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Regiospecific Transglycolytic Synthesis and Structural Characterization of 6-*O*-α-Glucopyranosyl–Glucopyranose (Isomaltose)

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The enzymatic synthesis of 6-*O*-α-glucopyranosyl–glucopyranose (isomaltose) was achieved. The regiospecific transglycosylation reaction was catalyzed by a crude preparation of α-D-glucosidase from *Aspergillus niger,* using *p*-nitrophenyl α-D-glucopyranose as the donor and glucopyranose as the acceptor. The yield of the reaction was 59% on a molar basis with respect to the donor. The structural identity of the product was fully determined by HPLC, HPAEC-PAD, ionspray mass spectrometry and ^{13}C NMR. © 2000 Academic Press

Key Words: **transglycosylation; isomaltose; 6-*O*-α-glucopyranosyl–glucopyranose; *Aspergillus niger*.**

In the field of carbohydrate biochemistry the use of enzymes has become a common tool for the synthesis of oligosaccharides (1–5). This goal can be achieved either with the help of glycosyltransferases or by the use of glycosidases (6–9). Indeed, many glycosidases may very efficiently catalyze the stereospecific formation of glycosidic bonds using simple substrates, i.e., disaccharides and/or glycosides, as donors. It is now well established that the proper choice of the enzyme is a general rule to reach the best performances of the synthesizing catalyst to optimize both yield and regioselectivity. Among the various glycosidases, those from *A. niger* seem to be very efficient showing a very good regiospecificity when used in transglycosylation reactions (10, 11).

The general scope of the present report was to contribute to assess the regioselectivity performance of glycosidases as catalysts for transglycosylation reactions. More specifically, a project has been recently started aiming at obtaining, by the only use of the latter reactions, different oligosaccharides of α-D-glucopyranose, varying as to the sequence of linkages. Glucooligosaccharides are extremely relevant for their nutritional value as well as structural motifs in complex carbohydrates. It was decided to start by investigating the possibility of using *A. niger* α-D-glucosidase for the regiospecific synthesis of the 6-*O*-α-glucopyranosyl–glucopyranose (isomaltose) (Fig. 1). From a biological point of view, the presence of this disaccharide has been associated with metabolic and renal illness (12, 13). Isomaltose can be obtained in a mixture with higher oligomers by enzymatic hydrolysis of dextran using a bacterial dextranase (14, 15). However, to get pure isomaltose, a tedious separation procedure must be undertaken. The chemical synthesis, both of the free disaccharide and of its methyl glycosides, have also been described (16, 17). In spite of its inherent structural simplicity, commercially available isomaltose is not very pure, nor its NMR spectrum in the underivatized form has yet been reported and fully assigned.

MATERIALS AND METHODS

Materials. *o*-Nitrophenyl α-D-glucopyranose (Glcα-*O*-*o*NP), *p*-nitrophenyl α-D-glucopyranose (Glcα-*O*-*p*NP), glucopyranose (Glc), 6-*O*-α-glucopyranosyl–glucopyranose, 1-phenyl-3-methyl-5-pyrazolone (PMP) were obtained from Sigma (St. Louis, MO). Transglucosidase L "Amano" as a crude preparation of α-D-glucosidase (EC 3.2.1.20) from *Aspergillus niger* was a kind gift of Amano Pharmaceutical Co. (Nagoya, Japan). Bio-Gel P2 was from Bio-Rad (Richmond, CA). Sep-Pak C_{18} (Vac 35 cc) cartridges were from Waters (Milford, MA). Acetonitrile for HPLC (HyperSolv) was from BDH (Poole, UK). TLC Silica Gel plates were from E. Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Assay of A. niger α-D-glucosidase. α-D-Glucosidase was assayed as follows: 50 μL of the enzyme solution were added to 450 μL of 4 mM Glcα-O-*o*NP in 50 mM acetate buffer, pH 5.0, and the solution was incubated for 5 min at 37°C. To stop the reaction, 1 mL of 0.2 M Na_2CO_3 was added. The liberated *o*-nitrophenol was determined spectrophotometrically at 410 nm. The molar extinction coefficient used for *o*-nitrophenol was 4600 cm^{-1} M^{-1}. One unit of α-D-glucosidase was defined as the amount of the enzyme releasing 1 μmol of *o*-nitrophenol per minute.

Kinetic analysis of the synthesis of isomaltose. 33.2 μmol of Glcα-O-*p*NP and 555 μmol of Glc were dissolved in 980 μL of 50 mM

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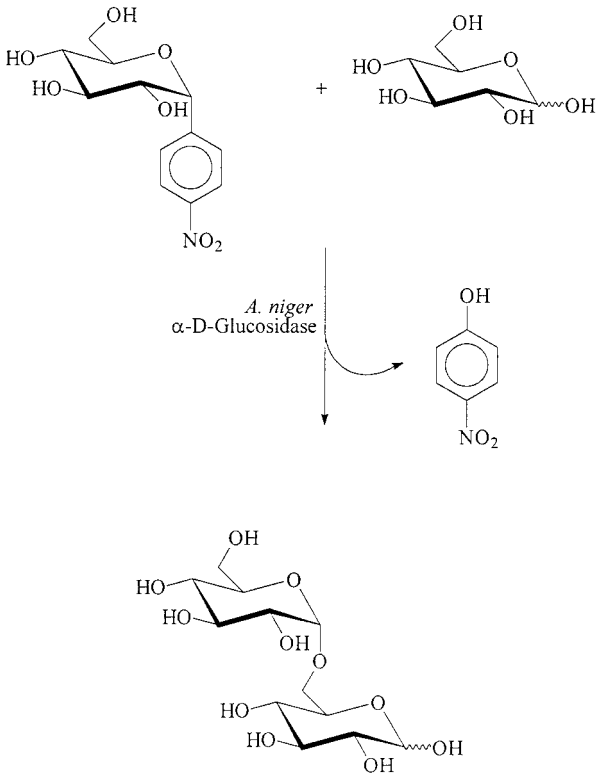

FIG. 1. Schematic representation of the transglycolytic synthesis of isomaltose.

K_2HPO_4/KH_2PO_4 pH 6.5. 0.2 units (20 μL) of *A. niger* α-D-glucosidase were added to each solution and then incubated for 8 h at 37°C. During incubation, on every 1 h, 50 μL of sample were collected, added to 450 μL of distilled water and heated in a boiling water bath for 10 min and then immediately cooled in ice. 10 μL of each sample were derivatized with PMP according the method of Honda *et al.* (18) and analyzed by a Jasco HPLC system consisting of a BIP-I pump equipped with a UVIDEC-100-V UV-visible detector monitoring at 245 nm and a Spherisorb-ODS column (5 μm, 250 × 4.00 mm i.d.). The column was eluted under isocratic conditions at a flow rate of 1 mL/min, using a mixture of K_2HPO_4/KH_2PO_4 (10 mM, pH 7.0): CH_3CN (85:15) as mobile phase.

Preparation and purification of isomaltose. 100 mg of Glcα-*O*-*p*NP and 1 g of Glc were dissolved in 10 mL of 50 mM K_2HPO_4/KH_2PO_4, pH 6.5. 2.0 units (200 μL) of *A. niger* α-*D*-glucosidase was added to the solution. After incubation at 37°C for 6 h, the mixture was heated in a boiling water bath for 10 min to inactivate the enzyme and then immediately cooled in ice. After centrifugation at 11,000 rpm for 5 min the supernatant was purified as described below.

Purification of isomaltose. The transglucosylation products were purified on a Sep-Pak C_{18} (Vac 35 cc) cartridge. Transglucosylation products and glucose were eluted by washing with water. The less polar compounds Glcα-*O*-*p*NP and *p*-nitrophenol were eluted with methanol. The water-eluted fraction was dried under reduced pressure, redissolved in 5 mL of water and then purified by gel permeation chromatography on two serial columns (2.0 × 100 cm each) of Bio-Gel P2 equilibrated in water. The elution was followed by TLC developed with 6:2:1 *n*-propanol:1 M ammonia:water, and detected with 0.2% orcinol in 2 M H_2SO_4. The analysis of the transglucosylation products involved the use of the same HPLC system as described above. The fractions containing the isomaltose were pooled, dried under reduced pressure, and redissolved in 2 mL of water and freeze-dried.

Structural identification methods. HPLC analysis of isomaltose was carried out as described above in the section on "kinetic analysis". The HPAEC-PAD system (Dionex, Sunnyvale, CA) was equipped with a metal-free isocratic pump (Model IP20), a pulsed amperometric detector (Model ED40), and a metal-free rotary injection valve with a 10 μL injection loop. Separations were performed with a Dionex CarboPac PA1 anion-exchange column (250 × 4.00 mm i.d.) plus guard column (50 × 4.00 mm i.d.). The flowthrough detection cell (Dionex) contained a gold working electrode (1.0-mm diameter) and an Ag/AgCl reference electrode; the counter electrode was the titanium cell body across the 25-μm thin layer channel from the working electrode. Pulsed amperometric detection was carried out with the following waveform parameters: $E_{det} = +0.05$ V, $t_{det} =$ 440 ms ($t_{int} = 240$ ms); $E_{ox} = +0.8$ V, $t_{ox} = 180$ ms; $E_{red} = -0.2$ V, $t_{red} = 360$ ms. The response time was 1 s. The eluent used was 0.2 M NaOH flowing at 1.0 mL/min. All chromatography data acquisition and processing were performed using the software PeakNet 5.1 (Dionex).

Proton decoupled ^{13}C NMR spectrum was recorded at room temperature (22.5°C) and at 100 MHz (^{13}C) with a Jeol EX 400. The sample concentration was 33 mg/mL in deuterium oxide, pD ~6. A 5-mm glass tube was used. The acquisition time was 0.55 s, the spectral width was 30 kHz with 16K original data points to give after zero filling a digital resolution of 0.9 Hz/pt. The ^{13}C chemical shifts were expressed relative to external Me_4Si and the chemical shift scale calibrated with respect to the methyl signal of CD_3OD used as internal standard (49.06 ppm from Me_4Si).

The mass spectrum was recorded on a API-I PE SCIEX quadrupole mass spectrometer equipped with an articulated ion spray and connected to a syringe pump for sample injection. The instrument was calibrated using a polypropylene glycol (PPG) mixture (3.3×10^{-5} M PPG 425, 1×10^{-4} M PPG1000 and 2×10^{-4} M PPG 2000), 0.1% acetonitrile and 2 mM ammonium formate in 50% aqueous methanol. The samples were dissolved in 50% aqueous acetonitrile to a final concentration of 0.2×10^{-4} M. Ammonium acetate 0.6×10^{-4} M was used as ionizing. The injection flow rate was 0.3 μL/min. The ionspray voltage (ISV) was 5600 V and the OR was 90 V.

RESULTS AND DISCUSSION

Before undertaking the enzymatic synthesis of Isomaltose, a preliminary kinetic investigation of the enzyme action was performed. The results of such kinetics analysis are reported in Fig. 2. The maximum of the synthesis of isomaltose was reached comparatively soon (6 h), before the prevailing hydrolytic process took over. No other isomer was produced in the time interval considered for the kinetic studies shown in Fig. 2 (8 h) and even after more than 24 h of incubation (data not shown). From a parallel set of kinetic experiments it has been observed that $T = 37$°C and pH 6.5 were the optimal reaction conditions. The value of pH 6.5 is slightly higher than the reported optimal pH for hydrolysis (i.e., 5.0) (19). Indeed, it is well established that pH and temperature as well as the structure of the acceptor and of the donor are key factors for addressing and controlling both regioselectivity and yield of transglycosylations (20–26).

The synthesis of isomaltose was then carried out on a preparative scale, according to the kinetic results. The compound obtained was purified by a sequential

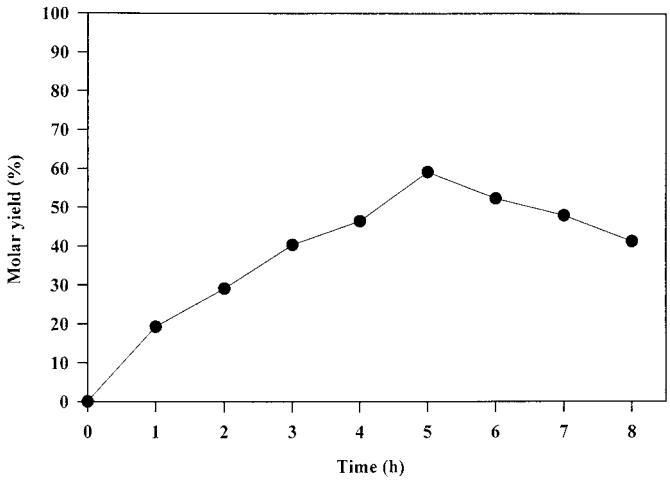

FIG. 2. Kinetic curve of the enzymatic synthesis of the isomaltose, using *A. niger* α-D-glucosidase. $T = 37°C$, pH 6.5.

use of solid phase extraction (SPE) and gel permeation chromatography (GPC) procedures (see Materials and Methods). The efficiency of the purification procedure was tested by following the yield and the sample purity through during the course of the process. The high efficiency of the purification procedure is indicated by the observation that only 6% of loss took place on passing from the initial crude mixture to the final pure product.

After purification, the synthesized product was characterized by HPLC, HPAEC-PAD, ionspray mass spectrometry and ^{13}C NMR.

HPLC measurements were performed on PMP derivatives of a commercial sample of isomaltose, used here as a standard, and of the synthesized disaccharide. The elution profiles are reported in Figs. 3A–3C). The reported curves clearly show that the retention time of the synthesized compound (Fig. 3B) coincides, within the experimental error, with that of the standard (Fig. 3C).

HPAEC-PAD measurements were performed on a commercial sample of Isomaltose and on the synthesized disaccharide. The elution profiles are reported in Figs. 4A and 4B. The reported curves clearly show that the retention time of the synthesized compound (Fig. 4A) coincides with that of the standard (Fig. 4B).

The positive ion mass spectrum of the purified disaccharide is reported in Fig. 5. The molecular peaks at 365.3 and 381.2 m/z, corresponding to the $[M + Na]^+$ and $[M + K]^+$ adducts, respectively, are in excellent agreement with the expected molar mass of isomaltose (i.e., 342.3).

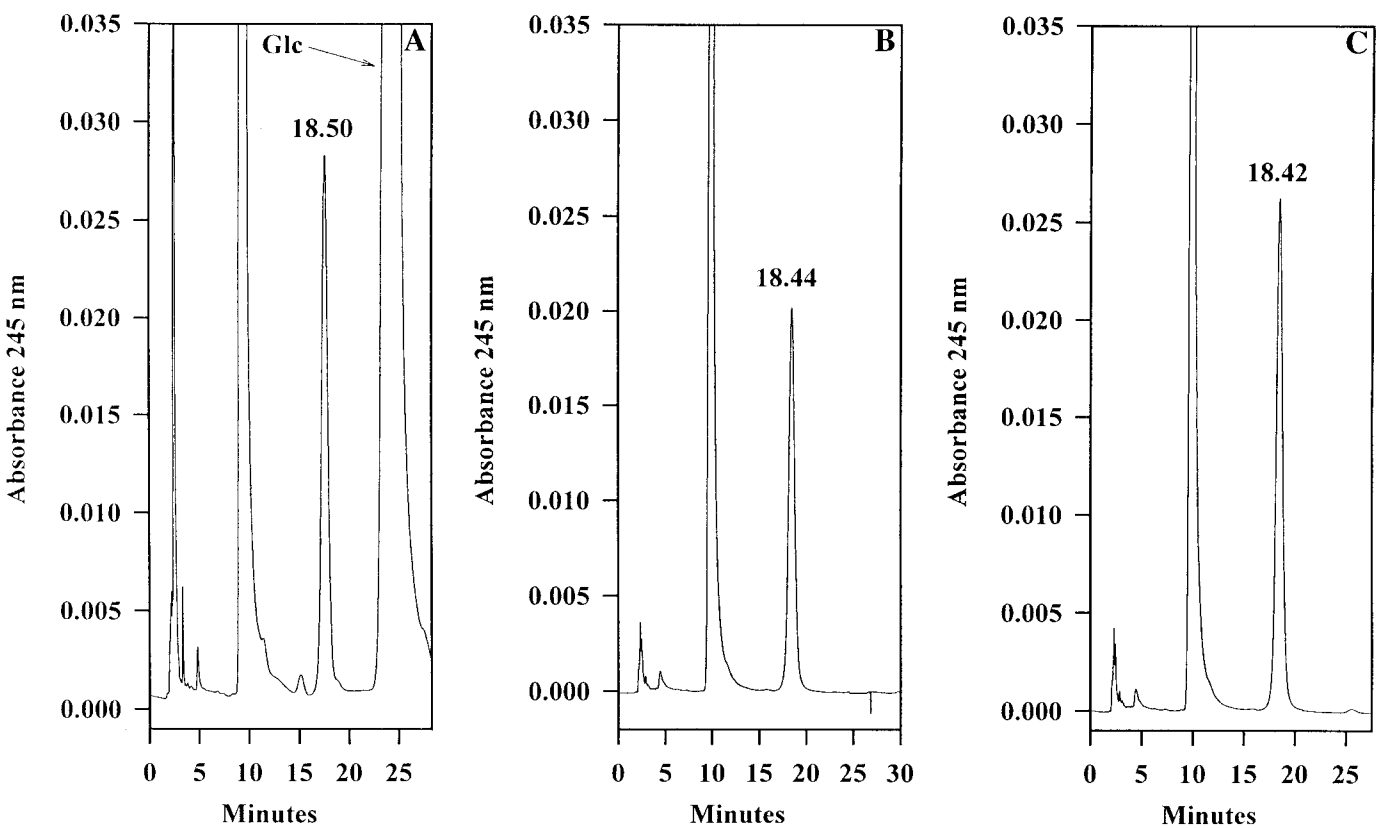

FIG. 3. HPLC elution profiles for the PMP derivatives of the crude preparation of isomaltose (A), for the synthesized compound (B), and for a commercial isomaltose sample (C).

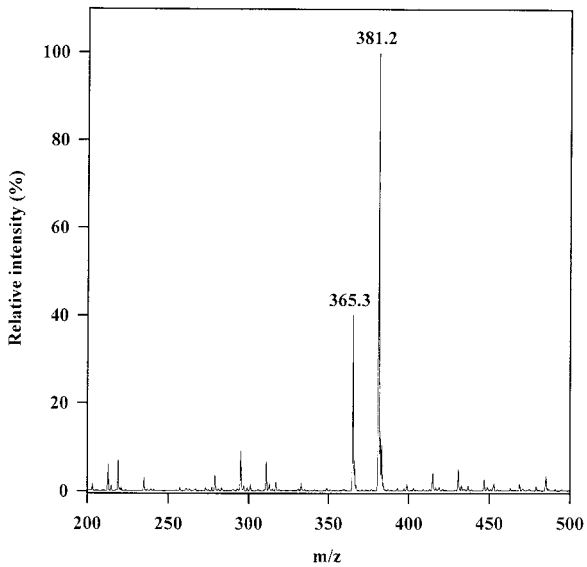

FIG. 4. HPAEC-PAD elution profiles for the synthesized compound, and for a commercial Isomaltose sample.

The proton-decoupled ^{13}C NMR spectrum was obtained from a deuterium oxide solution of the enzymatically synthesized disaccharide. ^{13}C chemical shifts are listed in Table 1 together with those obtained for a commercial isomaltose sample and already reported by Colson *et al.* (27) in their valuable carbon-13 study on several glucans. The chemical shifts are systematically ~1 ppm lower than those reported (27), were they were directly referred to TMS contained in a coaxial capillary. In the present case ^{13}C spectrum has been recorded at lower temperature and the chemical shift scale calibrated using an internal reference (see Materials and Methods). Nevertheless, the close agreement between the two set ^{13}C data of Table 2 leaves no much doubts on identify the structure of the synthesized compound with that of Fig. 1. In addition, the NMR data show also the high structural purity of the synthesized Isomaltose since no glycosidic linkage other than the (α1→6) is present.

The complexity of the signal pattern of ^{13}C spectrum obviously originates from the coexistence in solution of both α and β anomers of the glucose reducing unit. Unfortunately, no assignment was given for the β anomer in Ref. 27, where also the resonances corresponding to the carbon atoms at position 4 and 5 of both glucose residues were left unclear (see Table 1). To the knowledge of the authors the complete ^{13}C assignment of both the α and β forms of isomaltose had not been accomplished yet. The high purity of the synthesized disaccharide, as demonstrated by the experimental data so far presented, as well as the good resolution of the recorded ^{13}C spectrum, gave us the opportunity to produce the total assignment of carbon resonances. The ^{13}C assignments listed in Table 1 are the result of the correlation performed between the carbon signals ^{13}C spectrum and those of NMR studies reported for per-*O*-methyl-isomaltosides (α and β) and for isomaltose-related glucans such as glucopyranose residues (α and β), O-methylated glucopyranosides, melibiose (6-*O*-α-galactopyranosyl–glucopyranose) and maltose (4-*O*-α-glucopyranosyl–glucopyranose) (27–33). Beside the assignment of the signals corresponding to the C5 of the glucopyranose residue and to the C4 of both glucopyranosyl and glucopyranose sugars, it has been possible to revert the assignments of the C2 and C5 of the reducing and non reducing sugar, respectively, the latter one having been previously given at upper field with respect to the former (27). Taking into account that for simple sugars the carbon atom at position 2 is one of the most sensitive to pH changes (27), ~1 pH unit of difference between the present isomaltose solution and that previously used might account for the upfield shift of the C2 signal here observed. In Table 2 the resulted chemical shift difference between carbon-13 resonance of the α and β form of the synthesized Isomaltose, $\Delta_{(\beta\text{-}\alpha)} = \delta C_\beta - \delta C_\alpha$, are listed together with the corresponding $\Delta_{(\beta\text{-}\alpha)}$ values reported for a deuterium oxide solution of melibiose (6-*O*-α-galactopyranosyl–glucopyranose) (29, 31). Taking into account that these values are independent on the scale calibration and that isomaltose differs from melibiose only for having an equatorial OH group at position 4 of the non reducing sugar, the surprisingly close agreement between the two set of data strongly supports the carbon-13 atom assignments given in Table 1.

The combination of HPLC, HPAEC-PAD, ion-spray mass spectrometry and ^{13}C NMR unambiguously points to the identity of the synthesized product as isomaltose. Moreover, it indicates that the chemical and stereochemical purity is extremely high, i.e., corresponding to a possible presence of contaminants be-

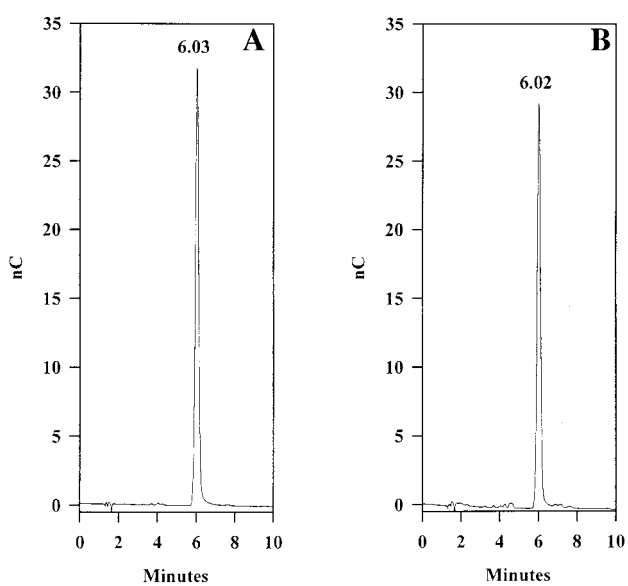

FIG. 5. Ion mass spectrum of the enzymatically synthesized isomaltose.

TABLE 1

^{13}C Chemical Shifts of the Enzymatically Synthesized Isomaltose[a] (pD = 6, T = 22.5°C), Commercial Isomaltose[b] (pD = 7, T = 32°C) and D-Glucose[c]

	Glcα1-[a]		-6Glc[a]				Glc[c]	
	α	β	α	β	Glcα1-[b]: α	-6Glc[b]: α	α	β
C1	98.19	98.14	92.41	96.29	99.25	93.40	92.89	96.71
C2	71.99	71.95	71.60	74.23	73.25	73.00	72.28	74.94
C3	73.25	73.25	73.25	76.15	74.30	74.30	73.58	76.74
C4	69.67	69.67	69.72	69.57	70.80 or 71.30*	70.80 or 71.30*	70.44	70.40
C5	71.65	71.65	70.21	74.48	72.70	71.30*	72.24	76.56
C6	60.62	60.62	65.92	65.48	61.80	67.00	61.40	61.57

[a] Present work.
[b] Data taken from Ref. 27.
[c] Data taken from Ref. 31.
* Assignment left uncertain in Ref. 27.

low the detection threshold of the used analytical techniques.

α-D-Glucosidases are hydrolytic enzymes acting on oligosaccharides containing a α-D-glucopyranose moiety at the nonreducing end. These enzymes are classified into three types (I, II, and III) on the basis of their substrate specificity (34). Many α-D-glucosidases have been reported to be present in microorganisms. α-D-Glucosidase from *A. niger* have been purified and characterized (19, 35). It belongs to type II (19, 35) and shows a good hydrolytic activity toward Glcα-*O*-*p*NP and isomaltose. In experiments using Isomaltose as donor, ^{14}C-labeled glucose as acceptor and the pure *A. niger* α-D-glucosidase as catalyst, it has been showed that this enzyme produced mainly ^{14}C-labeled isomaltose, together with minor isomeric components (36). Recently it has been demonstrated that the hydrolytic specificity of α-D-glucosidases is correlated with the regioselectivity of the transglycosylation reaction (37). Our results confirm those observations.

Although *A. niger* α-D-glucosidase has been extensively studied and characterized, only few reports deal with its use as catalyst for transglycosylation reaction, only to obtain glycosides (38, 39).

The data presented here clearly evidentiate that, using α-D-glucosidase from *A. niger*, the regiospecific transglycolytic synthesis of the disaccharide isomaltose can be achieved with good yield, comparatively short incubation time and use of very simple substrates. In addition, thanks to the high purity of the synthesized product enabled us to achieve the complete ^{13}C spectral assignment.

TABLE 2

Chemical Shift Difference $\Delta_{(\beta-\alpha)} = \delta C_\beta - \delta C_\alpha$ between Carbon-13 Atoms of the β and α Anomers of the Synthesized Isomaltose[a] and of Melibiose[b]

	Glcα1-		-6Glc	
	$\Delta_{(\beta-\alpha)}$[a]	$\Delta_{(\beta-\alpha)}$[b]	$\Delta_{(\beta-\alpha)}$[a]	$\Delta_{(\beta-\alpha)}$[b]
$C1_{\alpha,\beta}$	−0.05	−0.04	3.88	3.88
$C2_{\alpha,\beta}$	−0.05	0.00	2.63	2.62
$C3_{\alpha,\beta}$	0	0	2.90	2.94
$C4_{\alpha,\beta}$	0	0	−0.15	−0.15
$C5_{\alpha,\beta}$	0	0	4.27	4.25
$C6_{\alpha,\beta}$	0	0	−0.08	−0.09

[a] From Table 1.
[b] Refs. 28 and 30.

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