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The efficient enzymatic synthesis of *N*-acetyllactosamine in an organic co-solvent

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Abstract

In the presence of β-galactosidase from *Bifidobacterium bifidum*, *N*-acetyllactosamine was synthesized in significantly enhanced yield compared with earlier routes. Different proportions of the (1→4)- and (1→6)-linked forms were obtained depending on the choice of enzyme and reaction conditions, viz. the nature of added organic co-solvent (20–80% of 2-ethoxy ethyl ether, trimethyl phosphate, or acetone). The β-(1→4)-linked disaccharide was the major product and the β-(1→6)-linked disaccharide was the minor product. With β-galactosidases from *P. multicolor*, *A. oryzae*, *B. longum* the β-(1→6) linkage was exclusively synthesized. Procedures for optimising the yield of *N*-acetyllactosamine are discussed. An immobilized enzyme on a nylon powder column was used for the efficient recycling of enzyme and synthesizing the disaccharide.

Keywords: Immobilized β-galactosidase; *Bifidobacterium bifidum*; *N*-acetyllactosamine; Organic co-solvents; Nylon powder

1. Introduction

The use of enzymes for the synthesis of glycosidic linkages is of interest because of their regio- and stereoselectivity [1–3]. In addition, there are potential advantages in manipulating the water content in enzymatic reactions by introducing suitable organic solvents so as to favor synthesis over hydrolysis [4]. Although solvents of high polarity often cause enzyme deactivation in the synthesis of oligosaccharides, several successful applications by kinetic transglycosylation catalyzed with glycosidases have been reported in, e.g., 50% acetonitrile [5], 70% acetone [6], 60% diethylene glycol diethyl ether [7] and 90% acetonitrile [8]. Here, the specific aim is to study reactions in media of reduced water content through the addition of organic solvents which are water-miscible but do not contain functional groups (e.g., OH), which might participate in transglycosylation: and to increase the yield by reducing the extent of the competing hydrolysis and by enabling the dissolution of hydrophobic substrates. In order to determine and extend the range of conditions which might usefully be applied to the desired syntheses we have measured the activities of β-galactosidase from *Bifidobacterium bifidum* in wa-

Abbreviations: PNPGal, 4-nitrophenyl-β-D-galactopyranoside; GalF, fluoro-β-D-galactopyranoside; *N*-Acetyllactosamine, 2-acetamido-2-deoxy-4-*O*-β-galactopyranosyl-D-glucose; *N*-Acetylallolactosamine, 2-acetamido-2-deoxy-6-*O*-β-galactopyranosyl-D-glucose; EEE, 2-ethoxy ethyl ether; TMP, trimethyl phosphate; TEP, triethyl phosphate; MEA, 2-methoxyethyl acetate; GlcNAC, 2-acetamido-2-deoxy-*n*-glucose.

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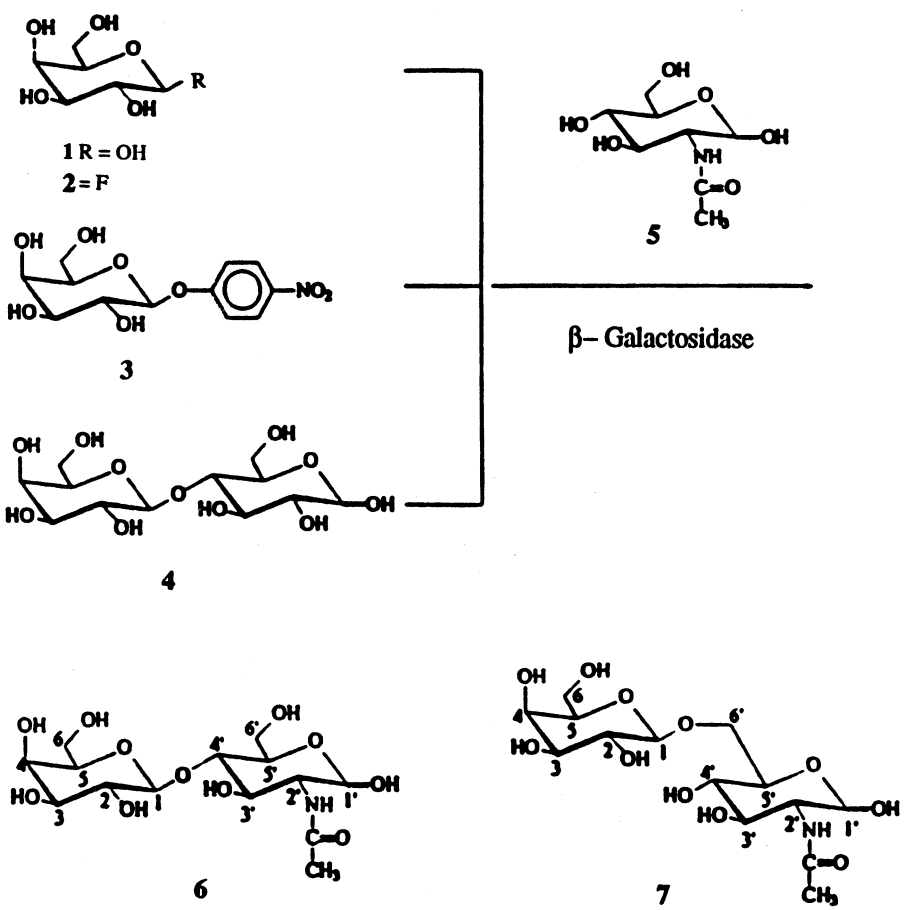
ter–solvent mixtures. The results indicate that the enzyme activity is maintained in different degrees in the presence of trimethyl phosphate, triethyl phosphate, acetone, acetonitrile and 2-ethoxy ethyl ether. In addition, we describe two syntheses of *N*-acetyllactosamine with β-galactosidase from *B. bifidum* using (i) the free form of the enzyme with various donors in a system incorporating 2-ethoxy ethyl ether, trimethyl phosphate, and an acetone-buffer and (ii) the immobilized form of the enzyme on nylon powder in the buffer system

2. Results and discussion

Among β-galactosidases from a number of microbes screened, those from *P. multicolor*, *A. oryzae*, and *B. longum* produced mainly (1→6) linkage. β-Galactosidase from *B. bifidum* however mainly produced β-(1→4)-linked disaccharide, with the β-(1→6)-linked disaccharide as minor product (see Scheme 1). The high-performance liquid chromatography (HPLC) chromatogram of the reaction products from PNPGal and *N*-acetylglucosamine (1:3 donor–acceptor) with *B. bifidum* β-galactosidase (25 units) in 20% of 2-ethoxy ethyl ether in an sodium acetate buffer (50 mM, pH 5.1) system after 2 h of reaction, is shown in Fig. 1.

As the use of organic solvents solubilises non-polar substrates and increases the yield of product by reducing the extent of the competing hydrolysis, we explored this option. The activities of the β-galactosidase from *B. bifidum* in solvent–buffer systems were measured as a function of time and expressed as a percentage of the initial activity in the buffer (see Fig. 2). With these solvents biphasic decay was apparent from the marked change in the slopes of the reaction plot after 6–20 h. These results were consistent with structural



Scheme 1. The formation of *N*-acetyllactosamine with β-galactosidase from *B. bifidum*.

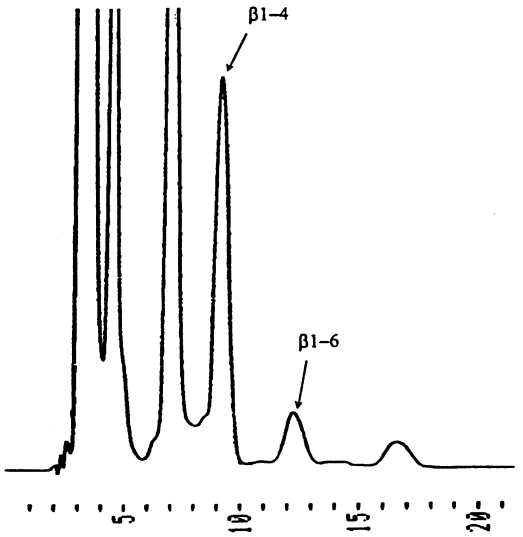


Fig. 1. HPLC chromatogram of *N*-acetyllactosamine reaction mixture.

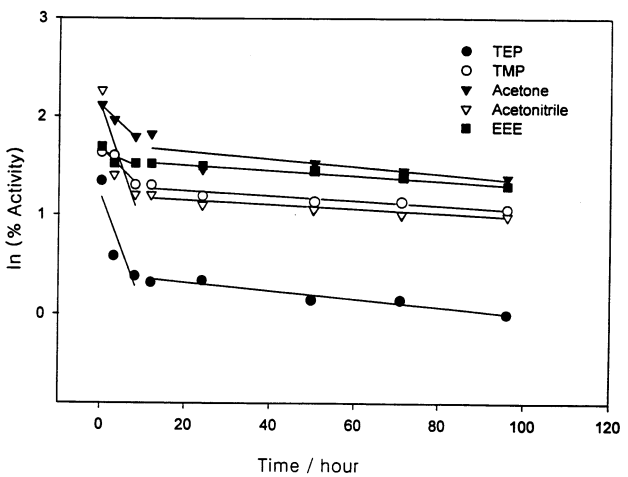


Fig. 2. Plot of % enzyme activity vs. time for *B. bifidum* β-galactosidase.

changes taking place within the protein when in contact with 50% (v/v) organic solvent–buffer mixtures. A more stable protein conformation might be adopted in the absence of substrate after several hours incubation. Alternatively, two distinct enzyme forms may be present in solutions, which possess different activity decay rates. Of the five different solvents tested, TMP, EEE and acetone were selected for examination of their synthetic potential.

The solvent effects with different concentrations (20, 50, 60, 80%) of EEE, TMP and acetone, with PNPGal or lactose as the donor on β-D-galactosidase mediated transglycosylation were investigated. Reaction conditions and yields are listed in Table 1. The yield of the major product, **6** with PNPGal as the donor reached a maximum (41%) after 8 h in a 50% EEE co-solvent system and prolonged incubation reduced the yield in favor of the *allo* isomer. Other products were also formed in small amounts. In the 20% co-solvent system, the maximum yield of **6** and **7** was reached after 2 h and the hydrolysis of products was more rapid than in the 50% solvent system (data not shown). In an 80% co-solvent system the observed rate of product formation was much slower than in the system with lower co-solvent. With both TMP and acetone the maximum yield was obtained in a 20% solvent system.

Comparison of the three different solvents showed the rate of hydrolysis to be in the order, acetone > EEE > TMP, but the yield of **6** was in a slightly different order, EEE > acetone > TMP. EEE buffer systems (*B. bifidum*, 20 and 50%) generated a better yield (41%) of **6** compared with previously reported yields of *N*-acetyllactosamine in 20% of triethylphosphate, 31% (*D. pneumoniae*) and 37% (*B. circulans*), respectively [9].

Using GalF as the donor, the yield of **6** (19%) was lower than with PNPGal. In view of the complications in synthesising GalF an investigation into the effects of different co-solvents on the yield of **6** was not pursued. The maximum yield of **6** with lactose as the donor in the buffer or in a 50% EEE co-solvent was reached, respectively, after 1 and 2 h and decreased thereafter. Kinetic results with lactose were similar to those with PNPGal. When different amounts of TMP, EEE and acetone (20, 50, 60 and 80%) with lactose were introduced, the maximum yields were not very different from those obtained with the aqueous buffer. With lactose as donor the introduction of organic solvents did not increase the yield of **6**. However, it was noticeable that with higher concentrations organic solvents (50–60%), the formation of many minor condensation products (data not shown) seems to follow the hydrolysis of lactose.

From these experiments it appeared that the solvent systems that confer the highest hy-

drolytic stability on the enzyme, e.g., acetone for β-galactosidase from *B. bifidum*, did not necessarily give the highest yield of the disaccharide. For example, it was the EEE co-solvent system (20–50%) which showed the highest yield of lactosamine. We have reported similar phenomenon [9] in the case of β-galactosidase from *K. fragilis* or *A. oryzae* which showed highest hydrolytic activity in actonitrile or MEA, respectively, and where no enzymic transformations were observed. However, with TMP and TEP both of which confer only reasonable hydrolytic activities, product yields were higher. It is concluded that solvents can have different effects on different reactions catalyzed by the same enzyme. Therefore, organic solvents which confer reasonable enzyme stability have to be assessed in terms of their hydrolytic activity and then in terms of their synthetic potential.

With the EEE co-solvent system, the ratio of **6** and **7** varies from 11:1 (20%) to 3:1 (50%). From this result, the perceived advantage in introducing organic co-solvents for the synthesis of oligosaccharides by enzymic transglycosylation is a change in the proportion of condensation products rather than the change in regioselectivity. Similar results were observed by Laroute and co-workers [10] who reported that although the overall regioselectivity in glucoamylase-catalysed glucose condensation (forming either maltose or isomaltose) was unaffected by added organic co-solvent, the relative ratio of the products varied from 0.56 (in 1,3-butanol) to 7.3 (in diethylene glycol diethyl ether). On the basis

Table 1
Reactants, conditions and products in enzymatic syntheses

Solvent	Donor	Donor/acceptor ratio	Reaction time (h)	Product (yield %)
EEE 20%	**3**	1:3	2	**6** (41) **7** (3.6)
50%			8	(41) (13)
60%				(39) (13)
80%				(27) (5.9)
TMP 20%	**3**	1:3	2	**6** (36) **7** (1.3)
50%			19	(21) (7.5)
60%			24	(15) (6.9)
80%			55	(3.9) (6.0)
Acetone 20%	**3**	1:3	8	**6** (33) **7** (1.6)
50%			8	(30) (6.6)
60%			24	(14) (7.8)
80%			8	(8.0) (5.0)
Buffer	**4**	1:3	2	**6** (21) **7** (3.5)
EEE 20%			2	(22) (1.1)
50%				(23) (1.2)
60%				(22) (1.3)
80%			6	(15) (4.4)
TMP 20%	**4**	1:3	2	**6** (15) **7** (3.1)
50%			4	(10) (3.6)
60%			24	(5.6) (6.2)
80%			1	(2.8) (0.5)
Acetone 20%	**4**	1:3	2	**6** (23) **7** (2.1)
50%			4	(19) (2.5)
60%			6	(11) (2.4)
80%			6	(2.2) (1.4)
EEE 20%	**2**	1:3	10	**6** (19) **7** (3.7)
Buffer immobilized enzyme	**4**	1:2.5	24	**6** (11) **7** (2.4)
Buffer immobilized enzyme	**1**	1:1	5 days	**6** (1.0)

of these observations, it appears that the source of the enzyme is the most important factor determining the nature and relative yield of products.

With high concentrations of organic solvents the reaction became slower; to achieve the same yield as in the medium containing less co-solvent the length of the incubation period and the enzyme concentration have to be increased. However, since both the formation and hydrolysis of minor products are also slow when organic solvents are used, desired minor products can be studied and isolated with greater ease. PNPGal in the EEE system produced a higher yield of **6** than with lactose because of its higher solubility in the presence of the organic co-solvent. Thus small amounts of organic co-solvent can be used without seriously affecting the enzyme activity. In general, if a regioselective specific enzyme is used with less polar substrates it is worthwhile determining the optimal content of organic solvents because greater amounts of organic solvents generally reduce enzyme activity further.

In order to use the enzyme repeatedly we immobilized the β-galactosidase (from *B. bifidum*) on a nylon powder column and activated carbon: lactose or galactose were chosen as donors because they are cheaper than PNPGal which gives a higher yield. When the lactose solution was circulated through a column containing immobilized system (see Section 3) the yield of product **6** was 11.1% in the first run and the yield was slightly lower in the second run. When unmodified substrate, galactose solution was used in the same system a yield of 1.0% (**6**) was obtained. Several other cases also have been reported in such low yields with galactose [11,12]. With lactose as donor the yield of **6** using a free enzyme synthesis was twice that using the immobilized enzyme method. However, the method is useful because the immobilized enzyme could be cycled several times without replacement, and the activity of the enzyme immobilized on nylon powder did not change over 2 months at 4 °C. Nylon is an attractive matrix because it is cheap and available in many different forms.

3. Experimental

General methods.—Chemicals were from Sigma and Aldrich (St Louis, MO, USA) and were used without further purification unless otherwise stated. Nylon powder was from Portex Ltd (Hythe, Kent, UK). *P. multicolor* (K.I. Chemicals, Shizuoka, Japan), *A. oryzae* and *B. longum* (Toyobo, Co., Osaka, Japan) were commercial products.

A 150 mM ammonium bicarbonate buffer at pH 7.5 (buffer A, to neutralize released HF) and 50 mM sodium acetate buffer at pH 5.1 (buffer B) were used. One unit of activity is the amount of enzyme that will liberate 1 μmol of 4-nitrophenol from 4-nitrophenyl-β-D-galactopyranoside per min under the given conditions. ^{1}H and ^{13}C NMR spectra were recorded in D_2O on a Bruker AMX 600 spectrometer (^{1}H, 600.14 MHz; ^{13}C 150.92 MHz) with TSP (trimethylsilyl-1-propanesulfonic acid, sodium salt) as a reference. Peak assignments were based on COSY and HSQC experiments. An HPLC equipped with an evaporative light-scattering detector (ELSD) was used with an Asahipak NH2P-50 column (4.6 × 250 mm, eluted with 1:3 water–acetonitrile, v/v, 1 mL/min)

Preparation of galactosyl fluoride.—To penta-*O*-acetyl-β-galactopyranoside (2.5 g) in a polyethylene bottle was added pyridine–HF (70%, 12 mL) and the mixture was stirred for a few minutes at −15 °C until a homogeneous solution was obtained. It was kept for the appropriate time at room temperature (rt) and CH_2Cl_2 (20 mL) was then added. The mixture was washed with water (3 × 20 mL) and with $NaHCO_3$ (20 mL), dried and evaporated, leaving the crude fluoride. This peracetylated galactosyl fluoride mixture was purified by flash column chromatography with the elution system of 4:1:1 toluene–EtOAc–Et_3N. The purified product was treated with methanolic NaOMe to give deacetylated galactosyl fluoride (yield 1.3 g, 52%) [13].

Immobilization of β-galactosidase with nylon powder.—All experimental steps were carried out under nitrogen gas. Nylon 6 powder (10 g, 88.7 mmol) was suspended in 100 mL of CH_2Cl_2. TTFB (Triethyloxonium tetrafluoroborate, 10 mL, 10 mmol) and cold

MeOH (200 mL) were added and after a few hours reaction time, the nylon powder was collected and then incubated with 30% PEI (polyethyleneimine, 100 mL) solution in $NaHCO_3$ buffer (0.1 M, pH 9.4) overnight (18 h). Then a further reaction was carried out with glutaraldehyde (5%) for 3 h at rt. After repeated washing with buffer the enzyme solution was stirred with activated nylon powder at 4 °C for 24 h (activity: 0.003 units/mg) [14].

Preparation of β-galactosidase from B. bifidum.—β-galactosidase from *B. bifidum* (DSM 20456) was cultured in Briggs liver broth for 7 days under anaerobic conditions (components of Briggs liver broth: tomato juice 400 mL, neopeptone 15 g, yeast extract 6 g, liver extract 75 mL, glucose 20 g, soluble starch 0.5 g, Tween 80 1 g, L-cysteine/HCl, 0.2 g, distilled water 505 mL, pH 6.8). The culture broth was centrifuged at 6000 rpm for 30 min at 4 °C. The supernatant so obtained had a β-galactosidase activity of 101 units/mL and was used without further purification [15].

Enzyme activity stability experiments.—Mixtures of enzyme (*B. bifidum*) in 50% organic solvent–buffer mixtures were stored at rt for a total of 4 days. Aliquots (0.1 mL) of the enzyme solution were removed at various time intervals and were added to a solution of PNPGal (2.9 mL, 5 mM, in the same solvent–buffer mixtures) and the enzyme activity measured as described above.

*2-Acetamido-2-deoxy-4-O-β-D-galactopyranosyl-D-glucose (***6**, *Scheme 1)*

Method A. β-Galactosidase from *B. bifidum* culture broth (0.25 mL, 25 units) was incubated with GalF (**2**) or PNPGal (**3**, each 300 mg, 1.5 mmol, 1 mmol) as the donor and GlcNAc (**5**, 600 mg, 3 mmol) as the acceptor in a suitable A, or B buffer containing 20% 2-ethoxy-ethyl ether (total 2 mL). The system was stirred at 37 °C for 10 h. The reaction was then stopped by deactivating the enzyme at 100 °C for 5 min; it was centrifuged and the filtrate was applied to an activated carbon column (2.0 × 50 cm). After washing the column with distilled water for 3 h at a flow rate of 2 mL/min to remove monosaccharides, the disaccharides were eluted with the linear gradient of water (2 L) and 30% aq EtOH (2 L). Disaccharide fractions were detected by the phenol–H_2SO_4 method and peaks were identified by the following method; after the fractions corresponding the disaccharides elution (Fractions numbered 40–45, **7** and Fractions numbered 53–67, **6**) were removed and the solution had been concentrated, *N*-acetyllactosamine (**6**) was obtained (148 mg, 41%). The minor product was identified as *N*-acetylallolactosamine (**7**, 22.4 mg, 5.9%). The same enzyme in various aliquots of EEE, trimethyl phosphate and acetone (20, 50, 60, 80%) were used with PNPGal (**3**, 300 mg, 1 mmol) as the donor and GlcNAc (**5**, 600 mg, 3 mmol) as the acceptor. Reaction conditions and procedures were as the same as above. The yields are summarized in Table 1. ^{13}C NMR: C1 (104.1) C1 α,β (90.5, 94.5), C2 (70.8), C2′α,β (53.5, 56.4), C3 (72.4), C3′ α,β (69.2, 72.3), C4 (68.4), C4 α,β (78.7, 78.4), C5 (75.3), C5′ α,β (70.2, 74.8), C6 (61.0), C6′ α,β (60.0, 60.6), CO α,β (174.8, 174.5), CH_3 α,β (22.2, 22.0).

Method B. To a solution of lactose (**4**, 500 mg, 1.5 mmol) and GlcNAc (**5**, 1 g, 4.5 mmol) in buffer B (0.7 mL), β-galactosidase from *B. bifidum* (30 units, 0.3 mL) was added. The reaction mixture was incubated at 37 °C for 10 h. The subsequent procedures were the same as described in (A). The yield of *N*-acetyllactosamine (**6**) was 21% (109 mg). The other product was isolated as *N*-acetylallolactosamine (**7**, 8 mg, 1.4%).

Method C. A substrate solution containing galactose (**1**, 4 g, 22 mmol) and GlcNAc (6 g, 27 mmol, total 40 mL) was circulated continuously through two columns containing the immobilized enzyme (β-galactosidase of *B. bifidum* (3 units/g) on nylon powder (45 g) and activated carbon (20 g). After 5 days, the activated carbon column was removed from the system and washed first with water and then the disaccharides and oligosaccharides were eluted with 35% EtOH. The sample was analysed by HPLC. Next, the residual solution of the monosaccharides was circulated continuously again in the same system, but with the activated carbon column replaced.

Method D. A solution containing lactose (**4**, 4 g, 11 mmol) and GlcNAc (**5**, 6 g, 27 mmol, total 40 mL) was circulated continuously in the system consisting of a column of β-galactosidase (*B. bifidum*, 3 units/g, 45 g) immobilized on nylon powder. The reaction was carried

out for 24 h and subsequent procedures were the same as described in (A). The yield of **6** (*N*-acetyllactosamine) was 11.1% (58 mg). The other product was isolated as **7** (*N*-acetylallolactosamine) (6.4 mg, 1.2%). The same procedure was repeated several times.

*2-Acetamido-2-deoxy-6-*O*-β-*D*-galactopyranosyl-*D*-glucose*.—β-Galactosidase from *P. multicolor*, *A. oryzae*, *B. longum* culture broth (0.25 mL, 25 units) was incubated with PNPGal (**3**) (300 mg, 1 mmol) as the donor and GlcNAc (**5**) (660 mg, 3 mmol) as the acceptor in 60% trimethyl phosphate–buffer B system (total, 2 mL) at 37 °C for 10 h. Samples (5 μL) were taken at various time intervals. Each sample was heated at 100 °C for 5 min, and made up 10 μL with deionized water. After centrifugation, 5 μL aliquots were applied to the HPLC and eluted with 80% aqueous acetonitrile at a flow rate of 1.0 mL/min The major peak was isolated and identified as *N*-acetylallolactosamine (**7**) by ^{13}C NMR: C1 (104.1) C1′ α,β (90.5, 94.5), C2 (71.2), C2′α,β (53.8, 56.6), C3 (72.6), C3′ α,β (70.4, 73.6), C4 (68.8), C4 α,β (70.1), C5 (75.0), C5′ α,β (70.8, 75), C6 (60.8), C6′ α,β (68.6, 68.2), CO α,β (174.8, 174.5), CH_3 α,β (22.2, 22.0).

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