyltransferase-based [6,7] strategies, or by the exclusive use of transglycolytic reactions [8–13].

The general aim of the present report was to confirm the convenience of the use of glycosidases only in the catalysis of transglycosylation reactions, also aiming at the highest regioselectivity. More specifically, it was interesting to investigate the possibility of using β-D-xylosidase for the regiospecific synthesis of 6-*O*-β-D-xylopyranosyl-2-acetamido-2-deoxy-D-glucopyranose (*N*-acetylprimeverosamine), a structural analog of a relevant disaccharide, 6-*O*-β-D-xylopyranosyl-D-glucopyranose (primeverose). The latter one has been so far obtained only by enzymatic hydrolysis of some natural glycosides (gaultherin, primeverin, rhamnicoside, etc.) from plant sources. An isomeric form of this molecule, known as isoprimeverose (6-*O*-α-D-xylopyranosyl-D-glucopyranose), has been

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obtained from the hydrolysis of tamarind seed polysaccharide [14].

The rational of the choice of Glc*p*NAc as the acceptor was, at the same time, both to use a molecule structurally very close to glucose and to take advantage of the presence of the *N*-acetyl group. This chromophore absorbs at 210 nm, thereby permitting an easy detection during the different experimental steps.

We have previously observed that the *Aspergillus niger* α-L-fucosidase catalyzes the regiospecific synthesis of the disaccharide 3-*O*-α-L-fucopyranosyl-2-acetamido-2-deoxy-D-glucopyranose [15]. A similar behaviour is hereafter shown for the β-D-xylosidase from the same microorganism. Preliminary reactions carried out in different experimental conditions indicated that pH 5.5 and T 37 °C were the optimal ones. In all cases only one reaction product was apparent besides the peaks of unreacted acceptor (Glc*p*NAc). The simple purification procedure, based on the use of solid phase extraction on C_{18} cartridges, increased the speed of the analysis of the reaction mixture by TLC. The same method was used in the kinetic study which followed the preliminary one. It was found that the reaction appeared to be rather slow. The maximum production of *N*-acetylprimeverosamine was obtained only after 20 h. After that time a reduction in the synthesis of the product was observed (Fig. 1) due to prevailing hydrolysis. The product

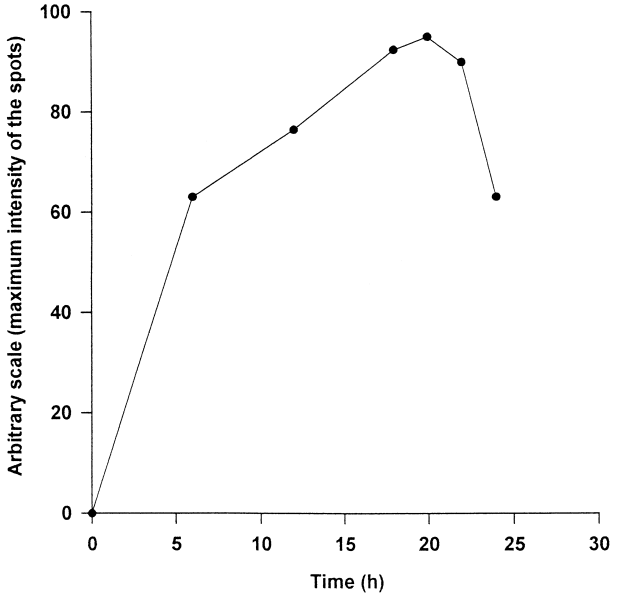

Fig. 1. Time-course production of 6-*O*-β-D-xylopyranosyl-2-acetamido-2-deoxy-D-glucopyranose at 37 °C in aqueous buffer solution, pH 5.5.

of repeated synthesis on a larger scale under optimum reaction conditions underwent an easy further purification by gel-permeation chromatography (GPC) on Bio-Gel P-2 exploiting the difference in molecular weight between the product and both the substrate (Glc*p*NAc) and the hydrolysis product (Xyl*p*), the latter if present to a detectable extent. A yield of 36%, on a molar basis with respect to the acceptor could be calculated from the GPC chromatographic data. HPLC analysis was performed on the reaction mixture after solid phase extraction. The product disaccharide appeared as a peak with a retention time of 6.84 min, different from that of Glc*p*NAc, the only other molecule detectable under the separation conditions used, which showed a retention time of 5.19 min.

The chemical characterization of the disaccharide isolated after GPC separation was at first performed by ionspray mass spectrometry. The positive ionspray mass spectrum of the purified product showed peaks at 354.0, 371.0, 376.0, and 392.0, corresponding to $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$, and $[M+K]^+$, respectively, in excellent agreement with the expected relative molar mass.

Further chemical identification of the product was performed by ^1H and ^{13}C NMR spectroscopy, whose signal assignments are reported in Tables 1 and 2, respectively. The assignments of the signals of C-6 of both the anomers of Glc*p*NAc were confirmed by means of a DEPT 135 experiment (data not shown). In fact, only the methylene groups (CH_2) show negative signals in this experiment. It is interesting to note that their frequences are 8.13 and 8.17 ppm higher than the corresponding signals for both the anomers of Glc*p*NAc. The comparison between the ^{13}C signals of the disaccharide and those of free Glc*p*NAc show much lower differences for all the other positions (about 1 ppm for C-5 and less than 0.2 ppm for all the other carbon atoms). This effect is typical of glycosylation products, and confirms the chemical identity of the obtained disaccharide with that of 6-*O*-β-D-xylopyranosyl-2-acetamido-2-deoxy-D-glucopyranose.

In conclusion the data presented demonstrate that the regiospecific, totally transglycolytic synthesis of the disaccharide *N*-acetylprimeverosamine (6-*O*-β-D-xylopyranosyl-2-acetamido-2-deoxy-D-glucopyranose) can be achieved with a good yield using β-D-xylosidase from *A. niger*. Although β-D-xylosidases from different sources have been extensively studied for their mechanism of action

Table 1
^{1}H chemical shifts (ppm) and coupling constants (J_{HH}) of 6-*O*-β-D-xylopyranosyl-2-acetamido-2-deoxy-D-glucopyranose

H-atoms	Xyl*p*(β1–	–6)Glc*p*NAcα	–6)Glc*p*NAcβ
H-1	4.438/4.457 (7.5)	5.192 (3.4)	4.712 (8.3)
H-2	3.305 (9.2)	3.886 (10.3)	3.685 (9.6)
H-3	3.446 (8.8)	3.756 (8.5)	3.534 (7.3)
H-4	3.629 (10.2, 5.1)	3.560 (9.5)	3.509
H-5a	3.962 (5.2, −11.3)	~3.95	~3.95
H-5b	3.318 (10.2, −11.3)		
H-6a		4.104 (1.5, −11.3)	4.158 (1.5, −11.5)
H-6b		3.874 (5.7, −11.3)	3.848 (5.7, −11.5)
CH_3		2.02	2.02

[16–19], only few reports deal with their use to catalyze transglycosylation reactions [20–24]. Among them, only three examples refer to *A. niger* β-D-xylosidase, namely for the synthesis of alkyl xylosides in the presence of various alcohols [22,23] and for the synthesis of some xylooligosaccharides [24], in both cases with a high transglycolytic efficiency. The present result confirms the high transfer tendency of this enzyme and its high regioselectivity as well.

1. Experimental

Materials.—*O*-Nitrophenyl β-D-xylopranoside (Xyl-O-*o*NP), *p*-nitrophenyl β-D-xylopyranoside (Xyl-O-*p*NP), and Glc*p*NAc were obtained from Sigma (St. Louis, MO, USA). Rhozyme HP150 as a crude preparation of β-D-xylosidase (E.C. 3.2.1.37) from *Aspergillus niger* was bought from InterSpex Products Inc. (Foster City, CA, USA), Sep-Pak C_{18} (light and Vac 35cc) cartridges from Waters (Millford, MA, USA), and Bio-Gel P-2 from Bio-Rad (Richmond, CA, USA). TLC Silica Gel plates and a LiChrosorb-NH_2 HPLC column were obtained from E. Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Table 2
^{13}C chemical shifts (ppm) of 6-*O*-β-D-xylopyranosyl-2-acetamido-2-deoxy-D-glucopyranose

C-atoms	Xyl*p*(β1–	–6)Glc*p*NAcα	–6)Glc*p*NAcβ
C-1	104.78/104.74	92.16	96.26
C-2	74.25	55.30	57.88
C-3	76.91	71.83	75.08
C-4	70.48	71.14	71.01
C-5	66.44	71.91	76.18
C-6		69.98	70.18
CO		175.78	176.04
CH_3		23.20	23.47

Assay of A. niger β-D-*xylosidase*.—β-D-xylosidase activity was assayed as follows. Fifty μL of the enzyme solution (10 mg/mL of crude enzyme in 50 mM NaOAc buffer, pH 5.5) were added to 450 μL 4 mM Xyl-O-*o*NP in the same buffer. The solution was incubated for 4 min at 37 °C. To stop the reaction, 1 mL 0.2 M Na_2CO_3 was added; the liberated *o*-nitrophenol was determined spectrophotometrically at 410 nm. One unit of β-D-xylosidase was defined as the amount of the enzyme releasing 1 μmol of *o*-nitrophenol per min.

Preparation of N-*acetylprimeverosamine*.—370 μmol Xyl-O-*p*NP and 4.5 mmol Glc*p*NAc were dissolved in 9.50 mL 50 mM NaOAc buffer, pH 5.5. 2.3 Units (500 μL of a 10 mg/mL solution) of *A. niger* β-D-xylosidase were added and the solution was incubated for 20 h at 37 °C. After incubation, the mixture was heated in a boiling water bath for 10 min, immediately cooled in ice, then purified.

Purification of transxylosylation products.—The mixture containing transxylosylation products was subjected to a solid phase extraction on a Sep-Pak C_{18} (Vac 35cc) column previously conditioned with 3 vols of MeOH followed by 3 vols of water. The volume of the water-eluted fraction was reduced and then the mixture was purified by GPC on two serial columns (2.0×100 cm each) of Bio-Gel P-2 equilibrated in water. The elution was followed by reading the absorbance of the even numbered fractions at 210 nm; the positive ones were analyzed by TLC as described below. The fractions containing the transxylosylation product were pooled and freeze-dried.

Kinetics of the synthesis of N-*acetylprimeverosamine*.—37 μmol Xyl-O-*p*NP and 450 μmol Glc*p*NAc were dissolved in 950 μL 50 mM NaOAc

buffer, pH 5.5. A solution (50 μL, 0.23 U) of *A. niger* β-D-xylosidase was added and the solution was incubated for 24 h at 37 °C. During the incubation, about every 6 h 50 μL of the sample were collected, added to 450 μL of distilled water, heated in a boiling water bath for 10 min, and then immediately cooled in ice. All the fractions were separately passed through a Sep-Pak C_{18} (light) cartridge prepared as described before. In this way, Glc*p*NAc, Xyl*p* and the transglycosylation product were eluted with water, while Xyl-O-*p*NP and free *p*-nitrophenol were eluted with MeOH. The eluted water fractions were freeze-dried. The residues were resuspended in 500 μL distilled water; 2.5 μL thereof were analyzed by TLC as described below. After development and detection, the TLC plate was scanned and the image obtained was digitalized by means of a personal computer home-made program and the data analyzed by data handling software.

Structural identification methods.—TLC was performed on Silica gel plates developed with 6:2:1 *n*-propanol–1 M ammonia–water, and detected with 0.2% orcinol in 2 M H_2SO_4. HPLC was carried out using a Jasco system equipped with a UV-visible detector, monitoring at 210 nm (*N*-acetyl group) and a LiChrosorb-NH_2 HPLC column eluted under isocratic conditions using 85:15 acetonitrile–water as the mobile phase.

Mass spectra were recorded in the positive-ion mode on a API-I PE SCIEX quadrupole mass spectrometer. The sample was dissolved in aq 50% acetonitrile at a final concentration of 20 μM. 60 μM Ammonium acetate was used as the ionising agent. The injection flow rate was 0.3 μL/min. The ionspray voltage was 5000 V and the orifice potential was 50 V. The spectra were recorded using a step size of 0.1 amu.

^{1}H and ^{13}C NMR experiments were performed on a Bruker AC 200 spectrometer equipped with a 5 mm multinuclear probe. ^{1}H measurements were performed at 297 K and chemical shifts were referred indirectly to acetone (2.225 ppm). All the samples were dissolved in D_2O at a concentration of 15 mg/mL. The spectral width was 3500 Hz and the digital resolution was 0.25 Hz/pt; the acquisition time was 2.3 s. The assignment of ^{1}H signals was made by means of a two-dimensional correlated spectroscopy (2D COSY45) experiment with presaturation of the residual HOD signal. The 2D spectrum was acquired using 64 scans per series with 1 and 0.5 K data points in F2 and F1, respectively, with zero-filling in F1. Centred sine-bell multiplication functions were applied prior to Fourier transformation. In order to properly assign some overlapping signals, selective one-dimensional total correlated spectroscopy (1D TOCSY) experiments were performed. These measurements were obtained in the inverse mode to calibrate the optimal pulse power. Selective 90 and 180° pulses were obtained using a modified DANTE pulse sequence [25]. Several experiments were stored with increasing mixing time to follow the magnetisation transfer along the selected spin system.

Decoupled ^{13}C spectra and the DEPT 135 experiment were obtained at 297 K; chemical shifts were referred indirectly to Me_4Si using 1,4-dioxane as internal standard. The width of ^{13}C spectra was 16.000 Hz with a digital resolution of 1 Hz/pt.

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