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En route to deoxygenated *N*-acetyllactosamine analogues employing uridyl and galactosyl transferases

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Dedicated to Professor Johannis P. Kamerling on the occasion of his 65th birthday

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ABSTRACT

All monodeoxygenated galactoses were treated with galactokinase, and for the 2-, 3-, and 4-deoxy compounds, transformation into the corresponding galactopyranosyl phosphates could be observed. In case of the 2-deoxy derivative, further reaction via UDP-2-deoxy-D-*lyxo*-hexose (UDP-2-deoxygalactose), which was also obtained chemically, the multiple enzymatic system could be employed to prepare 2′-deoxy-*N*-acetyllactosamine.

1. Introduction

Due to their prominent role in a large number of biological processes such as cell–cell and cell–pathogen interactions, glycostructures are in the focus of intense research as important examples.[1–5] Syntheses of highly complex saccharide structures were achieved successfully both by classical and by enzymatic approaches.[6–9] However, elaborate blocking group chemistry paired with usually cumbersome workup procedures is a disadvantage of classical oligosaccharide synthesis, and in several cases the use of enzymes turned out to be quite convenient and effective to overcome these difficulties. The majority of enzymes used in oligosaccharide synthesis to date reside among the subclasses of glycosylhydrolases and glycosyltransferases. By use of the former in transglycosylations, mixtures of regioisomers are usually obtained, which require intricate separation procedures. The latter are employed with nucleotide-activated sugar donor substrates that display an advantageously well-defined regio-, stereo-, and acceptor substrate specificity unmatched by hydrolases. Another subclass of transferases defines those that transfer a nucleotide residue irrespective of the sugar unit, thus converting a sugar nucleotide donor into another one. Among the glycosyl transferases that utilize nucleoside diphosphohexoses, uridine-5′-diphosphogalactose transferase (GalT, E.C. 2.4.1.22), and for the sugar nucleotide-converting transferases, galactose-1-phosphate uridyl transferases (Gal-1-P-UT, E.C. 2.7.7.12) are comparatively easily accessible and hence are the most intensively studied.[8,9]

The use of such enzymes is described in the following investigation toward syntheses en route to several *N*-acetyllactosamine analogues that display a deoxy functionality in the galactose part at positions 2, 3, 4, or 6. Such disaccharide structures are related to components found in many oligosaccharides and glycoconjugates. The GalT transferase requires uridine-5′-diphosphogalactose (UDP-Gal) as the genuine donor substrate, and thus in this case formation of various deoxygenated UDP-galactoses was of interest. These were prepared both enzymatically in situ and in case of UDP-2-deoxy-D-*lyxo*-hexose (UDP-2-deoxy-Gal) also chemically utilizing the morpholidate coupling procedure.

2. Results and discussion

Initially, the starting α-glycosyl phosphates (**6**–**8**) were synthesized by reacting the various deoxygenated galactoses (**1**–**4**) and L-arabinose (**5**) with ATP and galactokinase. ATP was recycled by use of the phosphoenolpyruvate/pyruvate kinase system (Scheme 1). The reaction was monitored and quantified by a pyruvate assay. The commercially available 2-deoxy-D-*lyxo*-hexose (2-deoxy-Gal, **1**) proved to be convertible to the corresponding phosphates in

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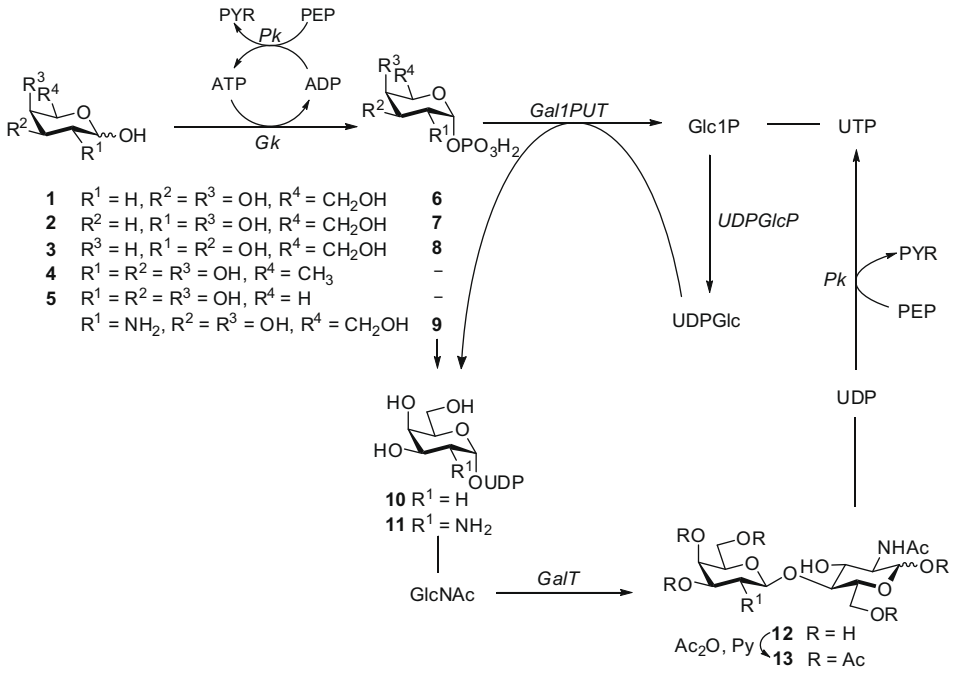


Scheme 1. Galactose-1-phosphate uridyltransferase-catalyzed formation of modified LacNAc derivatives. Enzymes: *GalT*: β-1→4)-galactosyltransferase; *Gal1PUT*: galactose-1-phosphate uridyltransferase; *Pk*: pyruvatekinase; *Gk*: galactokinase; *UDPGlcP*: UDP-glucose pyrophosphorylase.

excellent 91% yield after six days. This transformation was compatible for scaling up to a one gram scale.

3-Deoxy-D-*xylo*-hexose (3-D-Gal, **2**) was prepared in five steps from D-galactono-1,4-lactone via 2,5,6-tri-*O*-acetyl-3-deoxy-D-*xylo*-hexono-1,4-lactone[10] and its reduction with diisoamylborane, followed by deacetylation (for NMR data cf.[11]). Also 3-deoxy-Gal (**2**) displayed a reasonable activity toward galactokinase, which is expressed in a 45% yield for the corresponding phosphate **7** on a preparative scale.

4-Deoxy-D-*xylo*-hexose (4-D-Gal, **3**) was obtained following low-temperature benzoylation of galactose, which resulted in the 4-unblocked tetrabenzoate. Subsequent triflation, formation of the 4-iodo gluco derivative, reduction, and debenzoylation gave **3** (cf.[12]). In case of the 4-deoxy-Gal (**3**), the pyruvate assay showed a 5% turnover after 4 days into the phosphate **8**. However, neither D-fucose (**4**) nor L-arabinose (**5**) showed any reaction within six days as observed by the pyruvate assay analysis.

As previously shown by Nunez and Barker, GalT-catalyzed galactosylation transfer could be carried out efficiently on a preparative scale.[13] The rather expensive UDP-Gal was generated in situ starting from UDP-Glc by use of galactose-1-phosphate uridyl transferase, which transfers UMP from UDP-Glc to galactosyl phosphates. The readily available UDP-Glc is still relatively costly, but could be conveniently generated in situ from inexpensive α-D-glucose-1-phosphate and UTP catalyzed by uridine-5′-diphosphoglucose pyrophosphorylase (E.C. 2.7.7.9). The by-product pyrophosphate is subsequently hydrolyzed by inorganic pyrophosphatase (PPase, E.C. 3.6.1.1) to render the UDP-Glc formation irreversible. Upon galactosylation of GlcNAc that leads to LacNAc derivatives and UDP, the catalytic cycle is closed by formation of consumed UTP via phosphorylation of the released UDP with phosphoenolpyruvate (PEP) in the presence of pyruvate kinase (E.C. 2.7.1.4). By this enzymatic cycle the desired deoxygenated *N*-acetyllactosamines were obtained (Scheme 1). Thus, the intermediate nucleotides remain within the cycle and are generated as well as consumed in catalytic amounts, keeping the reaction within economically reasonable limits. In the case of the genuine galactose-1-phosphate, by applying this catalytic cycle Whitesides and co-workers reported the efficient preparation of UDP-Gal in 43% yield on a gram scale.[14]

In the case of 2-deoxy-D-*lyxo*-hexopyranosyl-1-phosphate (2-deoxy-galactopyranosyl-1-phosphate, **6**), the yield of 2′-deoxy-LacNAc (**12**) formed within this reaction could be improved to 29% on larger scale compared to previously reported results[15–17] by changing the buffer system from Tris to cacodylate buffer. Starting from 3-deoxy-D-*xylo*-hexopyranosyl-1-phosphate (3-deoxy-galactopyranosyl-1-phosphate, **7**) and 4-deoxy-D-*xylo*-hexopyranosyl-1-phosphate (4-deoxy-galactopyranosyl-1-phosphate, **8**), on the other hand, no formation of the corresponding LacNAc disaccharides could be observed, although their corresponding UDP derivatives were known to be donor substrates for galactosyl transferases.[18,19] Either both phosphates were no substrates for the Gal-1-P-uridyl transferase or the equilibrium of the galactosyl transferase-catalyzed reaction is so little favored that even the cleavage of pyrophosphate had no significant effect. The same applied to D-galactosaminyl phosphate (**9**), which also did not give the disaccharide D-GalNβ1-4GlcNAc. In previous work we showed that Gal-1-P-uridyl transferase tolerated an amino group but not an *N*-acetamido moiety in the 2-position of a galactopyranosyl phosphate.[20] The present findings expand the acceptance of a 2-deoxy functionality in galactopyranosyl phosphates, whereas the other hydroxyl functionalities seem to be critical for substrate recognition by the enzyme.

In order to further improve the economic exploitability of the reaction cycle shown in Scheme 1, in the case of the successful preparation of 2′-deoxy-LacNAc, the need for the relatively expensive UDP-Gal-1-P-uridyl transferase was bypassed by the chemical synthesis of the Leloir donor substrate UDP-2-deoxy-D-*lyxo*-hexose (UDP-2-deoxy-D-galactose, **10**) (cf.[21]). Starting by peracetylation of 2-deoxy-D-galactopyranose, subsequent Lewis acid-catalyzed thiophenylation and Zemplén deacetylation gave thiophenyl 2-deoxy-D-*lyxo*-hexopyranoside (thiophenyl 2-deoxy-galactopyranoside). Further perbenzylation and NBS-activated glycoside cleavage led to compound **14** by these five steps in 87–95% overall yield.

Employing dibenzyl diisopropyl phosphoamidite under 1*H*-tetrazole catalysis (cf.[22,23]), followed by in situ oxidation with mCPBA,[24] resulted in formation of the protected 2-deoxy-α-D-*lyxo*-hexopyranosyl dibenzylphosphate (**15**) in 52% overall yield. By hydrogenolysis with 10% palladium-on-charcoal, derivative **6**[25] was obtained in 65% yield. Compound **6** was transformed into its triethylammonium salt, treated with UMP-morpholidate and 1*H*-tetrazole (cf.[16,26,27]) and purified on Bio-Gel P2 to give the corresponding UDP-2-deoxy-galactose derivative **10** in 32% yield (Scheme 2).

3. Experimental

3.1. General methods

TLC was performed on Silica Gel 60-coated aluminum sheets (E. Merck) using the given eluent mixtures. Spots were visualized under UV light at 366 nm and by spraying with 10% H_2SO_4 in EtOH and subsequent heating. Column chromatography was performed on Silica Gel 60 (230–240 mesh, grain size 0.040–0.063 mm, E. Merck). Size exclusion chromatography and desalting procedures were performed on Bio-Gel P2 (Bio-Rad) with either aqueous 250 mM NH_4HCO_3 solution or deionized water as eluent. Optical rotations were measured on a Perkin–Elmer Polarimeter 243, with $[\alpha]_D$ values given in units of 10^{-1} deg cm^2 g^{-1}. NMR spectra were recorded on a Bruker AMX-400 (^{1}H: 400.13 MHz, ^{13}C: 100.61 MHz) and DRX-500 (^{1}H: 500.13 MHz, ^{13}C: 125.83 MHz) spectrometers. Chemical shifts are referred to the solvents used.

3.2. 2-Deoxy-α-D-*lyxo*-hexopyranosyl-1-phosphate (6)

2-Deoxy-D-*lyxo*-hexose (2-deoxy-D-galactose, **1**, 1.0 g, 6.1 mmol) was dissolved together with KCl (455 mg, 6.1 mmol), phosphoenol pyruvate (PEP, 1385 mg, 6.7 mmol), ATP (75 mg, 122 μmol), and dithiothreitol (DTT, 50 mg, 305 μmol) in 50 mM Tris buffer (61 mL, pH 7.5) and degassed with argon for 10 min. Then $MgCl_2{\cdot}6H_2O$ (125 mg, 610 μmol), bovine serum albumin (BSA 60 mg), a microspatulaful of NaN_3, galactokinase (*GK*, 10U), and pyruvate kinase (*PK*, 250) were added. The mixture was incubated at 30 °C for 6 days, then centrifuged, and the supernatant was freeze dried. The remaining material was washed with double-distilled water (150 mL) and then purified on Dowex 2x8 (Cl^-) by elution with a linear LiCl gradient (0–0.8 M). The monophosphate fraction eluting at 0.1–0.4 M LiCl was pooled and lyophilized. By gel-permeation chromatographic separation on Sephadex G10 (three times), the LiCl was completely removed and the material was lyophilized to give **6** as a colorless hygroscopic compound: yield 1.42 g (91%). ^{1}H NMR (400 MHz, D_2O): δ 5.42 (dd, 1H, 1-H); 4.02-3.96 (m, 1H, 3-H); 3.96–3.92 (m, 1H, 5-H); 3.64–3.51 (2m, 2H, 6a-, 6b-H); 3.71 (dd, 1H, 4-H); 1.82–1.70 (m, 2H, 2a-, 2e-H); $J_{1,P}$ 7.0, $J_{1,2a}$ 7.5, $J_{2a,3}$ 11.0, $J_{3,4}$ 2.5, $J_{4,5}$ 0.5 Hz. ^{13}C NMR (100 MHz, D_2O): δ 93.39 (C-1); 71.73, 68.08, 64.99 (C-3, C-4, C-5); 62.13 (C-6); 32.95 (C-2).

3.3. 3-Deoxy-α-D-*xylo*-hexopyranosyl phosphate (7)

3-Deoxy-D-*xylo*-hexose (3-deoxy-D-galactose, **2**, 100 mg, 610 μmol) was dissolved together with KCl (46 mg, 610 μmol), PEP (138 mg, 670 μmol), ATP (8 mg, 13 μmol) in 50 mM Tris buffer (6.1 mL, pH 7.5), and degassed with argon for 10 min. Then $MgCl_2{\cdot}6H_2O$ (13 mg, 61 μmol), BSA (6 mg) and NaN_3 (microspatulaful), *GK* (2 U), and *PK* (80 U) were added. After incubation at 30 °C for 7 days, workup and purification were done as for compound **6**. The glycosyl phosphate **7** was obtained as a colorless, hygroscopic material: yield 70 mg (45%). ^{1}H NMR (400 MHz, D_2O): δ 5.53 (dd, 1H, 1-H); 4.02-3.43 (m, 5H, 2-,4-,5-,6a-,6b-H); 1.95–1.79 (m, 2H, 3a-, 3e-H); $J_{1,P}$ 7.0, $J_{1,2}$ 3.5 Hz. ^{13}C NMR (100 MHz, D_2O): δ 93.56 (C-1); 71.13, 66.65, 64.38 (C-2, C-4, C-5); 61.98 (C-6); 33.31 (C-3).

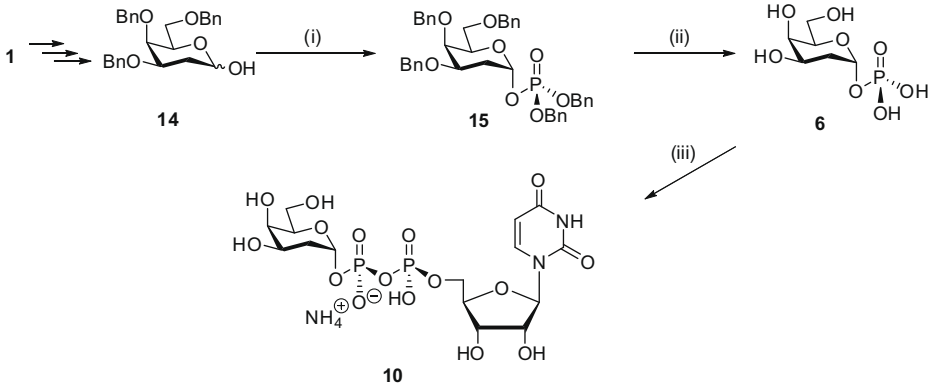
3.4. 4-Deoxy-α-D-*xylo*-hexopyranosyl phosphate (8)

4-Deoxy-α-D-*xylo*-hexose (4-deoxy-D-galactose, **3**, 100 mg, 610 μmol) and the same stoichiometric amounts of reagents, solvents, and enzymes as for the preparation of **7** were reacted for 6 days and worked up as above. By pyruvate assay a transfer of 5% could be observed. Partial purification led to a mixture of **3** and about 5% of **8**. ^{1}H NMR (400 MHz, D_2O): δ 5.54 (dd, 1H, 1-H); $J_{1,P}$ 7.5, $J_{1,2}$ 3.5 Hz.

Treatment of 6-deoxy-D-galactose (**4**, D-fucose) and L-arabinose (**5**) under similar conditions as for **1**–**3** did not show any glycosyl phosphate formation within 6 days.

3.5. Uridine 5′-(2-deoxy-α-D-galactopyranosyl) diphosphate (10)

The phosphate compound **6** (31 mg, 127 μmol) was dissolved in deionized water (1 mL) and run through a column of cation-exchange resin (Dowex 50W-X8, Et_3N form). Following lyophilization the colorless triethylammonium salt of **15** was treated with 4-morpholino-*N*,*N*′-dicylohexylcarboxamidinium salt (137 mg, 203 μmol) and 1*H*-tetrazole (45 mg, 642 μmol) in anhyd pyridine (10 mL) for 5 d. The solvents were evaporated and the residue taken up in water (5 mL), filtered, and purified by elution on Bio-



Scheme 2. Reagents and conditions: (i) (1) *i*Pr$_2$NP(OBn)$_2$, 1*H*-tetrazole, DCM; (2) mCPBA, 0 °C, 52%; (ii) (1) H_2, Pd/C (10%), 1:2:1 EtOAc–EtOH–H_2O, rt, 65%; (iii) (1) Dowex 50W-X8 (Et_3N form); (2) UMP-morpholidate, 1*H*-tetrazole, pyridine, 5 d; (3) 2. Bio-Gel P2, 250 mM NH_4HCO_3.

Gel P2 with aq NH_4HCO_3 (250 mM). After freeze drying the product, **10** was obtained as a colorless, very hygroscopic powder: 24 mg (32%). ^{1}H NMR (400 MHz, D_2O): δ 8.01 (d, 1H, H-6″); 6.03 (d, 1H, H-1′); 6.02 (d, 1H, H-5″); 5.48 (dd, 1H, H-1); 4.46 (m, 2H, H-2′, H-3′); 4.37 (m, 1H, H-4′); 4.26 (m, 2H, H-5a′, H-5b′); 4.01 (dd, 1H, H-3); 3.95 (dd, 1H, H-4); 3.88 (br t, 1H, H-5); 3.68 (m, 2H, H-6a, H-6b); 2.11 (ddd, 1H, H-2a); 2.05 (ddd, 1H, H-2e); $J_{1,P}$ 7.1, $J_{1,2a}$ 3.5, $J_{1,2e}$ < 1, $J_{2a,2e}$ 13.5, $J_{2a,3}$ 11.7, $J_{2e,3}$ 5.7, $J_{3,4}$ 3.2, $J_{4,5}$ < 1, $J_{5,6a}$ 5.6, $J_{5,6b}$ 6.1, $J_{6a,6b}$ 10.9, $J_{1',2'}$ 4.9, $J_{5'',6''}$ 8.1 Hz. ^{13}C NMR (100 MHz, D_2O): δ 166.6 (C-4″); 152.5 (C-2″); 142.0 (C-6″); 103.0 (C-5″); 92.7 (d, C-1); 88.8 (C-1′); 83.6 (d, C-4′); 74.1 (C-2′); 72.0 (C-5); 70.0 (C-3′); 68.9 (C-4); 66.5 (C-3); 65.3 (d, C-5′); 61.9 (C-6); 32.7 (d, C-2); $J_{C\text{-}1,P}$ 5.9, $J_{C\text{-}2,P}$ 8.3, $J_{C\text{-}4',P}$ 9.1, $J_{C\text{-}5',P}$ 5.3 Hz. ^{31}P NMR (202.5 MHz, D_2O): δ −10.5, −12.7.

3.6. 2-Deoxy-β-D-*lyxo*-hexopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose (12)

2-Deoxy-D-*lyxo*-hexose (2-deoxy-D-galactose, **1**, 200 mg, 1.22 mmol) was dissolved together with KCl (91 mg, 1.22 mmol), PEP (554 mg, 2.7 mmol), ATP (37 mg, 61 μmol), DTT (20 mg, 126 μmol), UTP (36 mg, 61 μmol), UDP-Glc (38 μg, 61 μmol), and GlcNAc (300 mg, 1.34 μmol) in 50 mM cacodylate buffer (12.2 mL, pH 7.5) and degassed with argon for 10 min. Then $MgCl_2{\cdot}6\ H_2O$ (12 mg, 61 μmol), BSA (12 mg), NaN_3 (microspatula-ful), $MnCl_2$ (12 mg, 61 μmol), *GK* (2U), *PK* (50 U) inorganic pyrophosphatase (50 U), UDP-glucose pyrophosphorylase (10 U), galactose-1-phosphate uridyltransferase (5 U), and galactosyltransferase (3 U) were added. The reaction proceeded at 30 °C for seven days under shaking, and the turnover was monitored by measuring the amounts of both phosphate[28] and pyruvate that formed.[29] Workup as above was followed by ion-exchange chromatography. Further purification was done by gel-permeation chromatography on Bio-Gel P2 with water as eluent. After freeze drying colorless amorphous compound **12** was obtained as an anomeric mixture: yield 131 mg (29%). ^{13}C NMR (63 MHz, D_2O): δ 173.31 ($NHCOCH_3$); 99.42 (C-1′); 93.93 (C-1, β); 89.59 (C-1, α); 77.87 (C-4, α); 77.44 (C-4, β); 74.65 (C-5′); 73.79 (C-5, β); 71.48 (C-3, β); 69.17 (C-5, α); 68.30 (C-3, α); 66.72 (C-3′); 65.73 (C-4′); 60.43 (C-6′); 59.18 (C-6); 55.29 (C-2, β); 52.80 (C-2, α); 32.54 (C-2′); 21.25 ($NHCOCH_3$, β); 20.97 ($NHCOCH_3$, α). FABMS: *m/z* 368 (M^++1), 390 (M^++Na).

3.7. 3,4,6-Tri-*O*-acetyl-2-deoxy-β-D-*lyxo*-hexopyranosyl-(1→4)-1,3,6-tri-*O*-acetyl-2-acetamido-2-deoxy-α/β-D-glucopyranose (13)

By peracetylation of **12** (Ac_2O, pyridine room temperature) and workup, the heptaacetate **13** was obtained as an amorphous colorless material. ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 7.15 (d, 1H, NH, α); 7.11 (d, 1H, NH, β); 6.06 (d, 1H, 1-Hα); 5.77 (d, 1H, 1-Hβ); 5.23 (m, 1H, 3-H); 5.23 (m, 1H, 4′-H); 5.04 (ddd, 1H, 3′-H); 4.83 (dd, 1H, 1′-H); 4.34 (dd, 1H, 6a-H); 4.33 (ddd, 1H, 2-Hα); 4.23 (dd, 1H, 6b-H); 4.20 (dd, 1H, 6a′-H); 4.11 (dd, 1H, 6b′-H); 4.07 (m, 1H, 2-Hβ); 4.00 (ddd, 1H, 5′-H); 3.95 (t, 1H, 4-H); 3.83 (ddd, 1H, 5-H); 1.95 (m, 1H, 2e′-H); 1.76 (ddd, 1H, 2a′-H); $J_{NH,2}$ 9.4 (α, β), $J_{1e,2}$ 3.6 (α), $J_{1a,2}$ 8.8 (β), $J_{2e',3'}$ 3.2, $J_{3',4'}$ 5.0, $J_{1',2a'}$ 9.6, $J_{1',2e'}$ 2.2, $J_{6a,6b}$ 12.2, $J_{2,3}$ 10.4, $J_{5',6a'}$ 3.6, $J_{5',6b'}$ 2.0, $J_{4',5'}$ 5.6, $J_{3,4}$ $J_{4,5}$ 9.6, $J_{5,6a}$ 4.4, $J_{5,6b}$ 2.2, $J_{2a',2e'}$ 12.4, $J_{2a',3'}$ 12.5 Hz.

3.8. 2-Deoxy-α-D-*lyxo*-hexopyranosyl-1-phosphate (6)

Dibenzyl (3,4,6-tri-O-benzyl-2-deoxy-α-D-*lyxo*-hexopyranosyl)-1-phosphate (**15**, 145 mg, 0.21 mmol) was dissolved in 1:2:1 water–MeOH–EtOAc (10 mL) and treated with Pd-on-charcoal (10%, 10 mg) under hydrogen (50 bar) at room temperature for 10 h. After filtration over Celite and evaporation of the solvent, the residue was lyophilized. The raw material was purified on Bio-Gel P2 with deionized water. After lyophilization 33 mg (65%) of the free-acid monoester **6** was obtained as a colorless amorphous foam: $[\alpha]_D^{20}$ 17.5 (*c* 1.0, D_2O); ^{1}H NMR (400 MHz, D_2O): δ 5.47 (m, 1H, H-1); 3.98 (ddd, 1H, H-3); 3.92 (dd, 1H, H-4); 3.84 (dt, 1H, H-5); 3.67 (m, 2H, H-6a, H-6b); 2.14 (ddd, 1H, H-2a); 2.08 (ddd, 1H, H-2e); $J_{1,P}$ 7.3, $J_{1,2a}$ 3.6, $J_{1,2e}$ < 1, $J_{2a,2e}$ 13.7, $J_{2a,3}$ 11.7, $J_{2e,3}$ 5.6, $J_{4,5}$ < 1, $J_{5,6a}$ 6.1, $J_{5,6b}$ 6.1, $J_{6a,6b}$ 10.7 Hz. ^{13}C NMR (100 MHz, D_2O): δ 92.3 (d, C-1); 71.5 (C-5); 68.6 (C-4); 66.2 (C-3); 61.9 (C-6); 32.1 (d, C-2); $J_{C\text{-}1,P}$ 5.7, $J_{C\text{-}2,P}$ 8.3 Hz. ^{31}P NMR (202.5 MHz, acetone-d_6): δ −0.21.

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