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Membrane-Permeant Derivatives of Mannose-1-phosphate

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Abstract—For treatment of congenital disorder of glycosylation type Ia (CDG-Ia) membrane-permeant derivatives of mannose-1-phosphate are required. Employing biologically cleavable phosphate protecting groups advantageous precursor derivatives could be synthesized following a facile approach. Their enzymatic cleavages using esterase from porcine liver (E.C. 3.1.1.1) were investigated.

Introduction

Congenital disorders of glycosylation, formerly called carbohydrate-deficient glycoprotein syndrome (CDG syndrome), belong to a new group of genetic, multisystemic, metabolic disorders. These autosomal recessive diseases were first described in 1980.[1]

The genetic defect influences the biosynthesis of N-linked glycans. CDG could be classified into two types based on the intracellular localization of the genetic defect. In type I the defect could be found in the synthesis or transfer of oligosaccharides in the endoplasmic reticulum (ER) whereas in type II the defect inhibits the processing of the N-linked glycoproteins in the Golgi.

In CDG-Ia phosphomannomutase 2 (PMM 2) is deficient, therefore the conversion of mannose-6-phosphate to mannose-1-phosphate fails, and thus the supply of GDP-mannose will be limited. Because GDP-mannose is the decisive mannose donor the biosynthesis of glycoproteins is significantly disturbed. One way of dealing with the PMM 2 deficiency would be treatment of patients with mannose-1-phosphate from external sources.

Due to the high polarity, mannose-1-phosphate is unable to penetrate through cellular membranes.[2,3] To overcome this limitation biologically reversible protecting groups previously used for carboxylic acids, phosphonates, nucleotides and inositoles[4–8] could be employed to form neutral phosphotriester. Suitable for this purpose seem to be the acetoxymethyl (AM) and the pivaloyloxymethyl (POM) esters. After penetration the acyloxy phosphate ester is expected to be converted into the parent compound by intracellular enzymatic cleavage by carboxylate esterases.[9,10] The acyloxy ester linkages should be hydrolyzed to the hydroxymethyl analogues. This intermediate in turn is chemically labile and looses one mole of formaldehyde[11,12] (Scheme 1). In order to test this in vitro, initial enzymatic deprotection experiments were conducted. For improving the lipophilicity the hydroxy groups of the mannose moiety were masked as different esters and carbonates.

Results and Discussion

Synthesis

Starting from mannose the benzyl mannopyranoside was obtained by Fischer glycosylation with benzyl alcohol.[13] This compound was converted into the appropriate substituted mannopyranosides using butyryl chloride, pivaloyl chloride or *iso*-propyl chloroformiate.[14,15] Subsequent hydrogenolysis of the benzyl groups on Pd/C (10%) yielded in the anomerically unblocked mannose derivatives **1**–**3**. Further reaction with dibenzyl di-*iso*-propylphosphoramidite using 1H-tetrazole gave the phosphite triesters which were oxidised in situ by *meta*-chloroperbenzoic acid (MCPBA) to the appropriate phosphate derivatives[16] **4**–**6**. Subsequently the benzyl groups were removed by hydrogenolysis on Pd/C (10%). The resulting phosphates **7**–**9** were converted into their acetoxymethyl (AM) and pivaloyloxymethyl (POM) esters **10**–**15** employing bromomethylacetate or iodomethylpivaloate,

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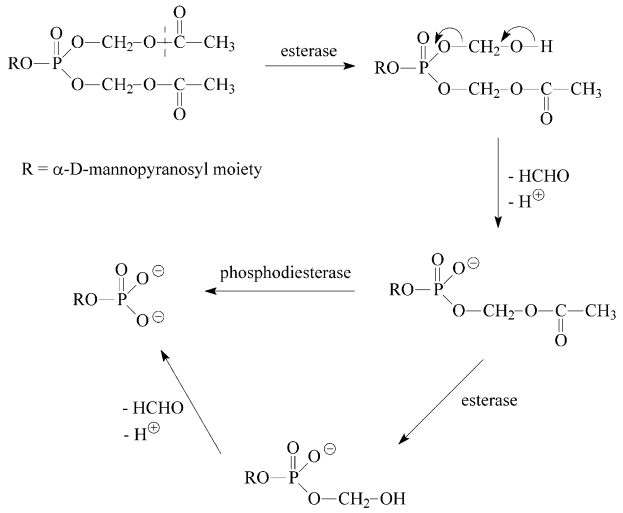


Scheme 1.

respectively, in the presence of *N*-ethyl-di-*iso*-propylamine (DIPEA) (Scheme 2).

The poor nucleophilicity of the phosphate and the lack of stability of the phosphate group obvious by the anomerically free mannose byproduct could be the reason for the relatively low yield in this step which requires improvements in further studies to come.

Enzymatic tests

The AM-ester of mannose protected with butyryl groups (**10**) was used as the model compound. First, a standard reaction was carried out without any enzyme to control the stability of the mannose phosphate under the reaction conditions. The ester was incubated in phosphate buffer at 37 °C overnight and the course of the reaction followed by TLC. Then MALDI-TOF analysis was carried out to show the stability of the ester under these conditions.

Subsequently, the ester was incubated in phosphate buffer in the presence of esterase (porcine liver, crude, EC 3.1.1.1) at 37 °C for 3 h and the reaction was controlled by TLC. Again MALDI-TOF analysis proved that the AM-groups were cleaved sucessively and that one butyryl group was removed. It may be presumed that the butyryl group in question is in position 6, as this would be the ester of a primary alcohol, and as such the most reactive site in the molecule. To our surprise, the remaining ester groups on the sugar moiety were not removed, however, as a side-product the phosphorus free mannose was detected. The same enzymatic test was conducted with pure enzyme (porcine liver, 3.2 M $(NH_4)_2SO_4$ solution, pH 8, EC 3.1.1.1). An ESI analysis was made, in addition to MALDI-TOF. It could thus be shown that again both AM-groups had been cleaved. Furthermore, the ESI spectrum demonstrated that all four butyryl protecting groups had been removed. The hydroxymethyl intermediates, produced during enzymatic cleavage, could also be detected. Further studies will be conducted in this area. Other possible substrates for the enzyme will be investigated and in vitro tests will be done.

Conclusion

In this study the preparation of various potential membrane-permeable derivatives of mannose-1-phosphate could be demonstrated combining biologically reversible phosphate and carbohydrate protecting groups. Enzymatic tests have revealed that AM-groups were cleaved to restore the original compound. It is envisaged to transfer this conception for membrane-permeant derivatives including biologically reversible protecting groups to other sugar phosphates and further biologically important components.

Experimental

General methods

NMR spectra were recorded on Bruker AC-250, AMX-400 and DRX-500. Chemical shifts of ^{1}H NMR and ^{13}C NMR are given relative to tetramethylsilane. Eighty-five per cent phosphoric acid was used as an external standard for ^{31}P NMR. Optical rotations were measured with Perkin–Elmer polarimeter 341. Melting points were determined with ST-apotec and are uncorrected. Elemental analyses were performed by the micro-analytical service of the Institute of Organic Chemistry of the University of Hamburg. MALDI-TOF spectra were recorded on Bruker Biflex III and ESI spectra on aHP series 1100 MSD. TLC were run on precoated plates, silica gel 60 GF_{254} (Merck). Detection was effected by observation under UV light at 254 nm, and by spraying with 10% ethanolic sulfuric acid and subsequent heating. Column chromatography was performed by flash technique using silica gel 60 (230–400 mesh, 0.040–0.063 mm, Merck).

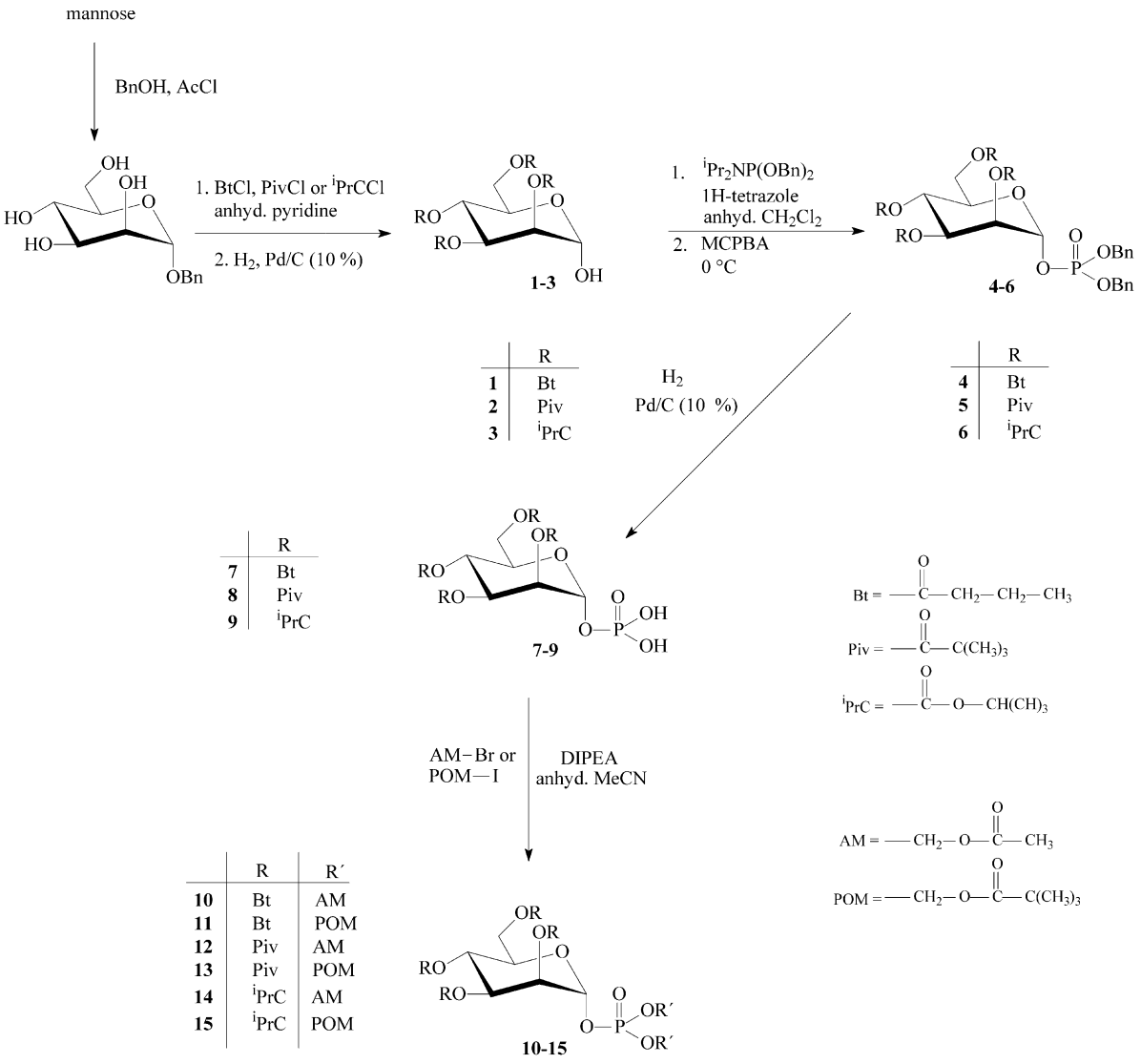
Reagents

Iodomethylpivaloate was synthesized following known procedure. Esterase (porcine liver, crude and 3.2 M $(NH_4)_2SO_4$ solution, EC 3.1.1.1) was purchased from Sigma. One unit (U) is defined as the hydrolysis of 1.0 μmol of ethyl butyrate to butyric acid and ethanol per min at pH 8.0 at 25 °C. DIPEA is stored over 4 Å molecular sieves.

Protection of benzyl mannopyranoside and hydrogenation[14,15]

Benzyl mannopyranoside was dissolved in dry pyridine (0.1 M solution) at 0 °C. Butyryl chloride (3 equiv/OH), pivaloyl chloride (3 equiv/OH) or *iso*-propylchlorformiate (1.5 equiv/OH, 1 M toluene) were added dropwise. The mixture was stirred overnight at room temperature.

Workup procedure for *butyryl chloride* and *pivaloyl chloride*: The reaction was quenched with methanol, then the solution concentrated and codistilled with toluene under reduced pressure. The residue was dissolved in dichloromethane, washed twice with saturated sodium



	R
1	Bt
2	Piv
3	iPrC

	R
4	Bt
5	Piv
6	iPrC

	R
7	Bt
8	Piv
9	iPrC

	R	R′
10	Bt	AM
11	Bt	POM
12	Piv	AM
13	Piv	POM
14	iPrC	AM
15	iPrC	POM

Scheme 2.

hydrogen carbonate and once with water, then dried over magnesium sulfate, filtrated, concentrated under reduced pressure and again codistilled with toluene. The crude residue was purified by column chromatography with petroleum ether/ethyl acetate (1:1).

Workup procedure for *iso-propyl chloroformiate*: The mixture was diluted with chloroform, washed twice with 1 M hydrochloric acid and once with water, then dried over magnesium sulfate, filtrated, concentrated under reduced pressure and codistilled with toluene. The crude residue was purified by column chromatography with petroleum ether/ethyl acetate (1:1). Subsequently, hydrogenation was in dry methanol (0.1 M solution) and Pd/C (10%) added cautiously and the mixture stirred at room temperature under normal H_2 pressure. After termination the solution was filtrated over Celite, concentrated under reduced pressure and purified by column chromatography with petroleum ether/ethyl acetate (3:1) to give compounds **1**, **2** or **3**.

2,3,4,6-Tetra-*O*-butyryl-α-D-mannopyranose (1). Compound **1** was synthesized in the manner described above. Yield: 0.85 g (1.85 mmol, 52% with respect to mannose, colourless sirup); R_f 0.49 in 1:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.44 (dd, 1H, H-3), 5.37 (dd∼t, 1H, H-4), 5.31 (dd, 1H, H-2), 5.24 (s, 1H, H-1), 4.27–4.16 (m, 3H, H-5, H-6a, H-6b), 3.12 (bs, 1H, OH), 2.42–2.16 (m, 8H, 4x-CO–CH_2–), 1.77–1.52 (m, 8H, 4x-CH_2–CH_3), 1.03–0.88 (m, 12H, 4x-CH_3); $J_{1,2}$ = 2.0, $J_{2,3}$ = 3.1, $J_{3,4}$ = 10.2, $J_{4,5}$ = 9.7 Hz; ^{13}C NMR (100.62 MHz, $CDCl_3$) δ 173.4, 172.7, 172.5, 172.3 (C=O), 92.4 (C-1), 69.7 (C-2), 68.8, 68.6 (C-3, C-5), 65.7 (C-4), 62.2 (C-6), 36.1, 36.0, 35.9 (–CO–CH_2–), 18.5, 18.3, 18.2 (–CH_2–CH_3), 13.7, 13.6 (–CH_3); $C_{22}H_{36}O_{10}$ (460.52).

2,3,4,6-Tetra-*O*-pivaloyl-α-D-mannopyranose (2). Compound **2** was synthesized in the manner described above. Yield: 1.38 g (2.63 mmol, 34% with respect to mannose, white crystals); mp 175.8 °C; R_f 0.23 in 3:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.53 (dd∼t, 1H, H-4), 5.46 (dd, 1H, H-3), 5.28 (dd, 1H,

H-2), 5.19 (bs, 1H, H-1), 4.29 (ddd, 1H, H-5), 4.22–4.13 (m, 2H, H-6a, H-6b), 3.17 (d, 1H, OH), 1.27, 1.24, 1.16, 1.12 (4xs, 36H, 4x-C(C$\underline{H}_3$)$_3$); $J_{1,2}=1.8$, $J_{2,3}=3.1$, $J_{3,4}=10.2$, $J_{4,5}=10.2$; ^{13}C NMR (100.62 MHz, $CDCl_3$) δ 178.3, 177.3, 176.7, 172.0 (C=O), 92.5 (C-1), 69.9 (C-2), 69.1 (C-3), 68.9 (C-5), 65.2 (C-4), 61.9 (C-6), 38.9, 38.8 (C_q,–$\underline{C}$(CH$_3$)$_3$), 27.2, 27.2, 27.1 (–C(C$\underline{H}_3$)$_3$); $C_{26}H_{44}O_{10}$ (516.63).

2,3,4,6-Tetra-*O*-*iso*-propylcarbonate-α-D-mannopyranose (3). Compound **3** was synthesized in the manner described above. Yield: 0.73 g (1.39 mmol, 73% with respect to mannose, colourless sirup); R_f 0.34 in 3:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.34 (s, 1H, H-1), 5.26–5.22 (m, 2H, H-2, H-3), 5.09 (dd∼t, 1H, H-4), 4.93–4.80 (m, 4H, 4x-C$\underline{H}$(CH$_3$)$_2$), 4.32–4.28 (dd, 3H, H-5, H-6a, H-6b), 1.34–1.26 (m, 24H, 8x-CH(C$\underline{H}_3$)$_2$); $J_{4,5}=9.7$ Hz; ^{13}C NMR (100.62 MHz, $CDCl_3$) δ 154.3, 153.9, 153.6, 153.4 (C=O), 92.1 (C-1), 73.0, 72.9, 72.7, 72.4 (–$\underline{C}$H(CH$_3$)$_2$), 72.5, 72.1 (C-2, C-3), 70.0 (C-4), 68.5 (C-5), 66.1 (C-6), 21.7, 21.7, 21.6 (–CH(C$\underline{H}_3$)$_2$; $C_{22}H_{36}O_{14}$ (524.52).

Phosphorylation[16]

Under argon atmosphere 1H-tetrazole (5 equiv) was suspended in dry dichloromethane (20 mL). After addition of dibenzyl di-*iso*-propylphosphoramidite (2.5 equiv) the mixture was stirred at room temperature for 15 min in order to form the tetrazolide intermediate. Then a solution of mannose derivatives **1**, **2** or **3** in dry dichloromethane (20 mL) was added and the mixture was stirred for further 3 h at room temperature before being cooled to 0 °C. MCPBA (3 equiv) was added and stirring was continued for 1 h. The solvents were removed under reduced pressure. Purification was by column chromatography with petroleum ether/ethyl acetate (3:1, 2:1) to give compounds **4**, **5** or **6**.

Dibenzyl-(2,3,4,6-tetra-*O*-butyryl-α-D-mannopyranosyl)-phosphate (4). Compound **1** (1.80 g, 3.92 mmol) was reacted in the manner described above. Yield: 2.51 g (3.48 mmol, 89%, sirup); $[\alpha]_D$ +13.7 (c 0.4, $CHCl_3$); R_f 0.45 in 1:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40–7.30 (m, 10H, Ph), 5.62 (dd, 1H, H-1), 5.36 (dd∼t, 1H, H-4), 5.31 (dd, 1H, H-3), 5.26 (dd∼t, 1H, H-2), 5.12–5.09 (m, 4H, 2x-C$\underline{H}_2$–Ph), 4.14 (dd, 1H, H-6a), 4.03 (ddd, 1H, H-5), 3.95 (dd, 1H, H-6b), 2.40–2.19 (m, 8H, 4x-CO–CH$_2$–), 1.75–1.53 (m, 8H, 4x-C$\underline{H}_2$–CH$_3$), 1.01–0.88 (m, 12H, 4x-CH$_3$); $J_{1,2}=1.5$, $J_{2,3}=3.1$, $J_{3,4}=10.2$, $J_{4,5}=9.7$, $J_{5,6a}=4.1$, $J_{5,6b}=2.0$, $J_{6,6}=12.2$, $J_{H\text{-}1,P}=6.1$ Hz; ^{13}C NMR (100.62 MHz, $CDCl_3$) δ 173.1, 172.3, 172.1, 172.0 (C=O), 130.2, 129.8 (C_q), 128.8–128.0 ($C_{arom.}$), 95.3 (d, C-1), 70.5 (C-5), 70.0 (d,–$\underline{C}$H$_2$–Ph), 69.9 (d,–$\underline{C}$H$_2$–Ph), 68.6 (d, C-2), 68.2 (C-3), 64.8 (C-4), 61.4 (C-6), 36.0, 35.9, 35.8 (–CO–$\underline{C}$H$_2$–), 18.4, 18.3, 18.2, 18.1 (–$\underline{C}$H$_2$–CH$_3$), 13.7, 13.6 (–$\underline{C}$H$_3$); $^2J_{C\text{-}1,P}=4.8$, 2x $^2J_{CH2,P}=6.1$, $^3J_{C\text{-}2,P}=10.9$ Hz; ^{31}P NMR (101.26 MHz, $CDCl_3$) δ −1.97. Anal. calcd for $C_{36}H_{49}O_{13}P$ (720.76): C 59.99, H 6.85; Found: C 60.01, H 6.74.

Dibenzyl-(2,3,4,6-tetra-*O*-pivaloyl-α-D-mannopyranosyl)-phosphate (5). Compound **2** (1.38 g, 2.63 mmol) was reacted in the manner described above. Yield: 1.43 g (1.84 mmol, 70%, sirup); $[\alpha]_D$ +22.1 (c 0.7, $CHCl_3$); R_f 0.52 in 1:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38–7.33 (m, 10H, Ph), 5.58 (dd, 1H, H-1), 5.52 (dd∼t, 1H, H-4), 5.31 (dd, 1H, H-3), 5.24 (dd∼t, 1H, H-2), 5.16–5.08 (m, 4H, 2x-C$\underline{H}_2$–Ph), 4.07–3.99 (m, 2H, H-5, H-6a), 3.89 (dd, 1H, H-6b), 1.25, 1.21, 1.14, 1.12 (4xs, 36H, 4x-C(C$\underline{H}_3$)$_3$); $J_{1,2}=1.9$, $J_{2,3}=3.2$, $J_{3,4}=10.4$, $J_{4,5}=10.1$, $J_{5,6a}=2.8$, $J_{5,6b}=1.3$, $J_{6,6}=12.6$, $J_{H\text{-}1,P}=6.3$ Hz; ^{13}C NMR (100.62 MHz, $CDCl_3$) δ 178.0, 176.6, 176.4, 172.0 (C=O), 133.7–127.5 ($C_{arom.}$), 95.6 (d, C-1), 70.6 (C-5), 70.1 (d,–$\underline{C}$H$_2$–Ph), 69.9 (d,–$\underline{C}$H$_2$–Ph), 68.7 (C-3), 68.6 (d, C-2), 64.2 (C-4), 61.0 (C-6), 38.9, 38.8 (C_q,–$\underline{C}$(CH$_3$)$_3$), 27.2, 27.1 (–C(C$\underline{H}_3$)$_3$); $^2J_{C\text{-}1,P}=5.6$, 2x $^2J_{CH2,P}=5.6$, $^3J_{C\text{-}2,P}=11.7$ Hz; ^{31}P NMR (101.26 MHz, $CDCl_3$) δ −1.76; MALDI-TOF-MS: *m*/*z* 799.53 $[M+Na]^+$, 815.46 $[M+K]^+$. Anal. calcd for $C_{40}H_{57}O_{13}P$ (776.86): C 61.84, H 7.40; Found: C 61.11, H 7.35.

Dibenzyl-(2,3,4,6-tetra-*O*-*iso*-propylcarbonate-α-D-mannopyranosyl)-phosphate (6). Compound **3** (1.19 g, 2.27 mmol) was reacted in the manner described above. 1.54 g (1.96 mmol, 87%, sirup); $[\alpha]_D$ +6.7 (c 0.5, $CHCl_3$); R_f 0.40 in 1:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37–7.32 (m, 10H, Ph), 5.75 (dd, 1H, H-1), 5.23 (dd∼t, 1H, H-2), 5.14–5.07 (m, 6H, H-3, H-4, 2x-C$\underline{H}_2$–Ph), 4.86 (m, 4H, 4x-C$\underline{H}$(CH$_3$)$_2$), 4.26 (dd, 1H, H-6a), 4.18–4.11 (m, 2H, H-5, H-6b), 1.33–1.23 (m, 24H, 8x-CH(C$\underline{H}_3$)$_2$); $J_{1,2}=1.6$, $J_{2,3}=2.2$, $J_{5,6a}=5.7$, $J_{6,6}=11.7$, $J_{H\text{-}1,P}=6.6$ Hz; ^{13}C NMR (100.62 MHz, $CDCl_3$) δ 154.3, 153.5, 153.4 (C=O), 128.7–128.1 ($C_{arom.}$), 94.9 (d, C-1), 73.3, 73.1, 72.8, 72.3 (–$\underline{C}$H(CH$_3$)$_2$), 71.7 (C-3), 71.5 (d, C-2), 70.2 (C-5), 70.0 (d,–$\underline{C}$H$_2$–Ph), 69.8 (d,–$\underline{C}$H$_2$–Ph), 69.1 (C-4), 65.3 (C-6), 21.7–21.6 (–$\underline{C}$H(CH$_3$)$_2$); $^2J_{C\text{-}1,P}=5.6$, 2x $^2J_{CH2,P}=5.6$, $^3J_{C\text{-}2,P}=11.7$ Hz; ^{31}P NMR (101.26 MHz, $CDCl_3$) δ −1.90; MALDI-TOF-MS: *m*/*z* 807.44 $[M+Na]^+$, 823.39 $[M+K]^+$. Anal. calcd for $C_{36}H_{49}O_{17}P$ (784.76): C 55.10, H 6.29; Found: C 55.23, H 6.45.

Hydrogenation

Pd/C (10%) was given cautiously to a solution of mannopyranosyl phosphate derivatives **4**, **5** or **6** in ethyl acetate/methanol/water (1:2:1). The mixture was stirred at room temperature under H_2 atmosphere (50 bar). After termination the solution was filtrated over Celite and concentrated under reduced pressure. The residue was purified by column chromatography with chloroform/methanol/water (6:3.5:0.5) to give products **7**, **8** or **9**.

2,3,4,6-Tetra-*O*-butyryl-α-D-mannopyranosyl phosphate (7). Compound **4** (2.43 g, 3.37 mmol) was reacted in the manner described above in 40 mL solvent for 5 h.

Yield: 1.35 g (2.50 mmol, 74%, yellow sirup); $[\alpha]_D$ +37.3 (c 1.0, $CHCl_3$); R_f 0.27 in 6:3.5:0.5 chloroform/methanol/water; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.60 (bs, 1H, H-1), 5.40 (dd, 1H, H-3), 5.36 (bs, 1H, H-2), 5.17 (dd∼t, 1H, H-4), 4.27–4.13 (m, 1H, H-5), 3.79–3.62 (m, 2H, H6a, H-6b), 2.41–2.24 (m, 8H, 4x-CO–CH$_2$–), 1.71–1.53 (m, 8H, 4x-C$\underline{H}_2$–CH$_3$), 1.0–0.87 (m, 12H, 4x-CH$_3$); $J_{2,3}=3.6$, $J_{3,4}=9.7$, $J_{4,5}=10.2$ Hz; ^{13}C NMR (100.62 MHz, $CDCl_3$) δ 174.9–172.5 (C=O), 94.9

(bs, C-1), 72.2 (C-5), 69.1 (C-2), 68.5 (C-3), 65.8 (C-4), 61.6 (C-6), 36.0, 35.9 (–CO–$\underline{C}H_2$–), 18.4, 18.3, 18.1 (–$\underline{C}H_2$–CH_3), 13.5 (–CH_3); $^3J_{C\text{-}2,P}=9.2$ Hz; ^{31}P NMR (101.26 MHz, $CDCl_3$) δ −1.71; MALDI-TOF-MS: *m/z* 563.61 $[M+Na]^+$, 579.50 $[M+K]^+$, 585.49 $[M-H+Na+Na]^+$, 601.41 $[M-H+Na+K]^+$. Anal. calcd for $C_{22}H_{37}O_{13}P$ (540.50): C 48.89, H 6.90; Found: C 48.90, H 6.60.

2,3,4,6-Tetra-*O*-pivaloyl-α-D-mannopyranosyl phosphate (8). Compound **5** (1.30 g, 1.67 mmol) was reacted in the manner described above in 32 mL solvent for 6 h.

Yield: 0.82 g (1.37 mmol, 82%, white solid); $[\alpha]_D$ +27.0 (c 1.0, $CHCl_3$); mp ~245 °C decomposition; R_f 0.34 in 6:3.5:0.5 chloroform/methanol/water; ^{1}H NMR (400 MHz, methanol-d_4) δ 5.58 (dd~t, 1H, H-4), 5.48 (dd~t, 2H, H-1, H-3), 5.32 (bs, 1H, H-2), 4.48–4.15 (m, 3H, H-5, H-6a, H-6b), 1.28, 1.23, 1.15, 1.10 (4xs, 36H, 4x-C($\underline{C}H_3$)$_3$); $J_{3,4}=10.4$, $J_{4,5}=10.1$ Hz; ^{13}C NMR (100.62 MHz, methanol-d_4) δ 179.8, 179.3, 178.8 (C=O), 95.2 (bs, C-1), 71.7 (C-2), 71.4 (C-3), 70.8 (C-5), 66.5 (C-4), 62.9 (C-6), 40.3, 40.2, 40.1, 40.0 (C_q,–$\underline{C}(CH_3)_3$), 28.0, 27.9, 27.8 (–C($\underline{C}H_3$)$_3$; $^3J_{C\text{-}2,P}=12.7$ Hz; ^{31}P NMR (101.26 MHz, methanol-d_4) δ −1.75; MALDI-TOF-MS: *m/z* 619.42 $[M+Na]^+$, 635.35 $[M+K]^+$, 641.40 $[M-H+Na+Na]^+$, 657.33 $[M-H+Na+K]^+$, 673.29 $[M-H+K+K]^+$. Anal. calcd for $C_{26}H_{45}O_{13}P$ (596.61): C 52.34, H 7.60; Found: C 45.28, H 6.61 (Material hygroscopic).

2,3,4,6-Tetra-*O*-*iso*-propylcarbonate-α-D-mannopyranosyl phosphate (9). Compound **6** (0.45 g, 0.57 mmol) was reacted in the manner described above in 8 mL solvent overnight.

Yield: 0.26 g (0.43 mmol, 75%, solid); $[\alpha]_D$ +23.4 (c 1.0, $CHCl_3$); mp 184.1 °C; R_f 0.30 in 6:3.5:0.5 chloroform/methanol/water; ^{1}H NMR (400 MHz, methanol-d_4) δ 5.58 (d, 1H, H-1), 5.26 (bs, 1H, H-2), 5.21 (dd, 1H, H-3), 5.12 (dd~t, 1H, H-4), 4.90–4.79 (m, 4H, 4x-C$\underline{H}$(CH_3)$_2$), 4.36–4.21 (m, 3H, H-5, H-6a, H-6b), 1.23–1.20 (m, 24H, 8x-CH(C$\underline{H}_3$)$_2$); $J_{2,3}=3.1$, $J_{3,4}=10.2$, $J_{4,5}=9.9$, $J_{H\text{-}1,P}=7.1$ Hz; ^{13}C NMR (100.62 MHz, methanol-d_4) δ 156.2, 155.5, 155.1 (C=O), 95.1 (d, C-1), 74.4, 74.2, 73.8, (–$\underline{C}H$(CH_3)$_2$), 74.3 (C-3), 74.3 (d, C-2), 70.9 (C-4), 70.4 (C-5), 66.5 (C-6), 22.3, 22.2 (–CH(C$\underline{H}_3$)$_2$); $^2J_{C\text{-}1,P}=3.6$, $^3J_{C\text{-}2,P}=9.7$ Hz; ^{31}P NMR (101.26 MHz, methanol-d_4) δ −1.02; MALDI-TOF-MS: *m/z* 627.35 $[M+Na]^+$, 643.29 $[M+K]^+$, 649.33 $[M-H+Na+Na]^+$, 665.28 $[M-H+Na+K]^+$. Anal. calcd for $C_{22}H_{37}O_{17}P$ (604.51): C 43.71, H 6.17; Found: C 44.32, H 6.06.

bis-Acetoxymethyl-(2,3,4,6-tetra-*O*-butyryl-α-D-mannopyranosyl)-phosphate (10). A solution of mannopyranosyl-1-phosphate **7** (131 mg, 0.24 mmol) in dry acetonitrile (3 mL) was evaporated to dryness. DIPEA (0.2 mL, 1.2 mmol) and dry acetonitrile (3 mL) were added and the solution was evaporated again and then in high vacuum. Subsequently, dry acetonitrile (3 mL), DIPEA (0.41 mL, 2.4 mmol) and bromomethylacetate (0.59 mL, 6.1 mmol) were added under argon. The mixture was stirred at room temperature overnight, the solvents evaporated and the residue purified by column chromatography with petroleum ether/ethyl acetate (1:1) to give **10** as a colourless sirup (68 mg, 0.1 mmol) in 41% yield; $[\alpha]_D$ +7.5 (c 0.5, $CHCl_3$); R_f 0.29 in 1:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.73–5.64 (m, 5H, H-1, 2x-CH_2–, AM), 5.41 (dd~t, 1H, H-4), 5.38 (dd~t, 1H, H-2), 5.35 (dd, 1H, H-3), 4.28–4.15 (m, 3H, H-5, H-6a, H-6b), 2.42–2.19 (m, 8H, 4x-CO–CH_2–), 2.17, 2.16 (2xs, 6H,–CH_3, AM), 1.75–1.52 (m, 8H, 4x-C$\underline{H}_2$–CH_3), 1.04–0.87 (m, 12H, 4x-CH_3); $J_{1,2}=1.9$, $J_{2,3}=3.2$, $J_{3,4}=9.9$, $J_{4,5}=9.9$, $J_{5,6a}=3.8$, $J_{5,6b}=1.5$, $J_{6,6}=11.7$, $J_{H\text{-}1,P}=7.7$ Hz; ^{13}C NMR (100.62 MHz, $CDCl_3$) δ 173.1, 172.2, 172.1 (C=O), 169.3, 169.2 (C=O, AM), 95.9 (d, C-1), 82.7 (dd~t,–CH_2–, AM), 70.8 (C-5), 68.3 (d, C-2), 68.1 (C-3), 64.7 (C-4), 61.4 (C-6), 35.9, 35.8 (–CO–$\underline{C}H_2$–), 20.6 (–CH_3, AM), 18.4, 18.3, 18.1 (–$\underline{C}H_2$–CH_3), 13.7, 13.6, 13.5 (–CH_3); $^2J_{C\text{-}1,P}=6.1$, $^2J_{CH2,P}=6.1$, $^3J_{C\text{-}2,P}=12.2$ Hz; ^{31}P NMR (101.26 MHz, $CDCl_3$) δ −5.05; MALDI-TOF-MS: *m/z* 707.29 $[M+Na]^+$, 723.19 $[M+K]^+$. Anal. calcd for $C_{28}H_{45}O_{17}P$ (684.51): C 49.12, H 6.63; Found: C 49.60, H 6.79.

bis-Pivaloyloxymethyl-(2,3,4,6-tetra-*O*-butyryl-α-D-mannopyranosyl)-phosphate (11). Mannopyranosyl-1-phosphate **7** (111 mg, 0.21 mmol) was suspended in dry acetonitrile (1 mL). DIPEA (0.11 mL, 0.62 mmol) and iodomethylpivaloate (0.15 g, 0.62 mmol) were added. The mixture was stirred at room temperature overnight, then the solvent removed and the residue dissolved in ethyl acetate. The mixture was washed twice with saturated brine, dried over sodium sulfate, filtrated and concentrated under reduced pressure. Purification of the crude was followed by column chromatography (petroleum ether/ethyl acetate + 1% Et_3N, 1:1) to give compound **11** (39 mg, 0.05 mmol, sirup) in 24% yield; $[\alpha]_D$ +4.1 (c 0.4, $CHCl_3$); R_f 0.88 in 1:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.67–5.58 (m, 5H, H-1, 2x-CH_2–, POM), 5.34 (dd~t, 1H, H-4), 5.31 (bs, 1H, H-2), 5.29 (dd, 1H, H-3), 4.22–4.07 (m, 3H, H-5, H-6a, H-6b), 2.33 (dt, 2H, –CO–CH_2–), 2.27 (t, 2H, –CO–CH_2–), 2.19 (dt, 2H, –CO–CH_2–), 2.12 (dt, 2H, –CO–CH_2), 1.68–1.46 (m, 8H, 4x-C$\underline{H}_2$–CH_3), 1.18 (s, 18H, –C(CH_3)$_3$, POM), 0.96–0.81 (m, 12H, 4x-CH_3); $J_{1,2}=1.5$, $J_{2,3}=3.1$, $J_{3,4}=9.2$, $J_{4,5}=9.7$, $J_{5,6a}=4.1$, $J_{6,6}=12.2$ Hz; ^{13}C NMR (100.62 MHz, $CDCl_3$) δ 173.5, 172.6, 172.5, 172.4 (C=O), 96.4 (d, C-1), 83.0 (d, –CH_2–, POM), 82.8 (d, –CH_2–, POM), 70.7 (C-5), 68.3 (d, C-2), 68.1 (C-3), 64.7 (C-4), 61.4 (C-6), 39.1 (C_q, –$\underline{C}(CH_3)_3$, POM), 36.4, 36.2 (–CO–$\underline{C}H_2$–), 27.2 (–C($\underline{C}H_3$)$_3$, POM), 18.8, 18.7, 18.5 (–$\underline{C}H_2$–CH_3), 14.1, 14.0, 13.9 (–CH_3); $^2J_{C\text{-}1,P}=6.1$, 2x $^2J_{CH2,P}=6.1$, $^3J_{C\text{-}2,P}=12.2$ Hz; ^{31}P NMR (101.26 MHz, $CDCl_3$) δ −4.92; MALDI-TOF-MS: *m/z* 791.32 $[M+Na]^+$, 807.29 $[M+K]^+$. Anal. calcd for $C_{34}H_{57}O_{17}P$ (768.81): C 53.12, H 7.47; Found: C 53.75, H 7.56.

bis-Acetoxymethyl-(2,3,4,6-tetra-*O*-pivaloyl-α-D-mannopyranosyl)-phosphate (12). Mannopyranosyl-1-phosphate **8** (269 mg, 0.45 mmol) was suspended in dry acetonitrile (3 mL) and dry toluene (0.5 mL). DIPEA (0.22 mL, 1.35 mmol) and bromomethylacetae (0.13

mL, 1.35 mmol) were added. The mixture was stirred overnight at room temperature, the reaction being monitored by TLC (petroleum ether/ethyl acetate, 1:1). After further addition of bromomethylacetate (0.1 mL, 1.02 mmol) and DIPEA (0.1 mL, 0.59 mmol) the suspension was stirred again at room temperature for 2 days. The solvents were removed, the residue dissolved in ethyl acetate (5 mL) and dichloromethane (5 mL). The mixture was washed twice with saturated brine, dried over sodium sulfate, filtrated and concentrated under reduced pressure. The crude residue was purified by column chromatography (petroleum ether/ethyl acetate, 2:1) to yield **12** (104 mg, 0.14 mmol) as yellowish solid in 31%; $[\alpha]_D$ +12.0 (c 0.5, $CHCl_3$); mp 88.5 °C; R_f 0.31 in 1:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.74–5.63 (m, 5H, H-1, 2x-CH_2–, AM), 5.58 (dd∼t, 1H, H-4), 5.39–5.35 (m, 2H, H-2, H-3), 4.29–4.16 (m, 3H, H-5, H-6a, H-6b), 2.17, 2.16 (2xs, 6H, –CH_3, AM), 1.28, 1.24, 11.16, 1.12 (4xs, 36H, 4x-C(C$\underline{H}_3$)$_3$); $J_{1,2}$ = 1.8, $J_{3,4}$ = 9.4, $J_{4,5}$ = 10.2 Hz; ^{13}C NMR (100.62 MHz, $CDCl_3$) δ 178.0, 176.4 (C=O), 171.2, 169.2, 169.1 (C=O, AM), 96.2 (d, C-1), 82.7 (d,–CH_2–, AM), 82.6 (d, –CH_2–, AM), 71.0 (C-5), 68.6 (C-3), 68.4 (d, C-2), 64.1 (C-4), 61.1 (C-6), 38.9, 38.8, 38.7 (C_q, –C(C$\underline{H}_3$)$_3$), 27.1, 27.0 (–C(C$\underline{H}_3$)$_3$), 21.1, 20.6, 20.5 (–C$\underline{H}_3$, AM); $^2J_{C\text{-}1,P}$ = 5.1, 2x $^2J_{CH2,P}$ = 5.1, $^3J_{C\text{-}2,P}$ = 12.2 Hz; ^{31}P NMR (101.26 MHz, $CDCl_3$) δ −5.47; MALDI-TOF-MS: *m/z* 763.52 $[M+Na]^+$, 779.46 $[M+K]^+$. Anal. calcd for $C_{32}H_{53}O_{17}P$ (740.74): C 51.89, H 7.21; Found: C 51.19, H 7.32.

bis-Pivaloyloxymethyl-(2,3,4,6-tetra-*O*-pivaloyl-α-D-mannopyranosyl)-phosphate (13). Compound **8** (232 mg, 0.39 mmol) was treated by the same procedure [dry acetonitrile (3 mL), dry toluene (1 mL), iodomethylpivaloate (0.57 g, 2.34 mmol), DIPEA (0.40 mL, 2.34 mmol), stirred for 3d] as for compound **11**. The obtained residue was purified by column chromatography (petroleum ether/ethyl acetate + 1% Et_3N, 3:1) to give **13** as a white solid (18 mg, 0.02 mmol) in 6% yield; $[\alpha]_D$ +6.1 (c 0.5, $CHCl_3$); mp 86.7 °C; R_f 0.62 in 1:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.75–5.64 (m, 5H, H-1, 2x-CH_2–POM), 5.58 (dd∼t, 1H, H-4), 5.41–5.36 (m, 2H, H-2, H-3), 4.32–4.22 (m, 2H, H-5, H-6a), 4.13 (d, 1H, H-6b), 1.27, 1.23, 1.16, 1.11 (4xs, 36H, 4x-C(C$\underline{H}_3$)$_3$), 1.24 (2xs, 18H, 2x-C(C$\underline{H}_3$)$_3$, POM); $J_{1,2}$ = 1.5, $J_{2,3}$ = 3.1, $J_{3,4}$ = 9.9, $J_{4,5}$ = 9.9, $J_{5,6a}$ = 2.8, $J_{6,6}$ = 11.2 $^3J_{H\text{-}1,P}$ = 5.6 Hz; ^{13}C NMR (100.62 MHz, $CDCl_3$) δ 177.9, 176.9, 176.7, 176.5 (C=O), 96.2 (d, C-1), 83.0 (d, –CH_2–, POM), 82.9 (d, –CH_2–, POM), 70.9 (C-5), 68.5 (d, C-2), 68.5 (C-3), 64.2 (C-4), 61.2 (C-6), 38.9, 38.8, 38.7 (C_q, –C(C$\underline{H}_3$)$_3$, Piv, POM), 27.1, 27.0 (–C(C$\underline{H}_3$)$_3$), 26.8 (–C(C$\underline{H}_3$)$_3$, POM); $^3J_{C\text{-}1,P}$ = 5.6, 2x $^2J_{CH2,P}$ = 5.1 $^3J_{C\text{-}2,P}$ = 12.7 Hz; ^{31}P NMR (101.26 MHz, $CDCl_3$) δ −5.38; MALDI-TOF-MS: *m/z* 847.33 $[M+Na]^+$, 863.30 $[M+K]^+$. Anal. calcd for $C_{38}H_{65}O_{17}P$ (824.90): C 55.33, H 7.94; Found: C 56.10, H 7.99.

bis-Acetoxymethyl-(2,3,4,6-tetra-*O*-*iso*-propylcarbonate-α-D-mannopyranosyl)-phosphate (14). Mannopyranosyl-1-phosphate **9** (25.6 mg, 0.04 mmol) was treated by the same procedure [dry acetonitrile (1 mL + 1 mL + 0.5 mL), DIPEA (0.02 mL, 0.12 mmol + 0.04 mL, 0.24 mmol), bromomethylacetate (96 μL, 0.98 mmol), stirred for 3d, column chromatography with petroleum ether/ethyl acetate (1:1)] as compound **10**. Product **14** (5.4 mg, 7.2 μmol) was obtained as a colourless sirup in 17% yield.%; $[\alpha]_D$ −1.6 (c 0.6, $CHCl_3$); R_f 0.22 in 1:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.79 (dd, 1H, H-1), 5.73–5.64 (m, 4H, 2x-CH_2–, AM), 5.51 (dd∼t, 1H, H-2), 5.16–5.13 (m, 2H, H-3, H-4), 4.92–4.81 (m, 4H, 4x-CH(C$\underline{H}_3$)$_2$), 4.33 (dd, 1H, H-6a), 4.28–4.22 (m, 2H, H-5, H-6b), 2.18, 2.16 (2xs, 6H, –CH_3, AM), 1.34–1.25 (m, 24H, 4x-CH(C$\underline{H}_3$)$_2$); $J_{1,2}$ = 1.5, $J_{2,3}$ = 2.0, $J_{5,6a}$ = 6.4, $J_{6,6}$ = 12.2 Hz; ^{13}C NMR (100.62 MHz, $CDCl_3$) δ 168.3, 168.2 (C=O, AM), 154.2, 153.5, 153.4, 153.3 (C=O), 95.5 (d, C-1), 82.8 (d, –CH_2–, AM), 82.7 (d,–CH_2–, AM), 73.5, 73.2, 72.9, 72.4 (–C$\underline{H}$(CH$_3$)$_2$), 71.5, 68.9 (C-3, C-4), 71.2 (d, C-2), 70.5 (C-5), 65.2 (C-6), 21.7, 21.6, 21.5 (–CH(C$\underline{H}_3$)$_2$), 20.6, 20.5 (–CH_3, AM); $^2J_{C\text{-}1,P}$ = 5.1, 2x $^2J_{CH2,P}$ = 5.1, $^3J_{C\text{-}2,P}$ = 12.2 Hz; ^{31}P NMR (101.26 MHz, $CDCl_3$) δ −5.30; MALDI-TOF-MS: *m/z* 771.08 $[M+Na]^+$, 787.03 $[M+K]^+$. Anal. calcd for $C_{28}H_{45}O_{21}P$ (748.51): C 44.92, H 6.06; Found: C 45.09, H 6.20.

bis-Pivaloyloxy-(2,3,4,6-tetra-*O*-*iso*-propylcarbonate-α-D-mannopyranosyl)-phosphate (15). Compound **9** (189 mg, 0.31 mmol) was suspended in dry acetonitrile (3 mL). DIPEA (0.16 mL, 0.94 mmol) and iodomethylpivaloate (0.23 g, 0.94 mmol) were added. The mixture was stirred overnight at room temperature and the course of the reaction followed by TLC (petroleum ether/ethyl acetate, 1:1). The mixture was stirred for a further 3 days after addition of DIPEA (0.16 mL) and iodomethylpivaloate (0.23 g). The solvents were removed, the residue was dissolved in ethyl acetate (5 mL) and dichloromethane (5 mL). The mixture was washed twice with saturated brine, dried over sodium sulfate, filtrated and concentrated under reduced pressure. The crude residue was purified by column chromatography (petroleum ether/ethyl acetate + 1% Et_3N, 3:1) to yield **15** (9.3 mg, 0.01 mmol) as colourless sirup in 4%; $[\alpha]_D$ +2.3 (c 0.5, $CHCl_3$); R_f 0.5 in 1:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.78 (dd, 1H, H-1), 5.75–5.65 (m, 4H, 2x-CH_2–, POM), 5.30 (dd∼t, 1H, H-2), 5.16–5.12 (m, 2H, H-3, H-4), 4.91–4.81 (m, 4H, 4x-C$\underline{H}$(CH$_3$)$_2$), 4.33 (dd, 1H, H-6a), 4.27–4.22 (m, 2H, H-5, H-6b), 1.32–1.26 (m, 24H, 4x-CH(C$\underline{H}_3$)$_2$), 1.24, 1.23 (2xs, 18H, 2x-C(C$\underline{H}_3$)$_3$, POM); $J_{1,2}$ = 1.8, $J_{2,3}$ = 4.9, $J_{5,6a}$ = 6.4, $J_{5,6b}$ = 2.5, $J_{6,6}$ = 12.2, $J_{CH,CH3}$ = 6.4, $^3J_{H\text{-}1,P}$ = 5.6 Hz; ^{13}C NMR (100.62 MHz, $CDCl_3$) δ 95.5 (d, C-1), 83.0 (d, –CH_2–, POM), 82.9 (d, –CH_2–, POM), 73.4, 73.1, 72.9, 72.4 (–C$\underline{H}$(CH$_3$)$_2$), 71.6 (C-3), 71.3 (d, C-2), 70.5 (C-5), 69.0 (C-4), 65.2 (C-6), 26.8 (–C(C$\underline{H}_3$)$_3$, POM), 21.7, 21.6 (–CH(C$\underline{H}_3$)$_2$); $^3J_{C\text{-}1,P}$ = 5.1, 2x $^2J_{CH2,P}$ = 5.1, $^3J_{C\text{-}2,P}$ = 12.2 Hz; ^{31}P NMR (101.26 MHz, $CDCl_3$) δ −5.23; MALDI-TOF-MS: *m/z* 855.26 $[M+Na]^+$, 871.22 $[M+K]^+$. Anal. calcd for $C_{34}H_{57}O_{21}P$ (8.32.79): C 49.04, H 6.90; Found: C 50.45, H 7.34.

Enzymatic tests

Crude enzyme. Compound **10** (10.1 mg) was incubated in phosphate buffer (pH 7.4, 0.05 M, 1 mL) with porcine

liver esterase (crude, EC 3.1.1., 1 U) at 37 °C for 3 h. The reaction was monitored by TLC (petroleum ether/ethyl acetate, 1:1). After termination by quenching with methanol (2 mL) the residue was centrifuged and desalted using $ZipTip_{C18}$ for direct spotting on a MALDI-TOF MS target. Methanol/water solution in various ratio was used for elution; MALDI-TOF-MS: *m*/*z* 706.98 $[M+Na]^+$, 722.92 $[M+K]^+$, 635.21 $[M–AM+Na]^+$, 651.19 $[M–AM+K]^+$, 657.27 $[M–AM–H+Na+Na]^+$, 673.24 $[M–AM–H+Na+K]^+$, 563.23 $[M–2AM+Na]^+$, 585.24 $[M–2AM–H+Na+Na]^+$, 601.21 $[M–2AM–H+Na+K]^+$, 587.26 $[M–AM–Bt–H+Na+Na]^+$, 515.31 $[M–2AM–Bt\text{-}H+Na+Na]^+$, 483.40 $[M–PO_3AM_2+Na]^+$/$[M–2Bt–2Ac+2H+Na]^+$, 499.31 $[M–PO_3AM_2+K]^+$/$[M–2Bt–2Ac+2H+K]^+$, 413.68 $[M–PO_3AM_2–Bt+Na]^+$, 429.63 $[M–PO_3AM_2–Bt+K]^+$.

Pure enzyme. Compound **10** (9.7 mg) was incubated in phosphate buffer (pH 7.4, 0.05 M, 1 mL) with porcine liver esterase (pure, EC 3.1.1.1, 1 U) at 37 °C for 3 h. The reaction was monitored by TLC (petroleum ether/ethyl acetate, 1:1). After termination by quenching with methanol (2 mL) the residue was centrifuged and desalted using $ZipTip_{C18}$ for direct spotting on a MALDI-TOF MS target and ESI analysis. Methanol/water solution in various ratio was used for elution; MALDI-TOF-MS: *m*/*z* 707.19 $[M+Na]^+$, 723.09 $[M+K]^+$, 635.22 $[M–AM+Na]^+$, 651.17 $[M–AM+K]^+$, 657.25 $[M–AM–H+Na+Na]^+$, 673.22 $[M–AM–H+Na+K]^+$, 611.19 $[M–Bt–Ac+H+K]^+$, 623.23 $[M–2Ac+H+Na]^+$, 563.21 $[M–2AM+Na]^+$, 585.22 $[M–2AM–H+Na+Na]^+$, 601.11 $[M–2AM–H+Na+K]^+$, 483.44 $[M–PO_3AM_2+Na]^+$/$[M–2Bt–2Ac+2H+Na]^+$, 499.22 $[M–PO_3AM_2+K]^+$/$[M–2Bt–2Ac+2H+K]^+$; ESI-MS: *m*/*z* 354 $[M–AM–4Bt+Na]^+$, 353 $[M–2AM–3Bt+Na]^+$, 283 $[M–2AM–4Bt+Na]$.

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