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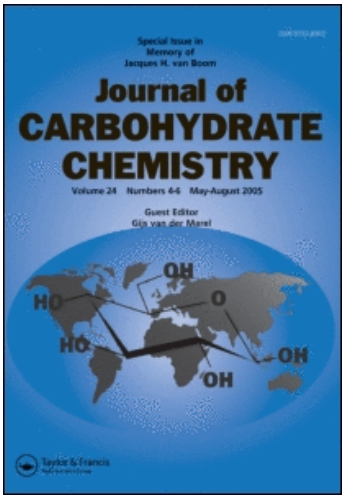

Journal of Carbohydrate Chemistry

Publication details, including instructions for authors and subscription information:
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Octa-*O*-Acetyl-Sucrose: Regioselective Deacetylations by Lipolytic Enzymes

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To cite this Article Kloosterman, M. , Weijnen, J. G. J. , De Vries, N. K. , Mentech, J. , Caron, I. , Descotes, G. , Schoemaker, H. E. and Meijer, E. M.(1989) 'Octa-*O*-Acetyl-Sucrose: Regioselective Deacetylations by Lipolytic Enzymes', Journal of Carbohydrate Chemistry, 8: 5, 693 — 704

To link to this Article: DOI: 10.1080/07328308908048031

URL: http://dx.doi.org/10.1080/07328308908048031

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J. CARBOHYDRATE CHEMISTRY, 8(5), 693-704 (1989)

OCTA-*O*-ACETYL-SUCROSE: REGIOSELECTIVE DEACETYLATIONS BY LIPOLYTIC ENZYMES

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Received March 15, 1989 - Final Form July 25, 1989

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ABSTRACT

Treatment of octa-*O*-acetyl sucrose (**1**) with a lipolytic enzyme preparation from the yeast Candida cylindracea in aqueous buffer resulted in the predominant formation of 1', 2, 3, 3', 4, 6, 6'-hepta-*O*-acetyl-sucrose (**2**). Treatment of **1** with lipase from wheat germ afforded a minor amount of 1', 2, 3, 3', 4, 6-hexa-*O*-acetyl-sucrose (**3**) together with 2, 3, 3', 4, 6-penta-*O*-acetyl-sucrose (**4**) as the major product. Effects of calcium ions, cosolvents, temperature and enzyme treatment on enzyme activity and selectivity were investigated. Results indicate that so called wheat-germ lipase is actually an esterase. Wheat germ lipase, immobilised on agarose, did not show appreciable activity on octa-*O*-acetyl-sucrose (**1**).

INTRODUCTION

Regio- and stereoselective hydrolysis of esters on racemic or prochiral substrates by lipolytic enzymes has been the subject of much research lately; e.g., in the area of

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agrochemicals, pharmaceuticals, flavours etc. [1,2] Nonetheless, for carbohydrates relatively few reports have been published since the pioneering work of Fink [3] and Leibowitz [4,5] in the early 1960' s, though recent progress is noted. [6-11]

Increasing research and development efforts have been directed to the preparation of various sucrose esters for use as fat substitutes, as intermediates to 4, 1', 6'-trichloro-4, 1', 6'-trideoxy-galactosucrose (an artificial sweetener) and as tobacco improvers.

Concerning octa-*O*-acetyl-sucrose (**1**), reports have appeared dealing with the chemical deacetylation. For instance, Ballard [12] used an aluminium oxide column in a chloroform solution to hydrolyse **1** into the prevailing 1', 2, 3, 3', 4, 4', 6-hepta-*O*-acetylsucrose (**5**) as well as 1', 2, 3, 3', 4, 6, 6'-hepta-*O*-acetyl-sucrose (**6**). Capek [13] subsequently found that deacetylation in a methanolic solution of octa-*O*-acetyl-sucrose by aluminium oxide, impregnated with potassium carbonate, afforded the hepta-*O*-acetyl-sucroses **2**, **5** and **6** and a mixture of 2, 3, 4, 4', 6, 6'-hexa-*O*-acetyl-sucrose (**7**) and 1', 2, 3, 4, 6, 6'-hexa-*O*-acetyl-sucrose (**8**).

Having already established that lipases and esterases exert regio-, stereo- and group-specificity on acylated monosaccharides and aminoalcohols, [14] we set out to determine if enzyme catalysed regioselective hydrolysis is feasible on the disaccharide octa-*O*-acetyl-sucrose.

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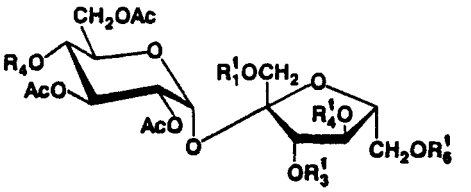
RESULTS AND DISCUSSION

Commercially available lipase and esterase preparations were screened for regioselective hydrolysis of octa-*O*-acetyl-sucrose (**1**). Screening was performed in shake-flasks (350 rpm) in potassium phosphate buffer (0.1 M, pH 7.5) incubated at 37°C, with a substrate/enzyme ratio of 2/1 (m/m). Conversion was monitored for five days using initially thin layer chromatography. For the majority of the enzyme preparations (virtually) no hydrolysis on **1** could be observed, whereas pig liver esterase (from Sigma) and experimental esterase (E 122, from NOVO) had converted **1** into a mixture of products without any product prevailing. With lipase from Candida cylindracea (A6, from Amano) complete hydrolysis of **1** into sucrose was noted.

Wheat germ lipase (L-3001, from Sigma) showed the preferential formation of one product (TLC estimation: 45 % yield). Subsequently, the wheat-germ lipase catalysed hydrolysis of octa-*O*-acetyl-sucrose was performed under pH-stat conditions. Titration with 2 M NaOH was a measure of enzyme activity and conversion. We found that the optimal amount of the major product remained constant during a considerable period of time (i.e. during addition of three to five equivalents of NaOH).



1 $R_1' = R_3' = R_4 = R_4' = R_6' = Ac$
2 $R_4' = H; R_1' = R_3' = R_4 = R_6' = Ac$
3 $R_4' = R_6' = H; R_1' = R_3' = R_4 = Ac$
4 $R_1' = R_4' = R_6' = H; R_3' = R_4 = Ac$
5 $R_6' = H; R_1' = R_3' = R_4 = R_4' = Ac$
6 $R_4 = H; = R_1' = R_3' = R_4' = R_6' = Ac$
7 $R_1' = R_3' = H; R_4 = R_4' = R_6' = Ac$
8 $R_3' = R_4' = H; R_1' = R_4 = R_6' = Ac$
9 $R_1' = R_3' = R_4 = R_6' = Ac; R_4' = CONHCOCCl_3$

Scheme I: Sucrose esters (1) to (9)

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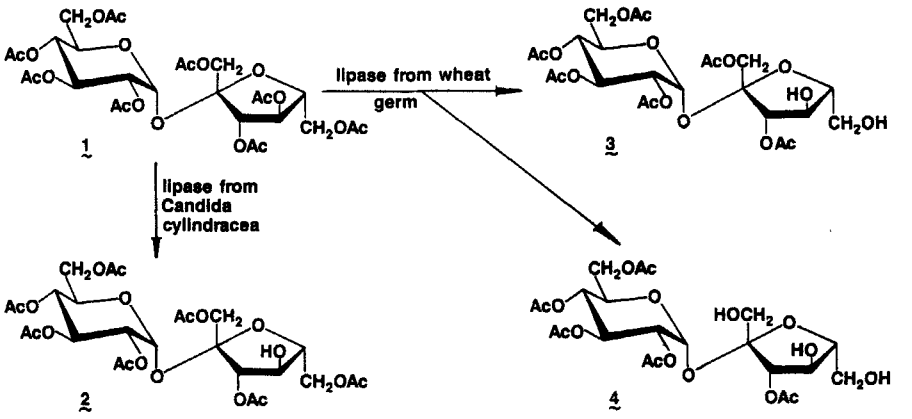
Nevertheless we encountered serious extraction problems when trying to extract the formed products from the reaction mixture due to the fact that a crude enzyme preparation was used.

Therefore, we investigated if simple further purification of the enzyme was possible without any loss in activity or selectivity.

Ninety-seven percent of the crude lipolytic enzyme preparation from wheat germ consists of protein, part of which is insoluble in water. By suspending the crude lipase preparation in aqueous buffer followed by centrifugation (30 min, 12.000 rpm), the supernatant could be separated from the pellet. The homogeneous supernatant proved to have the same specific activity of octa-*O*-acetyl-sucrose (**1**) as the crude lipase preparation.

Therefore it is advisable to remove the pellet from the crude lipase from wheat germ, as it proved to be responsible for initial problems during extraction of the penta-O-acetyl-sucrose from the reaction mixture by organic solvent.

After continuous extraction with dichloromethane, followed by column chromatography, the major product was isolated in 37 % yield, together with a minor product. The structures of these compounds proved to be 2,3,3',4,6-penta-O-acetyl-sucrose (**4**) and 1',2,3,3',4,6-hexa-*O*-acetyl-sucrose (**3**) (structural elucidation: see below).



Scheme II: Lipase-catalysed hydrolyses of octa-*O*-acetylsucrose.

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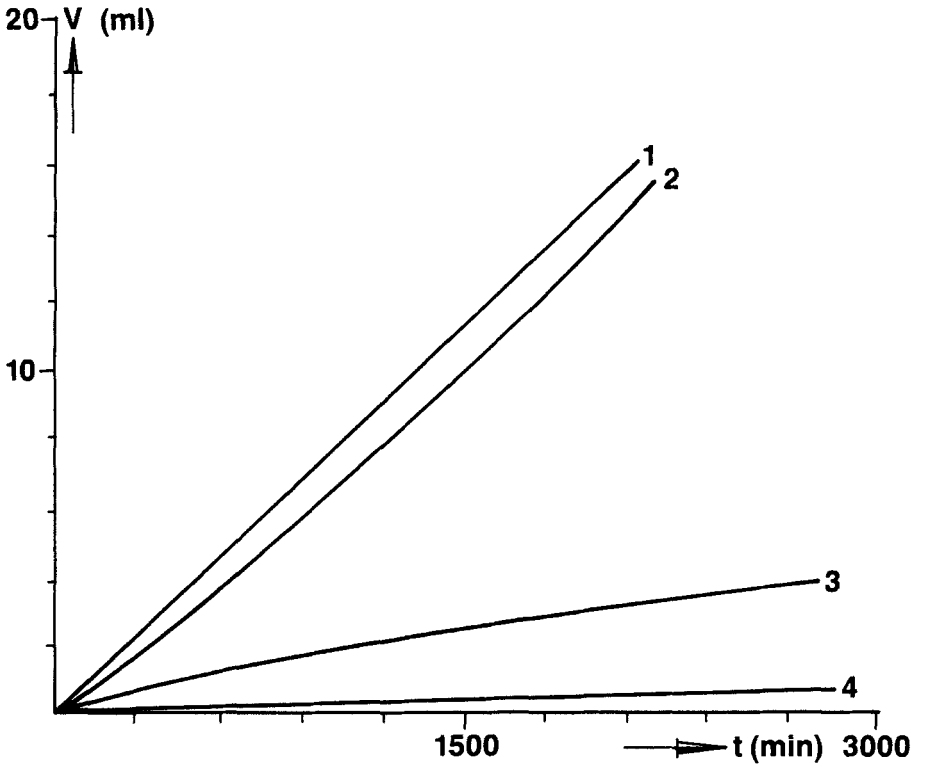
Addition of different amounts of calcium ions to the wheat germ lipase-catalysed hydrolysis medium of **1** was next investigated. Ca^{2+} is reported to affect Km but not v_{max}, suggesting that it aids the enzyme binding of substrate perhaps by keeping the interface free of products .[15] The presence of calcium ions in the solution has a dramatic effect on the apparent pKa of the fatty acids produced, thus changing the inflection point of the titration to a lower pH value. It is essential to be aware of this when estimating the course of the reaction by titration. We found that addition of different amounts of calcium ions, a known activator of several lipases, did not influence enzyme activity nor selectivity under the conditions investigated (0.3 and 3 mM $CaCl_2$).

Though Fe^{2+} and Fe^{3+} ions are known inhibitors of several lipases, use of millipore purified water, instead of demineralised water or tap water, had hardly any effect on the observed enzyme parameters.

Subsequently, the influence of variation in cosolvents, temperature, and immobilisation of the enzyme on the hydrolysis of compound **1** by wheat germ lipase was investigated. For instance, on a suspension of **1** (5 gram, 7.4 mmol) in potassium phosphate buffer (0.05 M, mL, pH 7.0) at 35°C, a specific activity of $5.8 \cdot 10^{-3}$ U/mn. mg enzyme (solid) was noted (see Scheme III, 2). Addition of lipophilic cosolvents [e.g. di-n-butylether (see Scheme III, 1)] did not markedly enhance the enzyme activity nor selectivity. This is interesting from a fundamental point of view, as we had already reported on the positive impact that lipophilic cosolvents may have on enzyme activity and selectivity for the conversion of methyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside (**10**) into methyl 2, 3, 4-tri-*O*-acetyl-α-D-glucopyranoside (**11**) by lipase from Candida cylindracea.[7]



Scheme III: Influence of addition of di-n-butyl ether [1], increase of temperature from 15°C to 35°C [3] and use of immobilised enzyme [4] compared with the standard enzyme preparation [2].

A plausible explanation for the observed effects of cosolvent, calcium and iron ions on the enzyme activity is that the enzyme in the crude wheat germ lipase preparation, responsible for the hydrolysis of compound **1**, is not a true lipase but perhaps an esterase.[3,15,17]

By lowering the temperature from 35°C to 15°C (see Scheme III, 3) the specific activity dropped from $5.7 \cdot 10^{-3}$ to $1.0 \cdot 10^{-3}$ U/min mg enzyme (solid) without any increase in selectivity.

The use of wheat germ lipase, immobilised on agarose was also investigated at 35°C. A slow conversion of **1** was observed when using the immobilised enzyme, thereby rendering this approach unattractive (see Scheme III, 4). Other methods of immobilisation may be of use here. By a somewhat different approach lipase from Candida cyclindracea

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(lipase AY, from Amano) was found to hydrolyse octa-*O*-acetyl-sucrose into a major product with a R_F value on TLC different from **3** and **4**. Thus, octa-*O*-acetyl-sucrose (**1**) (6.78 g, 10 mmol) was dissolved in 10 mL of toluene and added to a solution of 1 gram of lipase from Candida cylindracea dissolved in 90 mL phosphate buffer (0.1 M, pH 7.0). The reaction mixture was stirred for 48 hours at 45°C under pH stat conditions controlled by adding sodium hydroxide (0.3 N), and the major product was isolated by column chromatography. The isolated product proved to be 1', 2, 3, 3', 4, 6, 6'-hepta-*O*-acetyl-sucrose (**2**) (yield: 35 %).

The structures of the partially deacetylated sucrose derivatives **2**, **3** and **4** were established via one- and two-dimensional ^1H and ^{13}C NMR assignments. The recently published complete assignments of the sugar and acetyl ^1H and ^{13}C signals of octa-*O*-acetyl-sucrose were used as reference.[18] Results of NMR measurements are listed in Tables I and II.

^1H Chemical shifts for **2-4** were established via comparison with the data for octa-*O*-acetyl sucrose. As is evident from the NMR measurements, listed in Table I, chemical shift differences occur between sucrose octaacetate **1** and **2-4**, resulting from the deacetylation. On the basis of chemical shift increment data, one expects an upfield shift of 0.65 ppm for an α-proton on going from CH_2OAc to CH_2OH (1.15 ppm for CHOH), and an upfield shift of 0.25 ppm for a β-proton. All shifts are in accordance with the proposed structures. Because the $H_{4'}$ resonance of **2** was expected to be present in the crowded 4.3-4.4 ppm region, this was substantiated by the addition of trichloroacetyl isocyanate to the NMR-solution. This formed the appropriate carbamate **9** in situ.[19] The $H_{4'}$ signal was again deshielded to 5.53 ppm and the coupling constants corresponded to a fructofuranosyl moiety proton.

The results of the ^{13}C NMR measurements for **3** and **4** are listed in Table II. Assignments were again established via comparison with the data for **1**, and, for compound **4**, also via a proton-carbon correlation (Scheme IV).

For the ^{13}C shifts, an upfield shift of 3 ppm is expected for the α-carbon, and a downfield shift of 3 ppm for the β-carbon upon deacetylation.[20] As can be seen in Table II, these shifts are found for $C_{4'}$ and $C_{6'}$, but the value for $C_{1'}$ deviates from the expected value. To avoid incorrect assignment, we performed a COLOC (heteronuclear Correlation Long-range Coupling) experiment. This experiment was designed by Kessler to give two- and three-band correlations of quaternary carbon atoms with protons.[21] Thus, a correlation between the carbonyl group and the ring sugar proton (three bond correlation [22]) is direct evidence for the presence of an acetyl group at that position. The results of the COLOC experiment for compound **4** are shown in Scheme V. This experiment confirms that deacetylation occurred at positions $H_{1'}$, $H_{4'}$ and $H_{6'}$.

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TABLE I

^{1}H NMR data for compounds **2-4** and **9**
(Chemical shifts in ppm, coupling constants in Hz)

Compound	1a	2b	3	4	9b
H_1	5.69	5.84	5.62	5.58	5.82
H_2	4.87	5.07	4.83	4.82	4.98
H_3	5.44	5.81	5.38	5.38	5.72
H_4	5.08	5.30	5.15	5.02	5.28
H_5	4.28	4.48	4.18	4.19	4.48
H_{6a}	4.14-4.28	4.30-4.40	4.18-4.27	4.08-4.19	4.304.40
H_{6b}	4.14-4.28	4.20-4.40	4.18-4.27	4.08-4.19	4.30-4.40
$H_{1'a}$	4.17	4.30-4.40	3.97-4.40	3.47-3.57	4.30-4.40
$H_{1'b}$	4.17	4.30-4.40	3.97-4.40	3.47-3.57	4.30-4.40
$H_{3'}$	5.47	5.52	5.20	5.18	5.69
$H_{4'}$	5.36	4.30-4.40	4.45	4.43	5.53
$H_{5'}$	4.21	4.15	3.87	3.89	4.27
$H_{6'a}$	4.29-4.35	4.30-4.40	3.66-3.84	3.66-3.81	4.30-4.40
$H_{6'b}$	4.29-4.35	4.30-4.40	3.66-3.84	3.66-3.81	4.30-4.40
$J_{1,2}$c)	-d	3.8	3.7	3.6	3.6
$J_{2,3}$	-	10.2	10.3	10.2	10.3
$J_{3,4}$	-	9.8	9.6	9.6	9.9
$J_{4,5}$	-	9.8	10.3	9.9	9.8
$J_{1'a,1'b}$	-	-c	-11.8	-12.2	-c
$J_{3',4'}$	-	7.5	8.1	7.8	5.0
$J_{4',5'}$	-	7.7	8.8	7.9	5.3
$J_{6'a,6'b}$	-	-	-13.2	-12.8	-

a. Data taken from ref. 15
b. Compounds **2** and **9** measured in C_6D_6, **1**, **3** and **4** in $CDCl_3$
c. Other coupling constants could not be obtained due to chemical shift degeneracy
d. Data not reported in ref. 15

Concluding, treatment of octa-*O*-acetyl-sucrose with lipase from Candida cylindracea results in the removal of the secondary acetyl ester at C-4' in the fructose moiety, leaving three primary acetyl esters intact. This is quite remarkable in terms of regioselectivity. For instance, we previously reported the regioselective removal of the primary acetyl ester in methyl 2, 3, 4, 6-tetra-*O*-acetyl-α-D-glucopyranoside, a structural analogue of **1** by lipase from Candida cylindracea. A possible explanation for the results from **1** is that a

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TABLE II
^{13}C NMR chemical shifts (ppm) for compounds **1**, **3** and **4**

Compounds	**1**[a]	**4**	**3**
C_1	89.93	89.37	89.42
C_2	70.26	70.32	70.23
C_3	69.61	69.52	69.40
C_4	68.17	68.18	68.00
C_5	68.50	68.32	68.46
C_6	61.75	61.64	61.32
$C_{1'}$	62.85	64.09	64.58
$C_{2'}$	104.02	104.19	102.39
$C_{3'}$	75.68	78.77	78.65
$C_{4'}$	74.98	72.29	71.07
$C_{5'}$	79.14	82.85	82.37
$C_{6'}$	63.63	61.19	60.18

a. Data taken from ref. 15.

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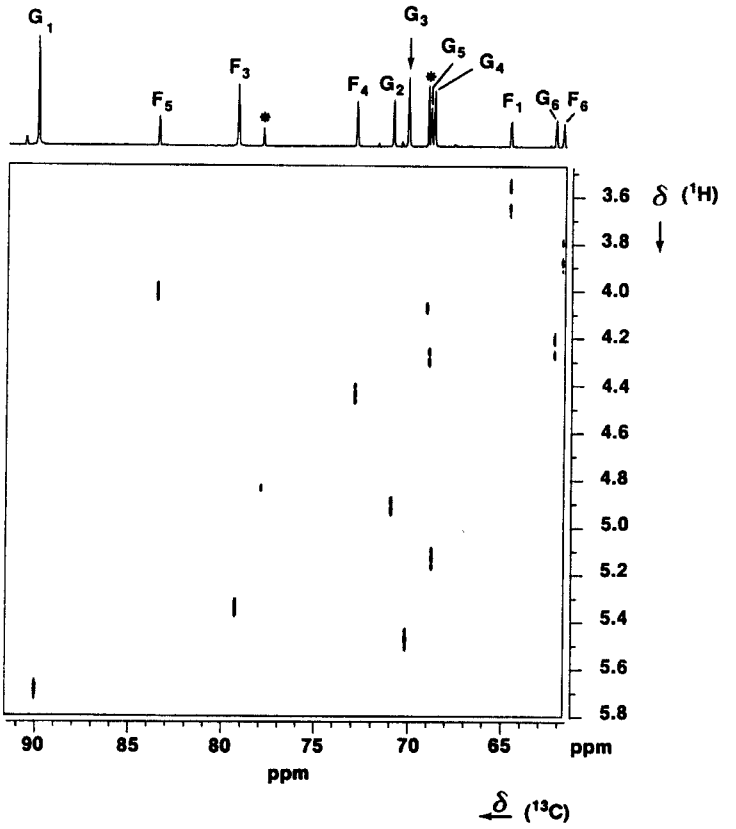
primary acetyl ester in the fructose moiety (i. e.-1') is actually being removed by the enzyme. There then a rapid acetyl migration from C-4' to C-1'. This could explain why for wheat germ lipase the secondary acetyl ester at C-4' is also being removed together with the primary acetyl esters at C-1' and C-6'. In contrast to these results, Capek clearly showed that the most reactive site in octa-*O*-acetyl-sucrose towards aluminium oxide is located at C-4', and that chemical deacetylation also occurred exclusively in the fructose moiety. Therefore, to resolve these different results unambiguously, a kinetic study of the deacetylation of 1', 2, 3, 3', 4, 4', 6, 6'-octa-*O*-acetyl-sucrose (**1**) and 1', 2, 3, 3', 4, 6, 6'-hepta-*O*-acetyl-sucrose (**2**) should be undertaken. Enzymatic trans-acetylation is not likely to occur, however, at the relatively low concentrations of acetic acid formed. This work is at present under investigation.

EXPERIMENTAL

1', 2, 3, 3', 4, 6, 6'-Hepta-*O*-acetyl-sucrose (**2**). Sucrose octaacetate **1** (6.78 g, 10 mmol) was dissolved in 10 mL of toluene and added to a solution of 1 g of lipase



Scheme IV: ^{13}C-^{1}H NMR correlation spectrum of **4** in $CDCl_3$

from Candida cylindracea [Lipase AY30 from AMANO PHARM. Co (Japan)] dissolved in 90 mL of a phosphate buffer (0.1 M, pH 7). The reaction mixture was stirred at 45°C and titrated automatically with NaOH (0.3 N) to keep the mixture at pH 7. After 48 h, the organic layer was concentrated and the residue was purified by silica gel column chromatography (hexane: ethyl acetate 20/80) to give **2** (2.22 g, 35 %); $[\alpha]^{25}_{D}$ + 53 8° (c 0.5, $CHCl_3$); lit[12] $[\alpha]_D$ + 54,3 (c 0.4, $CHCl_3$).

Anal. Calcd for $C_{26}H_{36}O_{18}$: C, 49.05; H, 5.70. Found: C, 48.99; H, 5.40.

For ^{1}H NMR data Brucker AM 300; C_6D_6 $(Me)_4Si$ int. stand) see Table I.

2, 3, 3', 4, 6-Penta-*O*-acetyl-sucrose (4). Wheat germ lipase (L-3001 from Sigma; 3.5 g) was suspended in a potassium phosphate buffer (0.1 M pH 7; 5 mL) and centrifuged for 30 min at 10,000 rpm. The supernatant was added to a suspension of

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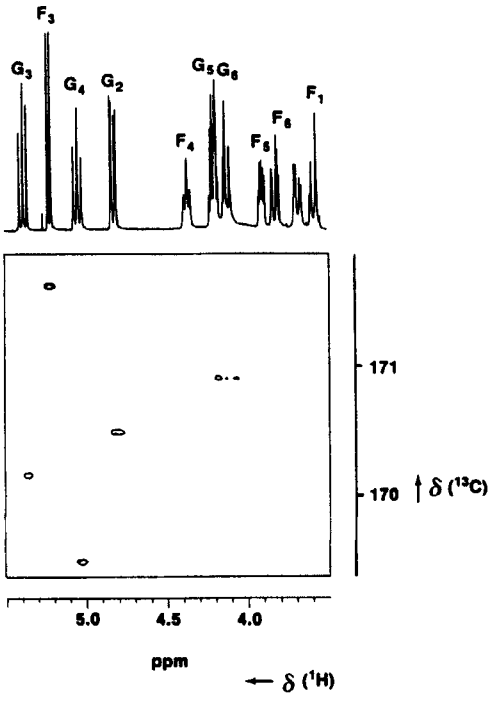

Scheme V: Three bond correlation between the carbonyl group and the ring sugar proton, obtained via a 2-D NMR COLOC experiment for **4**.

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octa-*O*-acetyl-sucrose (7 g, 1.03 mmol) in potassium phosphate buffer (0.05 M, pH 7.0) and the pH was kept constant using pH stat equipment (Titralab, Radiometer) by addition of 2 M NaOH. After addition of 4.5 equivalents of NaOH, the mixture was continuously extracted with dichloromethane. The organic layer was dried ($MgSO_4$), concentrated under reduced pressure and purified by silica gel column chromatography (Kieselgel 60, eluent CH_2Cl_2/MeOH, 9/1 v/v) to afford compound **4** (37 % yield), $[\alpha]_D = +31.5$ (c 1, CH_2Cl_2) and compound **3** (3.4 % yield). For NMR see Tables I and II.

NMR Spectra.- NMR spectra were measured on a Brucker AM-400 spectrometer, operating at 100.61 MHz for ^{13}C and 400.13 MHz for ^{1}H, or on an AM-300 (compounds **2** and **9**). Chemical shifts were measured relative to the $CDCl_3$ triplet at δ 77.0 (^{13}C) or to TMS (^{1}H) (compounds **3** - **4**). Compounds **2** and **9** were measured in C_6D_6 (internal TMS). The heteronuclear proton-carbon correlation [23] was used with delays Δ1 and Δ2 of 3.45 and 2.2 ms, respectively. The carbon spectral width was 4587

Hz, and the proton width was 1000 Hz. For each of 128 values of t, 128 transient were measured with 3 S delay. Total data matrix after transformation (no weighting functions) was 2 K x 512.

For the COLOC experiment, delays $\Delta 1$ and $\Delta 2$ were 30 and 22 ms. Spectral widths were 1000 Hz (f2) and 1600 Hz (f1); 64 transients were accumulated for each of 128 values of t_1. Data matrix after transformation was 1K x 256.

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