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Synthesis of *n*-alkyl glucosides by amyloglucosidase

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Amyloglucosidase from *Rhizopus* mold (3.2.1.3) has been employed for the synthesis of *n*-alkyl glucosides from alcohols of carbon chain lengths C1 to C18 by both shake flask and reflux methods. Glucoside yields obtained from the reflux method (5-44%) are better than those from the shake flask method (3-28%). While the shake flask method favoured glucosylation of medium chain length alcohols, the reflux method at *p*H 5.0, favoured glucosylation of all the chain lengths. *n*-Octyl-D-glucoside, *n*-octyl-maltoside and *n*-octyl-sucroside are also synthesized and optimum conditions for the synthesis of *n*-octyl-D-glucoside at both shake flask and reflux methods have been worked out.

Keywords: Amyloglucosidase, shake flask method, reflux method, 6-O-alkylation, glucosylation, *n*-octyl-glycoside

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Regioselective glycosylation involving carbohydrates is a quite challenging synthetic objective because of several hydroxyl groups in these molecules[1]. Many regio and stereo selective transformations of carbohydrate have been carried out recently using enzymes[2] and their use in glycoside synthesis avoids selective protection and deprotection and result in better control of configuration[3]. Enzymatic glycosylation is usually effected by glycosidases in both aqueous and organic media[4] under thermodynamically or kinetically controlled conditions[5,6] by employing non-aqueous, solvent free, high substrate, high temperature and moderate to high water activity conditions to yield glycosides[7-13]. Many of these glycosides are used as sweeteners, food additives, non-ionic surfactants, antibiotics in pharmaceutical preparations, artificial primers for glycogen biosynthesis and cosmetics[14-19].

The present study describes synthesis of *n*-alkyl glucosides of carbon chain lengths C1-C18 and few *n*-octyl glycosides using amyloglucosidase by both shake flask and reflux methods.

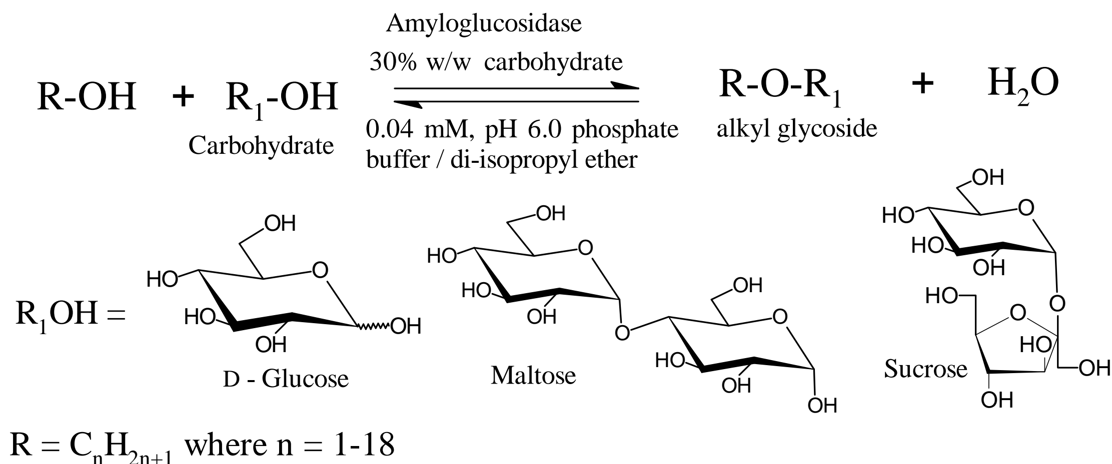
Results and Discussion

In the present work, enzymatic glycosylation was carried out using amyloglucosidase from *Rhizopus* mold, a commercially available enzyme known to cleave $\alpha(1\rightarrow4)$ glycosidic linkage of starch to give glucose. Glucosylation occurred with amyloglucosidase only in the presence of buffer. A general scheme for the glycosylation reaction is shown in **Scheme I**.

n-Octyl glucoside

Synthesis of *n*-octyl-D-glucoside was studied in detail. Comparative studies of the preparation in shake flasks under non-solvent conditions or in presence of very little solvents and by reflux method involving refluxing and stirring the reaction mixture in di-isopropyl ether solvent were carried out. Effects of incubation period, enzyme concentration, *p*H and buffer concentration were studied by both shake flask and reflux methods. At shake flask level, the optimum conditions were found to be 30% (w/w D-glucose) amyloglucosidase (**Table I**) concentration and 0.4 mL (0.8 mM) *p*H 4.0 acetate buffer at an incubation period of 72 hr. Similarly, the optimum conditions for the reflux method was also 30% (w/w D-glucose) amyloglucosidase (**Table I**) concentration and 0.4 mL (0.04 mM) *p*H 8.0 borate buffer at an incubation period of 72 hr. From the reflux method, the rate of glucosylation was found to be 4.74 µmole/hr.

n-Octyl maltoside (conversion yield 15%) and *n*-octyl sucroside (conversion yield 13%) were also



Scheme I

synthesized by this procedure. Out of 11 carbohydrates employed (aldohexoses-D-glucose, D-galactose, D-mannose, pentoses-D-ribose, D-arabinose, ketose-D-fructose, disaccharides-maltose, lactose, sucrose and carbohydrate alcohol-D-mannitol, D-sorbitol), only D-glucose, maltose and sucrose formed glycosides with *n*-octanol.

The reaction mixtures were analyzed by HPLC, which showed clear indication of glycoside formation both in case of *n*-octyl glycosides and various other alkyl glucosides. In the amino propyl column in $CH_3CN:H_2O$ (80:20 v/v) mobile phase, the free carbohydrate peak eluted first around 4.5 to 7.5 min (Experimental Section) followed by the glycosides around 7.0 to 10.5 min. The area under the glycoside peak with respect to that of the free carbohydrate gave the conversion yield. Some of the glucosides showed two separate peaks indicating that the C1 glycosylated and 6-O-alkylated products are formed. Chromatographic separation of the reaction mixtures on Sephadex G-15 gave good separation of the product glycosides from free carbohydrates. However, the product glycosides could not be separated into individual glycosides through chromatography. They were all solids, and were characterised spectroscopically.

UV-Vis spectra of *n*-octyl glycosides showed $\sigma \rightarrow \sigma^*$ bands in the range 194 nm to 206 nm (free *n*-octanol at 204 nm); IR spectral bands for the C-O-C symmetrical stretching in the 1033-1053 cm^{-1} range and the parent ion peaks in the mass spectra at *n*-octyl glucoside-294 $[M+2]^+$ and 317 $[M+2+Na]^+$, *n*-octyl maltoside-455 $[M+1]^+$ and *n*-octyl sucroside-455 $[M+1]^+$. Optical rotation of $+45.8^o$ for *n*-octyl glucoside indicated that the α:β anomeric composition of the glucosides formed was 70:30 (based on the specific rotation values of the *n*-octyl-α-O-glucoside and *n*-octyl-β-O-glucoside). This was further confirmed by the 2D-HSQCT spectrum from the area of C1 cross peaks for α : β anomers to be 75:25. The D-glucose employed was a 40:60 α and β anomeric mixture. Critical micellar concentration (CMC) for *n*-octyl glucoside was determined to be 16.1 m*M* (0.47%) by using Coomassie Blue reagent method at 620 nm[23].

Two-dimensional HSQCT spectra for the glycosides clearly established glycosylation. *n*-Octyl glycosides are surfactant molecules, which form micelles above certain critical micellar concentrations (CMC). Since the concentrations employed for NMR measurements are very much higher than their respective CMCs, the ^1H NMR signals were unusally broad such that, in spite of recording the spectra at 35°C, the individual coupling constant values could not be determined precisely. For the *n*-octyl glucoside, the two cross peaks at carbon chemical shift values 98.5 and 103.2 ppm apart from that of unreacted D-glucose indicated formation of C1α and C1β of *n*-octyl-D-glucoside. Straight chain proton signals from the *n*-octyl portion of the glycosides were observed in the range 1.11 to 1.32 ppm (C2′-C7′, CH_2), CH_2-O-at 3.1 ppm and CH_3 at 0.85 ppm[22]. 2D HSQCT NMR data showed that the major product was found to be the one glucosylated at the C1 position and a small amount of C6-O-alkylated product was also detected. NMR data showed formation of C1α glucoside, C1β glucoside and C6-

Table I — Effect of enzyme concentration, *p*H and buffer concentration on the synthesis of *n*-octyl-D-glucoside [a]

Experimental	Shake flask method Yield - % (μmole)	Reflux method Yield - % (μmole)
Enzyme %(w/w glucose)[b]		
10	26 (143)	11 (58)
20	28 (153)	8 (42)
30	28 (155)	46 (255)
40	28 (154)	26 (142)
50	27 (149)	40 (223)
60	25 (139)	-
75	-	27 (147)
80	19 (106)	-
100	10 (55)	-
*p*H[c], 0.01 M		
4.0	20 (109)	33 (183)
5.0	9 (52)	30 (164)
6.0	17 (96)	40 (222)
7.0	9 (52)	36 (199)
8.0	15 (86)	43 (238)
Buffer volume (mL)[d]		
0.1	15 (86)	5 (29)
0.2	17 (94)	8 (44)
0.4	20 (109)	40 (223)
0.6	4 (24)	31 (173)
0.8	4 (24)	No yield
1.0	-	No yield

[a] Conversion yields from HPLC with respect to 0.000555 mole of D-glucose. Yields are an average from two experiments. *n*-Octanol - 50 eq, temperature - 60°C for shake flask and 68°C for reflux method. [b] *p*H - 6.0, 0.01M, buffer - 0.04 mM for reflux method and 0.8 mM for shake flask method. [c] enzyme - 50% w/w D-glucose, buffer - 0.04 mM for reflux method and 0.8 mM for shake flask method. [d] enzyme - 50% w/w D-glucose, *p*H-4.0 (0.01 - 0.1 mM) for shake flask and *p*H-6.0 (0.2 - 2.0 mM) for reflux method. Error in yield measurements will be ± 5-10%. This applies to all the yields given in the subsequent Tables also.

O-alkylated products from *n*-octyl-D-glucose, C1α maltoside from maltose and C1-O-alkylated and C6-O-alkylated products from sucrose.

n-Alkyl-glycosides

n-Alkyl glucosides using alcohols of carbon chain length C1-C18 were synthesized with the following alcohols: methyl alcohol, ethyl alcohol, *n*-propyl alcohol, *n*-butyl alcohol, *n*-amyl alcohol, *n*-hexyl alcohol, *n*-heptyl alcohol, *n*-octyl alcohol, *n*-nonyl alcohol, *n*-decyl alcohol, lauryl alcohol, cetyl alcohol and stearyl alcohol.

Shake flask method

Alcohols of carbon chain lengths C2-C18 were employed for the synthesis of glucosides by shake flask method in presence of 0.4 mL (0.8 mM in the reaction mixture) of 0.01 M *p*H 4.0 acetate buffer **(Table II)**. The yields obtained (with respect to D-glucose) were found to be in the range 3% to 28%. The results showed that the yields are higher for ethanol (10%), *n*-propyl alcohol (13%) and *n*-butanol (9%). For other smaller chain length alcohols like *n*-amyl alcohol (3%), *n*-hexyl alcohol (9%) and *n*-heptyl alcohol (5%), the yields were much lower. However, from *n*-octyl alcohol to *n*-decyl alcohol, the yields were the highest (20% to 23%). The yields decreased slightly with further increase in alcohol chain lengths up to stearyl alcohol. The shake flask method gave lesser yields at *p*H 4.0 in general for the carbon chain lengths up to C7. From *n*-octyl alcohol onwards, the yields increased with increase in medium chain lengths upto C10.

Reflux method

Alkyl glucosides were also synthesized with various alcohols by the reflux method in presence of 0.4 mL (0.04 mM), *p*H 4.0 and 5.0 acetate buffer **(Table II)**. At *p*H 4.0 the yields were lower for methyl (13%), ethyl (5%) and *n*-propyl alcohols (7%). However, for the remaining alcohols, the yields were higher. The highest yield was observed for *n*-amyl alcohol (44%). In general, except lauryl alcohol (10%), the yields were higher for *n*-octyl alcohol (24%) onwards towards higher chain length alcohols.

At *p*H 5.0, the yields obtained were found to be in the range 12-44%. Higher yields were observed for ethyl alcohol (44%) and lauryl alcohol (36%). In general, the yields obtained by the reflux method were much higher than those by the shake flask method for all the alcohols studied. Further in the reflux method, the yields obtained at *p*H 5.0 were better compared to those observed at *p*H 4.0.

Cetyl and Stearyl glucosides

The effect of increasing enzyme concentration on the synthesis of cetyl and stearyl glucosides was

Table II — Synthesis of *n*-alkyl-D-glucosides by shake flask and reflux method [a]

Name of the alcohol	Yield - % (μmole) *p*H 4.0, 0.01 M	Yield - % (μmole) *p*H 4.0, 0.01 M	Yield - % (μmole) *p*H 5.0, 0.01 M
Methyl alcohol	-	13 (71)	25 (141)
Ethyl alcohol	10 (57)	5 (27)	44 (242)
n-Propyl alcohol	13 (73)	7 (39)	35 (197)
n-Butyl alcohol	9 (52)	28 (156)	16 (89)
n-Amyl alcohol	3 (16)	44 (245)	36 (199)
n-Hexyl alcohol	9 (47)	19 (107)	36 (198)
n-Heptyl alcohol	5 (26)	19 (105)	16 (87)
n-Octyl alcohol	20 (109)	24 (134)	30 (164)
n-Nonyl alcohol	28 (156)	22 (123)	22 (120)
n-Decyl alcohol	23 (127)	29 (160)	12 (65)
Lauryl alcohol	17 (94)	10 (55)	36 (200)
Cetyl alcohol	17 (96)	20 (111)	30 (128)
Stearyl alcohol	15 (85)	41 (230)	19 (96)

[a] Conversion yields from HPLC peak areas of glucoside and free D-glucose with respect to D-glucose concentration of 0.000555mole. Yields are an average from two experiments. Alcohol - 50 eq, enzyme - 50% w/w glucose, buffer - 0.4mL (0.04 mM in reaction mixture), 0.01M, temperature- 68°C.

investigated at enzyme concentrations ranging from 10% to 50% w/w D-glucose in presence of 0.4 mL of 0.01M (0.8 m*M*) *p*H 4.0 acetate buffer. In order to ensure proper mixing of the components, 5 mL of *n*-heptane was added to solublise the alcohols. The yields obtained at 40% (w/w D-glucose) enzyme concentration was higher in case of both cetyl (6%) and stearyl glucosides (19%) compared to other enzyme concentrations. The conversion yields for cetyl glucosides are: 10% enzyme-2% yield, 20% enzyme-6% yield, 30% enzyme-6% yield, 40% enzyme-6% yield, and 50% enzyme-6% yield. The conversion yields for stearyl glucosides are: 10% enzyme-13% yield, 20% enzyme-16% yield, 30% enzyme-12% yield, 40% enzyme-19% yield, and 50% enzyme-15% yield. The yields generally were higher at all the enzyme concentrations for stearyl alcohol compared to cetyl alcohol. This could be because the longer chain length alcohol functioned as a better nucleophile for the transfer of a glucose molecule than the shorter chain length alcohol.

Vic and Thomas[13] reported a glycoside yield of 13.1% for methanol, 9.8% for ethyl alcohol, 6.6% for *n*-butanol, 4.9% for *n*-hexanol and 3.6% for *n*-octanol in the reactions carried out with almond β-glucosidase by the shake flask method. The present work, especially by the reflux method with amyloglucosidase showed that the glucoside yields were much higher (methanol-25%, ethyl alcohol-44%, *n*-butanol-28%, *n*-hexanol-36% and *n*-octanol-46%) than those reported.

There are not many reports on the glycosylating potential of amyloglucosidase in the literature. The results from this investigation have shown conclusively that amyloglucosidase is an excellent enzyme for carrying out effective glucosylation of straight chain alcohols.

Experimental Section

All solvents and alcohols employed were distilled once before use. All solvents, alcohols and carbohydrates were purchased from S D Fine Chem. Ltd., India, Qualigens Fine Chemicals, India, and Loba Chem Pvt. Ltd, India. Amyloglucosidase (EC 3.2.1.3) from *Rhizopus* mold purchased from Sigma Chemical Company, USA was used. Acetate buffer (0.01M) for reactions, which required *p*H 4.0 and *p*H, 5.0 buffers, 0.01M Na_2HPO_4 for *p*H 6.0 and 7.0 and $Na_2B_4O_7$ (0.01M) for *p*H 8.0 buffer were employed.

Synthesis of glucosides

Shake flask method

Reactions were carried out in 25 mL stoppered conical flasks wherein D-glucose, 0.1g (0.000555 mole) and alcohol were taken in a molar ratio of 1:50.

Appropriate quantities of amyloglucosidase (10-50% w/w D-glucose) was added along with 0.4 mL of 0.01 M buffer of appropriate *p*H (corresponding to 0.8 m*M* in 5 mL reaction mixture) and incubated at 60 °C in a temperature control shaker at 150 rpm for 72 hr[20]. The reaction mixture was held in a boiling water bath for 5-10 min to denature the enzyme in order to avoid the hydrolytic reaction. Then 15-20 mL of water was added to dissolve the unreacted glucose and the product glucoside. The unreacted alcohol was separated in a separating funnel with petroleum ether or *n*-hexane. The bottom water layer was evaporated to get the unreacted glucose and the product glucoside.

Reflux method

In the reflux method, the reactions were carried out in a 150 mL two-necked flat-bottomed flask[21]. D-Glucose, 0.1g (0.000555 mole) and alcohol were taken in 1:50 molar ratio. An appropriate quantity of amyloglucosidase (10-75% w/w D-glucose) was added along with 0.4 mL of 0.01M buffer of appropriate *p*H (0.04 mM concentration in 100 mL reaction mixture). The reaction mixture was refluxed with 100 mL of di-isopropyl ether for 72 hr. The product work up was as described above.

The reaction mixtures were analyzed by HPLC using an amino-propyl column (3.9 × 300 mm length) and acetonitrile:water in 80:20 ratio (v/v) as the mobile phase at a flow rate of 1mL/min with refractive index detector. Retention times are: D-glucose-5.2 min, *n*-methyl-D-glucoside-7.0 min, *n*-ethyl-D-glucoside-7.1 min, *n*-propyl-D-glucoside-7.1 min, *n*-butyl-D-glucoside-7.1 min, *n*-amyl-D-glucoside-7.1 min, *n*-hexyl-D-glucoside-7.2 min, *n*-heptyl-D-glucoside-7.2 min, *n*-nonyl-D-glucoside-7.5 min, *n*-decyl-D-glucoside-7.6 min, laryl-D-glucoside-7.6 min, cetyl-D-glucoside-7.7 min, stearyl-D-glucoside-7.7 min, maltose-7.4 min, *n*-octyl maltoside-10.5 min, sucrose-6.4 min and *n*-octyl sucroside-9.1 min. Error measurements in HPLC yields will be ± 5-10%. In case of *n*-octyl glycosides, the product glycosides were isolated from the reaction mixture by using a 1×100 cm Sephadex G-15 column and eluting with water at 2 mL/hr. The isolated compounds were solids.

^{1}H, ^{13}C and two dimensional Heteronuclear Single Quantum Coherence Transfer Spectra (2-D HSQCT) NMR spectra were recorded on a Bruker DRX 500 MHz spectrometer (500.13 MHz proton and 125 MHz carbon frequencies). About 50 mg of the sample dissolved in DMSO-d_6 was used for recording the spectra. Chemical shift values were expressed in ppm relative to internal tetra-methyl silane (TMS).

Only resolvable signals are shown. Some assignments are interchangeable. *n*-Octanol signals are primed, non-reducing end glucose signals in maltose are double primed and D-glucose signals in sucrose are double primed.

***n*-Octyl-D-glucoside**: UV-Vis (H_2O, λ_{max}): 206 nm ($\sigma\rightarrow\sigma^*$, log$\varepsilon_{206}$-3.09 M^{-1}), 278 nm ($n\rightarrow\pi^*$, logε_{278}-1.86 M^{-1}); IR (stretching frequency): 1053 (glycosidic C-O-C symmetrical), 3605 cm^{-1} (OH); Optical rotation (C 1, H_2O) $[\alpha]_D$ at 25°C = +45.8°; MS: m/z 294 $[M+2]^+$, 317 $[M+2+Na]^+$; Critical micellar concentration = 16.1 m*M*; 2D HSQCT (DMSO-d_6) ***n*-Octyl-α-D-glucoside**: ^{1}H NMR: δ (500.13 MHz) 4.62 (H-1α), 3.18 (H-2α), 3.42 (H-3α), 3.74 (H-4α), 3.18 (H-5α), 3.42 (H-6α), 3.1 (CH_2-1′), 1.51 (CH_2-2′), 1.23 (CH_2-3′-7′), 0.85 (CH_2-8′); ^{13}C NMR: δ (125 MHz) 98.5 (C1α), 72.0 (C2α), 72.4 (C3α), 70.2 (C4α), 72.1 (C5α), 60.8 (C6 α), 14.0 (C8′), 23.0 (C7′), 31.2 (C6′), 29.8 (C3′), 30.0 (C5′), 70.2 (C1′). ***n*-Octyl-β-D-glucoside**: ^{1}H NMR: δ 4.17 (H-1β), 2.88 (H-2β), 3.12 (H-5β), 3.60 (H-6β), 3.1 (CH_2-1′), 1.51 (CH_2-2′), 1.23 (CH_2-3′-7′), 0.85 (CH_2-8′); ^{13}C NMR: δ 103.2 (C1β), 74.7 (C2β), 77.1 (C3β), 71.0 (C4β), 77.1 (C5β), 61.5 (C6 β), 14.0 (C8′), 23.0 (C7′), 31.2 (C6′), 30.0 (C5′), 70.5 (C1′). **C6-O-alkylated**: ^{1}H NMR: δ 4.90 (H-1α), 3.20 (H-2α), 3.10 (H-5α), 3.64 (H-6α), 3.1 (CH_2-1′), 1.51 (CH_2-2′), 1.21 (CH_2-3′-7′), 0.85 (CH_2-8′); ^{13}C NMR: δ 92.2 (C1α), 72.5 (C2α), 72.1 (C5α), 67.2 (C6 α), 14.0 (C8′), 29.5 (C3′), 30.0 (C5′), 70.5 (C1′).

***n*-Octyl maltoside**: UV-Vis (H_2O, λmax): 194 nm ($\sigma\rightarrow\sigma^*$, log$\varepsilon_{194}$-3.17 M^{-1}), 278.5 nm ($n\rightarrow\pi^*$, log$\varepsilon_{278.5}$-1.98 M^{-1}); IR (stretching frequency): 1033 (glycosidic C-O-C symmetrical), 1255 (glycosidic C-O-C asymmetrical), 3415 cm^{-1} (OH); Optical rotation (C 1, H_2O), $[\alpha]^D$ at 25°C = +91.1°; MS: m/z 455 $[M+1]^+$; 2D HSQCT (DMSO-d_6) ^{1}H NMR: δ (500.13 MHz) 4.63 (H-1α), 3.30 (H-5α), 3.66 (H-6α), 4.99 (H-1″α), 3.46 (H-2″α), 3.20 (H-3″α), 3.08 (H-4″α), 3.44 (H-6″α), 2.9 (CH_2-1′), 1.11-1.25 (CH_2-2′-7′), 0.85 (CH_2-8′); ^{13}C NMR: δ (125 MHz) 98.8 (C1α), 75.0 (C5α), 60.5 (C6α), 100.8 (C1″α), 71.8 (C2″α), 72.1 (C3″α), 70.2 (C4″α), 61.0 (C6″α), 14.1 (C8′), 23.0 (C7′), 31.5 (C6′), 29.8 (C3′), 29.0 (C2′), 70.3 (C1′).

***n*-Octyl sucroside**: UV-Vis (H_2O, λmax): 205 nm ($\sigma\rightarrow\sigma^*$, log$\varepsilon_{205}$-3.41 M^{-1}), 276 nm ($n\rightarrow\pi^*$, logε_{276}-

2.41 M^{-1}); IR (stretching frequency): 1054 (glycosidic C-O-C symmetrical), 1259 (glycosidic C-O-C asymmetrical), 3357 cm^{-1} (OH); Optical rotation (C 1, H_2O), $[\alpha]^D$ at 25°C = +13.3°; MS: m/z 455 $[M+1]^+$; 2D HSQCT (DMSO-d_6) **C1**-O-**alkylated**: ^{1}H NMR: δ (500.13 MHz) 3.76 (H-1α), 3.81 (H-4α), 3.79 (H-5α), 3.40 (H-6α), 5.18 (H-1″α), 3.10 (H-3″α), 3.03 (H-4″α), 3.54 (H-5″α), 3.62 (H-6″α), 3.01 (H-1′), 1.01-1.23 (H-2-7′), 0.84 (H-8′); ^{13}C NMR: δ (125 MHz) 62.8 (C1α), 104.0 (C2α), 75.4 (C4α), 83.0 (C5α), 62.0 (C6α), 91.5 (C1″α), 72.2 (C3″α), 70.5 (C4″α), 72.0 (C5″α), 61.0 (C6″α), 14.4 (C8′), 23.2 (C7′), 31.5 (C6′), 29.2 (C5′), 29.6 (C2′), 70.2 (C1′). **C6**-O-**alkylated**: ^{1}H NMR: δ 3.54 (H-1α), 3.87 (H-3α), 3.72 (H-4α), 3.72 (H-5α), 3.25 (H-6α), 4.90 (H-1″α), 3.17 (H-3″α), 3.11 (H-4″α), 3.44 (H-5″α), 3.48 (H-6″α), 3.01 (H-1′), 1.0-1.25 (H-2-7′), 0.85 (H-8′); ^{13}C NMR: δ 61.8 (C1α), 104.04 (C2α), 77.4 (C3α), 76.0 (C4α), 82.0 (C5α), 63.0 (C6α), 92.1 (C1″α), 72.2 (C3″α), 70.2 (C4″α), 72.0 (C5″α), 61.2 (C6″α), 14.6 (C8′), 23.3 (C7′), 31.3 (C6′), 29.6 (C4′), 29.7 (C2′), 70.0 (C1′).

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