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Total synthesis of uridine diphosphate-*N*-acetylmuramoyl-L-alanine

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ABSTRACT

The first total synthesis of uridine diphosphate *N*-acetylmuramoyl-L-alanine in 13% overall yield is presented. The 11-step synthetic route is based on the synthetic strategy used for the synthesis of uridine diphosphate *N*-acetylmuramic acid, the MurC ligase substrate. However, an unexpected amide bond cleavage under basic conditions demanded crucial modifications of the final synthetic steps. The total chemical synthesis of MurD ligase substrate provides an excellent alternative to chemoenzymatic synthesis.

1. Introduction

Antibiotic resistance represents an increasing threat to public health.[1] Multi-drug resistant strains of bacteria are stimulating research into antimicrobial compounds that inhibit the growth of bacteria by new mechanisms of action.[2] The inhibition of essential targets within the bacteria is a good strategy and the enzymes involved in the biosynthesis of the bacterial peptidoglycan constitute such a group of targets.[3–5] Biological evaluation of inhibitors with enzymatic assays requires large quantities of commercially unavailable substrates. These substrates have been obtained from bacterial extracts,[6] by enzymatic,[7–9] chemo-enzymatic[10,11] or, very rarely, by total chemical synthesis.[12–14] MurD enzyme[15,16] is the second consecutive Mur ligase which almost universally attaches D-glutamic acid to the growing peptide chain of the peptidoglycan precursor and uses UDP-*N*-acetylmuramoyl-L-alanine **15** as the nucleotide substrate.

We have previously reported an improved total synthesis of UDP-*N*-acetylmuramic acid,[17] the MurC ligase[18] nucleotide substrate. Our strategy was designed in such a way that, if needed, it would allow the total chemical synthesis of other Mur ligase substrates. Both the UDP-*N*-acetylmuramic acid and UDP-*N*-acetylmuramoyl-L-alanine **15** are highly functionalized structurally complex natural molecules with several stereogenic centers originating from enantiopure natural carbohydrates. However, over the course of the total synthesis several key stereogenic centers have to be introduced by stereoselective steps and any reactions which could lead to racemization must be avoided at all cost.

Herein, we report the synthetic route to UDP-*N*-acetylmuramoyl-L-alanine **15** with important modifications to our previous strategy towards Mur ligases' substrates which allowed the successful synthesis of MurD ligase substrate in 11 synthetic steps.

2. Results and discussion

The first three reaction steps (Scheme 1) were part of the established synthesis of UDP-*N*-acetylmuramic acid. The synthesis started from *N*-acetylglucosamine **1**, which was protected by benzylation with benzyl alcohol at the anomeric position. Even though this protecting group was removed later in the synthesis and, theoretically, the synthesis could proceed with the α/β mixture of benzyl anomer **2**, it was decided that a permanent α-anomeric configuration of the *N*-acetylglucosamine system would simplify the isolation and purification of the correct diastereoisomers after the stereoselective reactions still to come. Pure benzyl α-anomer **2** was obtained with thermodynamic control of the reaction by high reaction temperatures in refluxing toluene. The introduction of a benzylidene protection group to the 4′-and 6′-hydroxy positions was carried out according to Gross and Rimpler[19] to afford protected α-*N*-acetylglucosamine derivative **3** with a free 3′-hydroxy group. By treating **3** with racemic 2-chloropropionic acid, the Williamson stereoselective ether synthesis proceeded in good yield, to afford sodium salt **4** as a mixture of two diastereoisomers. ^{1}H NMR was used to determine the stereoselectivity of the reaction. Integration of the benzylidene protons at 5.66 and 5.55 ppm for the muramic and isomuramic acid derivatives, respectively, showed a diastereoisomeric ratio of 74:26 in favour of the muramic acid derivative **4** with the (*R*)-configuration of the lactoyl side-chain. This marks the dividing point of the synthetic pathways to substrates of different Mur ligases.

When the sodium salt **4** was reacted with methyliodide in DMF, this gave methyl ester **5** in quantitative yield and led towards UDP-*N*-acetylmuramic acid as previously reported.[17] On the other hand, when the methyl ester of L-alanine was coupled with **4**, this gave

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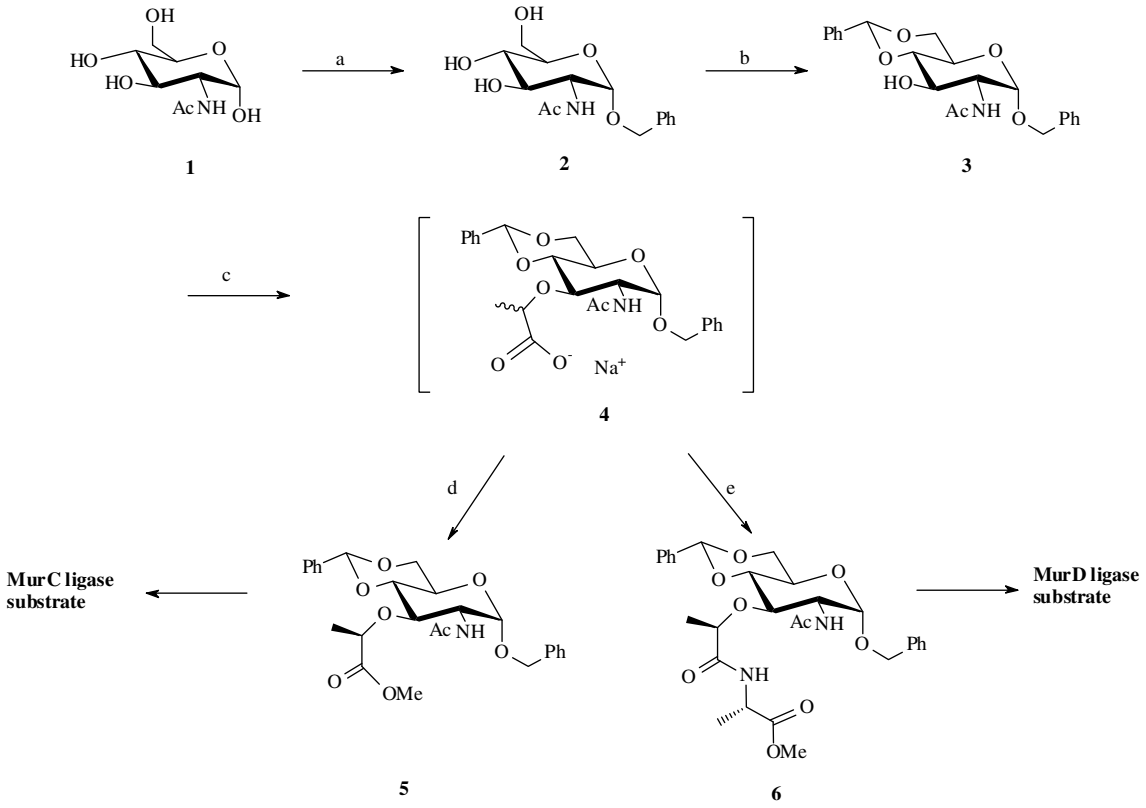


Scheme 1. Reagents and conditions: (a) BnOH, PTSA, toluene, reflux, 3 h, 80%; (b) $(CH_3CH_2O)_3CH$, PhCHO, dioxane, DMF, rt, 24 h, 76%; (c) 2-chloro-propionic acid, NaH, dioxane, 45 °C, 16 h; (d) (i) MeI, DMF, rt, 4 h; (ii) diastereoisomer separation, 75% (two steps); (e) (i) L-Ala-OMe, EDC, Hobt, TEA, CH_2Cl_2, rt, 12 h; (ii) diastereoisomer separation, 65% (two steps).

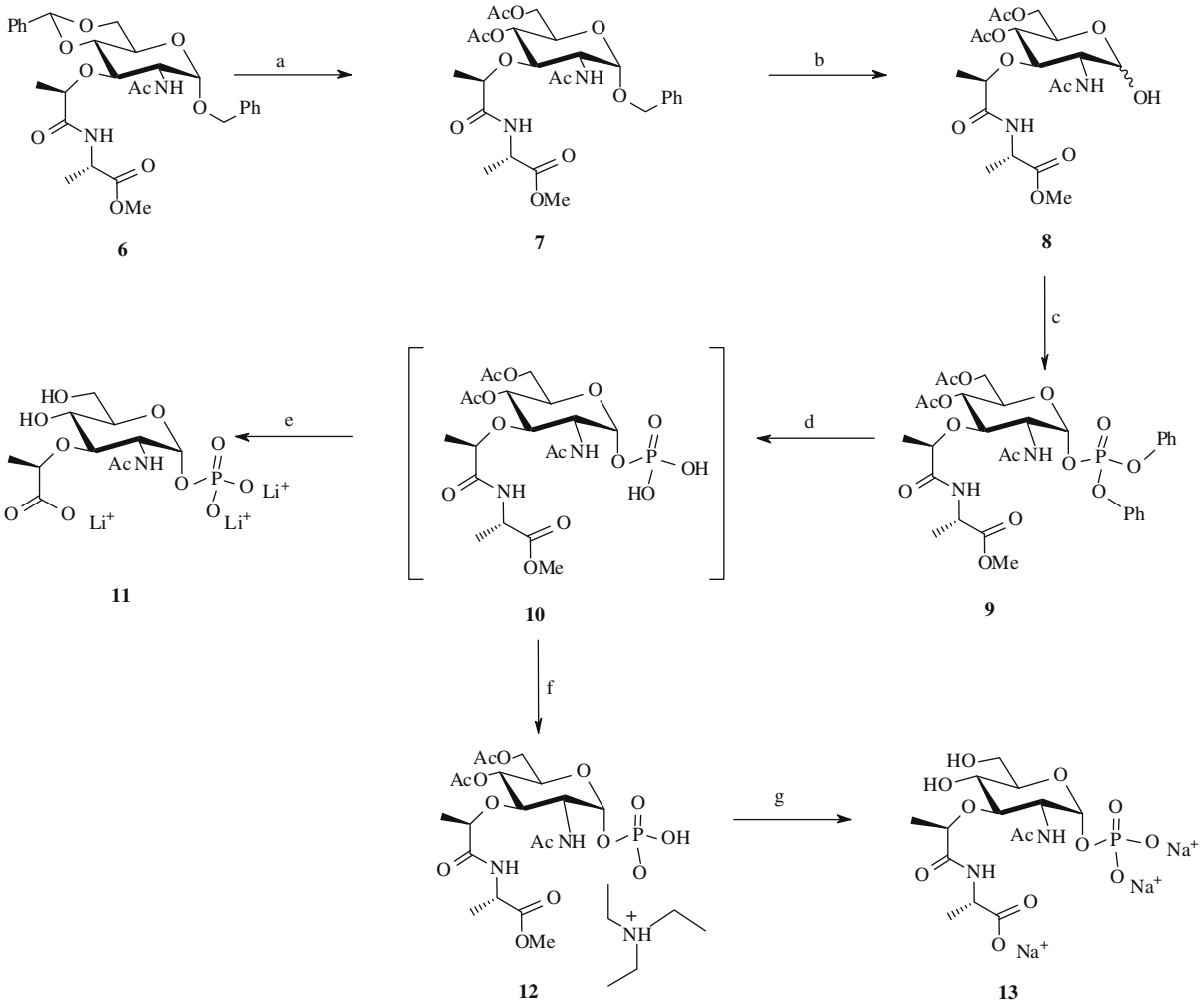
amide **6**, an intermediate towards the MurD substrate (Scheme 1). Careful temperature control and the appropriate addition of reagents resulted in no racemization being observed at this step, while the yield remained good at 65% for the two steps.[20] The isomuramic diastereoisomers of **5** and **6** with an (*S*)-configuration in the lactoyl group were removed by chromatography in both cases. Next, the benzylidene group was removed by acidolysis in aqueous 60% acetic acid and was replaced by a base-labile and hydrogenolysis resistant acetate ester protecting group, to afford the diacetyl intermediate **7** (Scheme 2). As expected, the overall yield for both reaction steps was high at 89%. This was followed by removal of the anomeric benzyl protecting group by hydrogenolysis in 98% yield.

It is noteworthy that this seemingly facile reaction proceeds very slowly, and with low yield, if the wrong reaction solvent is used.[21] In our hands the conversion of **7** to hemiacetal **8** was fast, reproducible and proceeded with quantitative yields only when appropriate Pd/C and THF were used as solvents (Scheme 2). The introduction of an α-phosphate was achieved according to the modified procedure of Sabesan and Neira.[22] The reaction was kinetically controlled and carried out at −25 °C in dry dichloromethane with the addition of 4-pyrrolidinopyridine as the catalyst, followed by slow addition of diphenylchlorophosphate. This afforded just the α-anomer of **9** in 72% yield, as evidenced from 300 MHz ^{1}H NMR spectrum which showed a dd at 6.75 with the coupling constants of 5.4 and 3.1 Hz characteristic of the anomeric proton of α-phosphates. Diphenylphosphate **9** had to be purified and handled with care because of its inherent instability. The diphenyl phosphate group was utilized because it could be introduced with a single reaction step and allowed convenient and high yielding deprotection under hydrogenation conditions. Compared to procedures which involve the one-pot dibenzyl phosphite introduction to the anomer position with subsequent oxidation,[23–25] this offered a significant advantage in simplicity, yield and stereoselectivity of the reaction.

Therefore, hydrogenation in THF, with PtO_2 used as a catalyst, allowed a straightforward conversion to unprotected phosphate **10**, which was quenched immediately by 1 M LiOH to stabilize the free α-phosphate group and remove the acetate and methyl ester protecting groups, according to the existing synthetic strategy (Scheme 2). Surprisingly, the reaction mostly afforded α-1-phospho-*N*-acetylmuramic acid **11**. Obviously, the amide bond between the muramic acid and L-alanine moieties was cleaved under these conditions.

We reasoned that steric and electronic effects brought about the otherwise unexpected cleavage of the amide bond since the amide bond of the *N*-acetylamide moiety remained intact under the same reaction conditions. The use of more diluted lithium hydroxide and THF mixtures did not improve the outcome of this reaction. Therefore, we assumed that the THF/water solvent system played an important role in this unwanted cleavage. However THF, present from the previous reaction step, could not be removed because of the instability of the free phosphate **10**, so changes in the deprotection and the final diphosphate coupling steps had to be introduced.

For this reason, diphenylphosphate **9** was not globally deprotected but stabilized by the direct addition of triethylamine to the hydrogenation reaction mixture. After purification on a short LH-20 Sephadex column, with methanol as eluent, the triethylamine salt of **12** was obtained as a crystalline solid. Phosphate **12** was then subjected to a variety of deprotection conditions, with the intention of finding conditions that would remove all the protecting groups in quantitative yield but not cause cleavage of the labile amide bond. When the reaction was carried out in 50 mM aqueous sodium hydroxide and the pH of the reaction mixture was allowed



Scheme 2. Reagents and conditions: (a) (i) 60% AcOH, 90 °C, 1 h; (ii) Ac_2O, Py, rt, 12 h, 89%; (b) H_2, Pd/C (10%), THF, rt, 24 h, 98%; (c) $ClPO(OPh)_2$ 4-pyrrolidinopyridine, CH_2Cl_2, −25 °C, 24 h, 72%; (d) H_2, PtO_2, THF, rt, 24 h; (e) 1 M LiOH, THF/H_2O, rt, 24 h; (f) TEA (3 equiv), 92%; (g) 50 mM aqueous NaOH, rt, 4 h, quant.

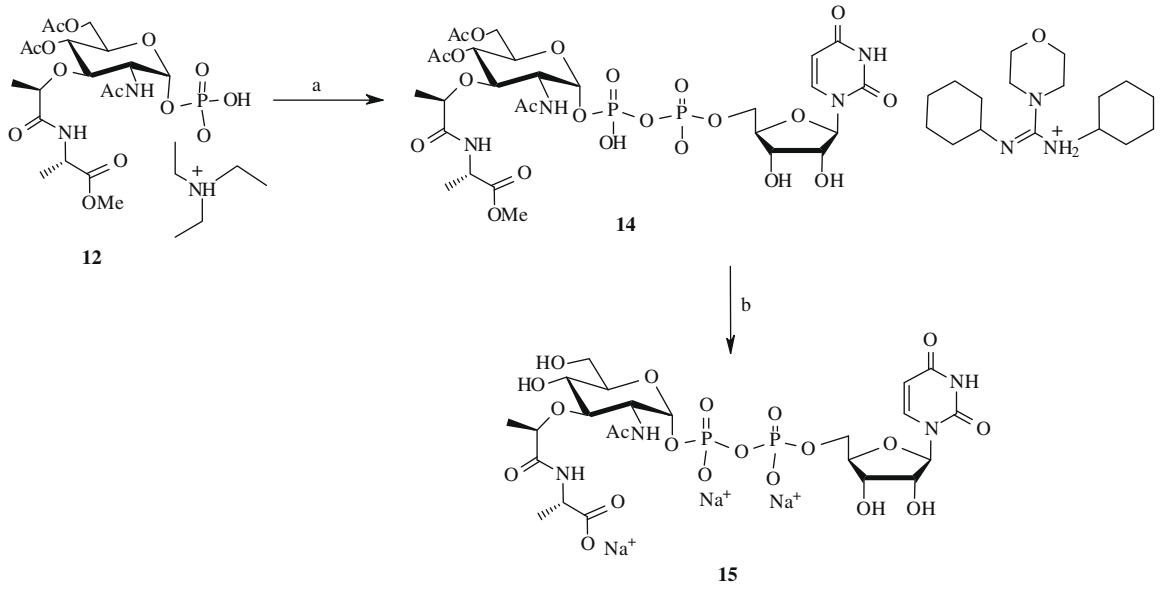
to drop as the reaction progressed, the target compound α-1-phospho-*N*-acetylmuramoyl-L-alanine **13** could be obtained without any side products and was stable, even if the reaction time was prolonged (Scheme 2).

In spite of this encouraging result, it was decided that the final deprotection of the acetate and methyl ester groups would be accomplished at the very end of the total synthesis for two reasons. It has been reported that the coupling reaction with uridine-5′-monophospho morpholidate proceeds much more efficiently if the coupled sugar-phosphate is still protected[26] and, furthermore, if the obtained protected diphosphate could be purified by flash chromatography, then the demanding final purification by gel filtration could be omitted. In view of this, the protected α-1-phospho-*N*-acetylmuramoyl-L-alanine was successfully coupled with uridine-5′-monophosphomorpholidate to afford protected UDP-*N*-acetylmuramoyl-L-alanine **14** without the addition of tetrazole as catalyst[27] (Scheme 3). Even though no catalyst was used, the protected α-phosphate **12** was reacted with equimolar amounts of uridine-5′-monophospho morpholidate, 61% yield was achieved for this demanding coupling reaction.

As anticipated, the use of protected α-1-phosphate **12** also made a big difference to the purification procedure. The crude diphosphate **14** could be effectively purified by flash chromatography in a fashion similar to that described by Dinev et al. for uridine diphospho($^{13}C_6$)glucose.[28] However, in our hands, flash chromatography did not allow for the exchange of the cations, so the protected diphosphate **14** was obtained in the form of the 4-morpholino-*N*,*N*′-dicyclohexylcarboxamidine salt. This presented no major problem since the cations could be conveniently exchanged after the final reaction step. As already described for the monophosphate **12**, 50 mM sodium hydroxide was used for deprotection of the diphosphate **14** in the final step. Since the sodium hydroxide was diluted enough to allow the pH to drop as the reaction proceeded, no amide bond hydrolysis occurred. After the straightforward desalting Sephadex G-10 column, which removed the unwanted 4-morpholino-*N*,*N*′-dicyclohexylcarboxamidine cations the trisodium salt of UDP-*N*-acetylmuramoyl-L-alanine **15** was obtained in very high yield.

3. Conclusion

In conclusion, we have presented the first total chemical synthesis of UDP-*N*-acetylmuramoyl-L-alanine. The synthetic strategy for UDP-*N*-acetylmuramic acid was employed until an unexpected hydrolysis of the amide bond occurred at the global deprotection step. This problem was successfully solved by reversing the order of diphosphate coupling and the final deprotection. The latter was carried out as the last step of the synthesis under controlled and increasingly milder basic conditions. The synthetic path dis-



Scheme 3. Reagents and conditions: (a) uridine-5-phosphomorpholidate, 4 Å molecular sieves, DMF, 60 °C, 20 h, 61%; (b) (i) 50 mM aqueous NaOH, rt, 4 h; (ii) Sephadex G-10 desalting column, 95%.

covered, with an overall yield of 13% provides a good alternative to the chemoenzymatic synthesis, especially if scale-up is required. This is currently in progress in our laboratory.

4. Experimental

All reactions were carried out under an argon atmosphere using anhydrous solvents, unless indicated otherwise. Chemicals from Sigma–Aldrich, Fluka and Merck (10% Pd/C) were used without further purification. Analytical TLC was performed on Merck Silica Gel (60F 254) plates (0.25 mm) and components visualized with ultraviolet light and stained with 20% sulfuric acid in ethanol, rhodamine G6, 2,4-dinitrophenylhydrazine, bromocresol green and ninhydrin. Flash chromatography was performed using Merck Silica Gel (0.040–0.063 mm). Gel filtration was performed using Sephadex G-10 and LH-20 stationary phases and methanol or bidistilled water as eluents. ^{1}H, ^{13}C, ^{31}P, DEPT-135, gradient COSY and gradient HSQC NMR spectra were recorded on a Bruker AVANCE DPX300 spectrometer at 302 K in $CDCl_3$, DMSO-d_6, D_2O and MeOH–d_4 solutions using TMS or residual undeuterated solvent as the internal standard. Microanalyses were performed on a Perkin–Elmer C, H, N analyzer 240 C. Optical rotation was measured using Perkin–Elmer 241 MC polarimeter at 589 nm. Reverse-phase HPLC analysis was performed using Agilent 1100 Series system and Phenomenex Luna C18 5u (250 × 4.60 mm) column. Mass spectra were obtained using a VG-Analytical Autospec Q and Q-TOF Premier mass spectrometers.

4.1. Benzyl-2-deoxy-2-acetylamido-α-D-glucopyranoside 2

N-Acetyl-glucosamine **1** (25 g, 113 mmol) and *p*-toluenesulfonic acid monohydrate (1.9 g, 10 mmol) were suspended in 300 mL of toluene and 180 mL of benzyl alcohol. The reaction mixture was refluxed in a Dean–Stark apparatus with water removal by azeotrope mixture. After 3 h, the reaction mixture was cooled to ambient temperature and sodium bicarbonate (1.26 g, 15 mmol) dissolved in water was added. Toluene was removed under reduced pressure and ether–hexane (2:1) mixture (900 mL) was added to the oily residue and stirred vigorously for 3 h. The amorphous precipitate was filtered off, washed with ether and the crude product recrystallized from ethanol to yield colourless crystals (28.1 g, 90.4 mmol, 80% yield). Mp 180–182 °C. IR (KBr, cm^{-1}): 3285, 2931, 1630, 1552, 1376, 1027, 734, 695. $[\alpha]_D^{20} = +265.6$ (*c* 0.08, DMF). ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) = 7.79 (d, 1H, *J* = 8.1 Hz, NH), 7.39–7.26 (m, 5H, Ph–H), 4.99 (d, 1H, *J* = 5.6 Hz, OH-4), 4.72–4.70 (m, 2H, H-1, OH-3), 4.67 (d, 1H, J_{gem} = 12.6 Hz, CH_{2a}–Ph), 4.52 (t, 1H, *J* = 5.8 Hz, OH-6), 4.42 (d, 1H, J_{gem} = 12.6 Hz, CH_{2b}–Ph), 3.72–3.62 (m, 2H, H-2, H-6), 3.57–3.43 (m, 3H, H-3, H-6′, H-5), 3.16 (ddd, 1H, *J* = 9.1, 9.1, 5.6 Hz, H-4), 1.84 (s, 3H, $COCH_3$). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 169.4, 137.9, 128.1, 127.5, 127.4, 95.9, 73.1, 70.9, 70.6, 67.7, 60.8, 53.7, 22.5. LRMS (FAB), *m*/*z* = 312 (M+H)$^+$, (ESI) *m*/*z* = 334.1 (M+Na)$^+$. HRMS (ESI), *m*/*z* calcd for $C_{15}H_{21}NO_6Na$ 334.1267 (M+Na)$^+$, found 334.1270.

4.2. Benzyl 4,6-*O*-benzylidene-2-acetylamido-2-deoxy-α-D-glucopyranoside 3

Compound **3** was synthesized according to the literature procedure.[11] Compound **2** (15 g, 48.2 mmol) was dried using absolute ethanol and toluene co-evaporation under reduced pressure. The dry solid was suspended in 50 mL of dry DMF, 50 mL anhydrous dioxane, 25 mL triethylorthoformate and 20 mL of freshly distilled benzaldehyde. *p*-Toluenesulfonic acid monohydrate (1.2 g, 6.31 mmol) was added and the reaction mixture stirred at ambient temperature. After 24 h, the reaction mixture was poured into an Erlenmeyer flask containing 300 mL of ether. The suspension was stirred at 0 °C and after 1 h the colourless precipitate was filtered off, washed with an additional 100 mL of ether and dried in vacuo. Crystallization from absolute ethanol afforded a colourless solid (14.6 g, 36.6 mmol, 76%). Mp >240 °C. IR (KBr, cm^{-1}): 3300, 3066, 2966, 2868, 1650, 1552, 1372, 1013, 928, 727, 592. $[\alpha]_D^{20} = +49.3$ (*c* 0.20, DMF). ^{1}H NMR ($CDCl_3$, 300 MHz): δ (ppm) = 7.52–7.26 (m, 10H, Ph–H), 5.79 (d, 1H, *J* = 8.7 Hz, NH), 5.57 (s, 1H, CH–Ph), 4.94 (d, 1H, *J* = 3.8 Hz, H-1), 4.78 (d, 1H, J_{gem} = 11.8 Hz, CH_{2b}–Ph), 4.50 (d, 1H, J_{gem} = 11.8 Hz, CH_{2a}–Ph), 4.28–4.20 (m, 2H, H-2, H-6), 3.96 (ddd, 1H, *J* = 9.2, 9.2, 3.0 Hz, H-5), 3.86 (dd, 1H, *J* = 9.5 Hz, 4.6 Hz, H-3), 3.79–3.70 (m, 1H, H-6′), 3.60 (app t, 1H, *J* = 9.2 Hz, H-4), 2.96 (d, 1H, *J* = 3.3 Hz, OH-3), 1.99 (s, 3H, CH_3CO). ^{13}C NMR ($CDCl_3$, 75 MHz): δ (ppm) 171.3, 137.0, 136.8, 129.2, 128.7, 128.3, 128.3, 128.1, 126.3, 101.9, 97.2, 82.1, 70.6, 70.1, 68.8, 62.8, 54.0, 23.2. LRMS (FAB), *m*/*z* = 400 (M+H)$^+$, (ESI) *m*/*z* = 422.2 (M+Na)$^+$.

HRMS (ESI), m/z calcd for $C_{22}H_{25}NO_6Na$ 422.1580 $(M+Na)^+$, found 422.1578.

4.3. (*S*)-Methyl 2-(2-(*R*)-(2-acetamido-1-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-3-α-D-glucopyranosyloxy) propanamido)propanoate 6

Compound **3** (10.0 g, 25.0 mmol) was suspended in 100 mL of benzene and the solvent was evaporated under reduced pressure to remove traces of water. The colourless solid was dissolved in 100 mL of dry dioxane and flushed with argon. Sodium hydride (4.20 g, 175 mmol) was slowly added at room temperature. The reaction mixture thickened significantly on heating at 50 °C for 1 h under argon atmosphere. The temperature was then lowered to 45 °C and racemic 2-chloro-propionic acid (8.0 g, 74 mmol) dissolved in dry dioxane (20 mL) was slowly added dropwise. After the addition, the mixture was heated at 45 °C for 16 h, when the TLC analysis showed total consumption of the starting material. The reaction mixture was cooled to ambient temperature and quenched carefully with water. The solvents were removed under reduced pressure and, after the addition of 100 mL of brine, the sodium salt was left overnight to precipitate at 0 °C. The amorphous precipitate was suction filtered and dried at 50 °C. A slightly brownish solid (15.0 g, 30.4 mmol, 125%) was obtained and used in the next reaction step without purification. The crude sodium salt was suspended in dry dichloromethane (500 mL) and cooled to 0 °C in an ice-bath under argon atmosphere. L-Alanine methyl ester hydrochloride (6.36 g, 45.6 mmol), 1-hydroxybenzotriazole (4.53 mg, 33.5 mmol) and finally 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (6.42 g, 33.5 mmol) were added to the stirred suspension. Triethylamine was used to adjust the pH value to 8 and the mixture was allowed to warm slowly to ambient temperature. After stirring overnight the suspension was diluted with dichloromethane (200 mL) and washed with sodium bicarbonate, dilute citric acid and finally brine. The organic phase was dried over sodium sulfate and concentrated to dryness under reduced pressure. The crude product was purified using flash chromatography (dichloromethane–ethyl acetate–hexane = 3:3:1). A colourless solid (9.1 g, 16.4 mmol, 65% yield) was obtained. Mp 220–222 °C. IR (KBr, cm^{-1}): 3090, 2931, 2870, 1742, 1657, 1554, 1499, 1451, 1372, 1321, 1214, 1156, 1124, 1090, 1055, 1028, 1006, 973, 733, 695. $[\alpha]_D^{20} = +44.6$ (c 0.21, DMF). ^{1}H NMR ($CDCl_3$, 300 MHz): δ (ppm) 7.90–7.26 (m, 10 H, Ph–H), 6.90 (d, 1H, J = 7.2 Hz, NH), 6.13 (d, 1H, J = 7.2 Hz, NH), 5.57 (s, 1H, CH–Ph), 4.97 (d, 1H, J = 3.7 Hz, H-1), 4.72 (d, 1H, J_{gem} = 11.8 Hz, CH_{2a}–Ph), 4.51–4.43 (m, 2H, CH_{2a}–Ph, NHC*H*CH_3), 4.33–4.29 (m, 1H, H-2), 4.24 (dd, 1H, J = 9.9, 4.5 Hz, H-6), 4.11 (q, 1H, J = 6.7 Hz, OC*H*CH_3), 3.87 (ddd, 1H, J = 9.3, 9.0, 4.5 Hz, H-5), 3.80–3.67 (m, 6H, H-6′, H-3, $COOCH_3$, H-4), 1.93 (s, 3H, $COCH_3$), 1.42 (d, 3H, J = 7.6 Hz, NHCHC*H*$_3$), 1.39 (d, 3H, J = 6.7 Hz, OCHC*H*$_3$). ^{13}C NMR ($CDCl_3$, 75 MHz): δ (ppm) 173.0, 172.9, 170.3, 137.1, 136.8, 129.0, 128.7, 128.3, 128.2, 125.9, 101.5, 97.6, 81.7, 78.4, 77.7, 77.2, 70.2, 68.8, 63.2, 53.1, 52.4, 48.0, 23.4, 19.4, 17.9. LRMS (FAB), m/z = 557 $(M+H)^+$, (ESI) m/z = 557.3 $(M+H)^+$, 579.2 $(M+Na)^+$. HRMS (ESI), m/z calcd for $C_{29}H_{36}N_2O_9Na$ 579.2319 $(M+H)^+$, found 579.2315. Microanalysis calcd for $C_{29}H_{36}N_2O_9$ (%): C, 62.58; H, 6.52; N, 5.03; found: C, 62.85; H, 6.73; N, 5.03.

4.4. (*S*)-Methyl 2-(2-(*R*)-(2-acetamido-4,6-di-*O*-acetyl-1-*O*-benzyl-2-deoxy-3-α-D-glucopyranosyloxy) propanamido)propanoate 7

Compound **6** (2.00 g, 3.60 mmol) was suspended in aqueous acetic acid (50 mL, 60%) and heated at 90 °C. After 1 h, the solvents were evaporated under reduced pressure. Repetitive toluene co-evaporation was used to remove traces of water and acetic acid. The colourless foam was dried for several hours in vacuo and used in the next reaction step. The crude product was dissolved in pyridine (40 mL), flushed with argon and cooled to 0 °C. While being stirred, acetic acid anhydride (5.0 mL, 45.4 mmol) was added dropwise. The reaction mixture was allowed to warm to ambient temperature and stirred overnight. Methanol (10 mL) was added slowly to quench the excess acetic acid anhydride and the solvents were evaporated under reduced pressure. Toluene co-evaporation was used to remove traces of pyridine. The crude product was purified using flash chromatography (dichloromethane–methanol = 15:1). A colourless solid (1.78 g, 3.22 mmol, 89% yield) was obtained. Mp 148–149 °C. IR (KBr, cm^{-1}): 2929, 1745, 1658, 1563, 1548, 1535, 1451, 1376, 1240, 1123, 1044, 668. $[\alpha]_D^{20} = +77.2$ (c 0.18, DMF). ^{1}H NMR ($CDCl_3$, 300 MHz): δ (ppm) 7.24–7.27 (m, 5H, Ph–H), 6.87 (d, 1H, J = 7.2 Hz, NH), 6.95 (d, 1H, J = 9.5 Hz, NH), 5.08 (app t, 1H, J = 9.7 Hz, H-4), 4.97 (d, 1H, J = 3.6 Hz, H-1), 4.70 (d, 1H, J_{gem} = 11.7 Hz, CH_{2a}–Ph), 4.50 (d, 1H, J_{gem} = 11.7 Hz, CH_{2b}–Ph), 4.43–4.33 (m, 2H, NHC*H*CH_3, H-2), 4.20 (dd, 1H, J = 12.4, 4.6 Hz, H-6), 4.07 (dd, 1H, J = 12.4, 2.4 Hz, H-6′), 3.98 (q, 1H, J = 6.7 Hz, OC*H*CH_3), 3.91 (ddd, 1H, J = 10.2, 4.4, 2.4 Hz, H-5), 3.71 (s, 3H, $COOCH_3$), 3.66 (app t, 1H, J = 9.7 Hz, H-3), 2.11 (s, 3H, $COCH_3$), 2.08 (s, 3H, $COCH_3$), 1.89 (s, 3H, NHCOC*H*$_3$), 1.44 (d, 3H, J = 7.2 Hz, NHCHC*H*$_3$), 1.32 (d, 3H, J = 6.7 Hz, OCHC*H*$_3$). ^{13}C NMR ($CDCl_3$, 75 MHz): δ (ppm) 172.8, 172.1, 170.7, 170.1, 169.3, 136.6, 128.7, 128.4, 128.3, 97.0, 78.7, 78.4, 70.2, 69.5, 68.5, 62.1, 53.0, 52.3, 48.0, 23.3, 20.8, 20.8, 18.7, 17.4. LRMS (ESI), m/z = 553.2 $(M+H)^+$, 575.2 $(M+Na)^+$, HRMS (ESI), m/z calcd for $C_{26}H_{37}N_2O_{11}$ 553.2397 $(M+H)^+$, found 553.2400. Microanalysis calcd for $C_{26}H_{36}N_2O_{11}$ (%): C, 56.51; H, 6.57; N, 5.07; found: C, 56.15; H, 6.72; N, 4.94.

4.5. (*S*)-Methyl 2-(2-(*R*)-(2-acetamido-4,6-di-*O*-acetyl-2-deoxy-3-D-glucopyranosyloxy)propanamido) propanoate 8

Compound **7** (1.60 g, 2.89 mmol) was dissolved in freshly distilled dry THF (200 mL). Palladium on charcoal (700 mg, 10%) was added after the solution was flushed with argon for 10 min. The reaction mixture was first flushed with hydrogen for 30 min and then stirred under positive hydrogen pressure at ambient temperature. After 24 h, the catalyst was filtered off and washed with a small amount of THF. The solvent was evaporated under reduced pressure giving a colourless foam (1.32 g, 2.86 mmol, 98% yield). The product was used without purification in the next reaction step. Mp 84–87 °C. IR (KBr, cm^{-1}): 2957, 1746, 1659, 1547, 1452, 1376, 1242, 1121, 1043, 601. $[\alpha]_D^{20} = +40.3$ (c 0.12, MeOH). ^{1}H NMR ($CDCl_3$, 300 MHz): δ (ppm) 6.97 (d, 1H, J = 7.2 Hz, NH), 6.90 (d, 1H, J = 8.9 Hz, NH), 5.26 (s, 1H, OH), 5.14 (d, 1H, J = 3.2 Hz, H-1), 4.99 (app t, 1H, J = 9.7 Hz, H-4), 4.44–4.30 (m, 1H, $NHCHCH_3$), 4.19–4.00 (m, 5H, H-2, H-6, H-5, $OCHCH_3$, H-6′), 3.72 (app t, 1H, J = 9.6 Hz, H-3), 3.68 (s, 3H, $COOCH_3$), 2.05 (s, 3H, $COCH_3$), 2.04 (s, 3H, $COCH_3$), 1.92 (s, 3H, $NHCOCH_3$), 1.40 (d, 3H, J = 7.2 Hz, $NHCHCH_3$), 1.27 (d, 3H, J = 6.6 Hz, $OCHCH_3$). ^{13}C NMR ($CDCl_3$, 75 MHz): δ (ppm) 172.8, 172.6, 171.0, 170.9, 169.5, 91.7, 78.0, 77.3, 69.9, 67.8, 62.4, 53.7, 52.4, 48.0, 23.2, 20.8, 20.7, 18.6, 17.4. LRMS (ESI), m/z = 463.2 $(M+H)^+$, 445.2 $(M-H_2O)^+$, 485.2 $(M+Na)^+$, HRMS (ESI), m/z calcd for $C_{19}H_{31}N_2O_{11}$ 463.1928 $(M+H)^+$, found 463.1949. Microanalysis calcd for $C_{19}H_{30}N_2O_{11}$(%): C, 49.35; H, 6.54; N, 6.06; found: C, 48.99; H, 6.69; N, 5.80.

4.6. (*S*)-Methyl 2-(2-(*R*)-(2-acetamido-2-deoxy-4,6-di-*O*-acetyl-1-*O*-diphenoxyphosphoryl-3-α-D-glucopyranosyloxy)-propanamido)propanoate 9

Compound **8** (335 mg, 0.73 mmol) and 4-pyrrolidinopyridine (536 mg, 3.62 mmol) were dissolved in dry dichloromethane

(30 mL). The reaction flask was flushed with argon and cooled to −25 °C. After being stirred for 20 min, diphenyl chlorophosphate (450 μL, 2.16 mmol) was added dropwise using a syringe. After 24 h, at −25 °C under an argon atmosphere the reaction mixture was diluted with dichloromethane (30 mL) and washed consecutively with cold citric acid (5%), saturated sodium bicarbonate solution and brine. The organic phase was dried over sodium sulfate and the solvent evaporated under reduced pressure at a temperature not exceeding 25 °C. The crude product was purified using gradient flash chromatography (ethyl acetate–hexane = 50:50 to 100:0) yielding a transparent oil which turned to a colourless foam (362 mg, 0.52 mmol, 72% yield) after treatment with ether and drying in vacuo. IR (KBr, cm^{-1}): 3072, 2360, 2340, 1748, 1662, 1592, 1498, 1455, 1373, 1212, 1164, 1111, 1045, 964, 922, 778, 691, 617, 539. $[\alpha]_D^{20} = +61.4$ (*c* 0.22, DMF). ^1H NMR ($CDCl_3$, 300 MHz): δ (ppm) 7.39–7.20 (m, 10H, Ph–H), 6.79 (d, 1H, *J* = 8.1 Hz, NH), 6.72 (d, 1H, *J* = 7.2 Hz, NH), 6.75 (dd, 1H, *J* = 5.4, 3.1 Hz, H-1), 5.14 (app t, 1H, *J* = 9.6 Hz, H-4), 4.51–4.41 (m, 1H, NHC*H*CH_3), 4.41–4.34 (m, 1H, H-2), 4.13 (dd, 1H, *J* = 12.4, 3.9 Hz, H-6), 4.05 (q, 1H, *J* = 6.7 Hz, OC*H*CH_3), 4.02–3.97 (m, 1H, H-5), 3.91 (dd, 1H, *J* = 12.4, 2.9 Hz, H-6′), 3.74 –3.69 (m, 4H, $COOCH_3$, H-3), 2.09 (s, 3H, $COCH_3$), 2.00 (s, 3H, $COCH_3$), 1.78 (s, 3H, NHCOC*H*$_3$), 1.43 (d, 3H, *J* = 7.2 Hz, NHCHC*H*$_3$), 1.32 (d, 3H, *J* = 6.9 Hz, OCHC*H*$_3$). ^{13}C NMR ($CDCl_3$, 75 MHz): δ (ppm) 172.7, 172.1, 170.7, 170.6, 169.0, 150.3, 150.2, 123.0, 125.8, 125.7, 120.1, 120.0, 119.9, 119.8, 97.8, 78.1, 76.4, 70.5, 69.0, 61.3, 53.3, 53.2, 52.4, 48.0, 22.9, 20.7, 20.6, 18.9, 17.6. ^{31}P NMR ($CDCl_3$, 121 MHz): δ (ppm) −13.20 (s, 1P). LRMS (ESI), *m*/*z* = 695.3 $(M+H)^+$, 717.2 $(M+Na)^+$, 733.2 $(M+K)^+$, HRMS (ESI), *m*/*z* calcd for $C_{31}H_{39}N_2O_{14}PNa$ 717.2037 $(M+Na)^+$, found 717.2045. Microanalysis calcd for $C_{31}H_{39}N_2O_{14}P$ (%): C, 53.60; H, 5.66; N, 4.03; found: C, 53.71; H, 5.80; N, 4.22.

4.7. Triethylammonium salt of (*S*)-methyl 2-(2-(*R*)-(2-acetamido-2-deoxy-4,6-di-*O*-acetyl-1-*O*-phosphoryl-3-α-D-glucopyranosyloxy)propanamido)propanoate 12

Compound **9** (300 mg, 0.44 mmol) was dissolved in freshly distilled dry THF (50 mL). Platinum oxide (100 mg) was added after the solution was flushed with argon for 20 min. The reaction mixture was stirred vigorously while the hydrogen gas was bubbled through for 30 min and then left under hydrogen atmosphere at ambient temperature for 24 h. Triethylamine (250 μL, 1.78 mmol) was added and the solvent was evaporated under reduced pressure. The crude product was purified on an LH-20 Sephadex column using methanol as eluent, giving a very hygroscopic solid (261 mg, 0.41 mmol, 92% yield). IR (KBr, cm^{-1}): 2939, 2677, 2492, 1745, 1662, 1547, 1452, 1377, 1243, 1168, 1114, 1039, 967, 918, 847, 554. $[\alpha]_D^{20} = +39.0$ (*c* 0.09, MeOH). ^1H NMR (MeOH–d_4, 300 MHz): δ (ppm) 5.44 (dd, 1H, *J* = 6.9, 3.3 Hz, H-1), 5.08 (app t, 1H, *J* = 9.6 Hz, H-4), 4.35 (q, 1H, *J* = 9.6 Hz, NHC*H*CH_3), 4.31–4.22 (m, 3H, H-5, H-2, H-6), 4.17 (q, 1H, *J* = 6.8 Hz, OC*H*CH_3), 4.14–3.91 (dd, 1H, *J* = 11.9, 2.3 Hz, H-6′), 3.89 (app t, 1H, *J* = 9.8 Hz, H-3), 3.73 (s, 3H, $COOCH_3$), 3.14 (q, 6H, *J* = 6.8 Hz, N*H*$_2$$CH_3$), 2.12 (s, 3H, $COCH_3$), 2.07 (s, 3H, $COCH_3$), 1.96 (s, 3H, NHCOC*H*$_3$), 1.44 (d, 3H, *J* = 7.3 Hz, NHCHC*H*$_3$), 1.32 (d, 3H, *J* = 6.8 Hz, OCHC*H*$_3$), 1.31 (q, 9H, *J* = 7.3 Hz, NCH$_2$*CH*$_3$). ^{13}C NMR (MeOH–d_4, 75 MHz): δ (ppm) 175.0, 174.3, 173.5, 172.5, 171.6, 95.3, 79.2, 78.8, 70.8, 70.0, 63.2, 55.2, 52.8, 47.7, 23.1, 21.0, 20.8, 19.3, 17.3, 9.2. ^{31}P NMR (MeOH–d_4, 121 MHz): δ (ppm) −0.17 (s, 1P). LRMS (ESI−), *m*/*z* = 541.1 $(M-H)^-$; (ESI+), *m*/*z* = 565.1 $(M+Na)^+$, 581.1 $(M+K)^+$. HRMS (ESI+), *m*/*z* calcd for $C_{19}H_{31}N_2O_{14}PNa$ 565.1411 $(M+Na)^+$, found 565.1409. HPLC: Column C_{18} Phenomenex Luna 5μ; mobile phase: 20% acetonitrile, 80% aqueous trifluoroacetic acid (0.01%), flow rate 1.0 mL/min; injection volume: 10 μL; retention time: 8.2 min (97.6% @ 210 nm).

4.8. 4-Morpholino-*N*,*N*′-dicyclohexylcarboxamidine salt of (*S*)-methyl 2-(2-(*R*)-(2-acetamido-2-deoxy-4,6-di-*O*-acetyl-1-*O*-(uridine-5′-diphosphoryl)-3-α-D-glucopyranosyloxy)-propanamido)propanoate 14

Compound **12** (140 mg, 0.22 mmol) was suspended in dry benzene (10 mL) and the solvent was evaporated. After the starting material was dried in vacuo for 1 h, dry DMF stored over molecular sieves, the 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine salt of uridine monophosphate morpholidate (160 mg, 0.23 mmol) and 4 Å molecular sieves were added. The reaction mixture was flushed with argon and first stirred at room temperature for 30 min and then heated at 60 °C. After 24 h, water (1 mL) was added and the reaction was stirred for an additional 24 h. The solvents were evaporated under reduced pressure at a temperature not exceeding 30 °C. The crude product was purified by gradient flash chromatography (5–20% of water in acetonitrile). A colourless solid was obtained (175 mg, 0.13 mmol, 61% yield). Mp 164–165 °C. IR (KBr, cm^{-1}): 2936, 2858, 1745, 1680, 1620, 1564, 1452, 1377, 1243, 1116, 1040, 1002, 929, 520. $[\alpha]_D^{20} = +105.3$ (*c* 0.08, DMF). ^1H NMR (MeOH-d_4, 300 MHz): δ (ppm) 8.05 (d, 1H, *J* = 8.2 Hz, CH), 5.99 (d, 1H, *J* = 4.9 Hz, H-1$_r$), 5.87 (d, 1H, *J* = 8.3 Hz, CH), 5.60 (dd, 1H, *J* = 7.4, 2.9 Hz, H-1$_g$), 5.10 (app t, 1H, *J* = 9.6 Hz, H-4$_g$), 4.38–4.17 (m, 10H, H-2$_r$, H-3$_r$, H-4$_r$, H-5$_r$, H-5′$_r$, H-2$_g$, H-5$_g$, H-6$_g$, H-6′$_g$, OC*H*CH_3, NHC*H*CH_3), 3.98 (app t, 1H, *J* = 9.8 Hz, H-3$_g$), 3.77 (app t, 4H, *J* = 4.8 Hz, 2CH_2O), 3.73 (s, 3H, $COOCH_3$), 3.44 (app t, 4H, *J* = 4.8 Hz, 2CH_2N), 3.38–3.33 (m, 2H, 2CH), 2.11 (s, 3H, $COCH_3$), 2.07 (s, 3H, $COCH_3$), 2.03 (s, 3H, NHCOC*H*$_3$), 1.98–1.69 (m, 10H, 5CH_2), 1.44 (d, 3H, *J* = 7.3 Hz, NHCHC*H*$_3$), 1.32 (d, 3H, *J* = 6.6 Hz, OCHC*H*$_3$), 1.51–1.15 (m, 10H, 5CH_2). ^{13}C NMR (MeOH-d_4, 75 MHz): δ (ppm) 174.7, 174.3, 174.00, 172.5, 171.6, 166.0, 159.3, 152.6, 142.6, 103.3, 96.2, 90.0, 85.1, 79.1, 78.7, 75.6, 71.2, 70.5, 70.3, 67.3, 66.2, 63.2, 56.0, 55.1, 52.8, 49.8, 34.4, 26.3, 26.1, 23.2, 21.1, 20.8, 19.2, 17.4. ^{31}P NMR (MeOH-d_4, 121 MHz): δ (ppm) −9.8 (d, 1P, *J* = 20.0 Hz), −12.2 (d, 1P, *J* = 20.0 Hz). LRMS (ESI−), *m*/*z* = 847.2 $(M-H)^-$; (ESI+), *m*/*z* = 294.3 $(M+H)^+$. HRMS (ESI−), *m*/*z* calcd for $C_{28}H_{41}N_4O_{22}P_2$ 847.1688 $(M-H)^-$, found 847.1709. HPLC: Column C_{18} Phenomenex Luna 5μ; mobile phase: 20% acetonitrile, 80% aqueous trifluoroacetic acid (0.01%), flow rate 1.0 mL/min; injection volume: 10 μL; retention time: 15.0 min (99.1% @ 254 nm).

4.9. Trisodium (*S*)-2-(2-(*R*)-(2-acetamido-2-deoxy-1-*O*-(uridine-5′-diphosphoryl)-3-α-D-glucopyranosyloxy)-propanamido)propanoate 15

Compound **14** (73 mg, 0.064 mmol) was dissolved in bi-distilled water (4.5 mL) and 1 M sodium hydroxide (240 μL) was added. The reaction mixture was stirred at ambient temperature and monitored by HPLC. After 4 h, the pH was adjusted to 8 by the addition of 1 M HCl. After concentration to approx. 1 mL under reduced pressure at ambient temperature the crude product was purified by ion-exclusion chromatography on a G-10 Sephadex column equilibrated with bi-distilled water. The fractions containing the product were pooled and evaporated under reduced pressure and dried using toluene-coevaporation yielding a colourless solid (50 mg, 0.061 mmol, 95% yield). Mp 210–212 °C. IR (KBr, cm^{-1}): 3422, 1664, 1406, 1250, 1120, 930. $[\alpha]_D^{20} = +40.7$ (*c* 0.04, MeOH). ^1H NMR (D_2O, 300 MHz, MeOH standard): δ (ppm) 8.35 (d, 1H, *J* = 9.5 Hz, NH), 7.90 (d, 1H, *J* = 8.1 Hz, CH), 7.79 (d, 1H, *J* = 7.1 Hz, NH), 5.94 (d, 1H, *J* = 3.6 Hz, H-1r), 5.93 (d, 1H, *J* = 8.3 Hz, CH), 5.44 (dd, 1H, *J* = 7.2, 3.2 Hz, H-1$_g$), 4.35–4.30 (m, 2H, H-2$_r$, H-3$_r$), 4.26–4.22 (m, 1H, H-4$_r$), 4.20–4.14 (m, 4H, OC*H*CH_3, H-5$_r$, H-5′$_r$, NHC*H*CH_3), 4.11–4.04 (m, 1H, H-2$_g$), 3.94–3.89 (m, 1H, H-5$_g$), 3.82–3.75 (m, 2H, H-6, H-6′) 3.74 (app t, 1H, *J* = 9.5 Hz H-3$_g$) 3.60 (t, 1H, *J* = 9.5 Hz, H-4$_g$), 1.97 (s, 3H, NHCOC*H*$_3$), 1.35 (d, 3H,

J = 6.6 Hz, OCHCH_3), 1.34 (d, 3H, J = 7.3 Hz, NHCHCH_3), ^{13}C NMR (D_2O, 75 MHz, MeOH standard): δ (ppm) 178.7, 175.2, 174.4, 166.3, 151.9, 141.8, 102.8, 94.7, 88.7, 83.3, 79.7, 78.1, 73.9, 73.1, 69.8, 68.4, 65.2, 60.5, 53.4, 50.0, 22.3, 18.6, 17.2. ^{31}P NMR (D_2O, 121 MHz): δ (ppm) −10.1 (d, 1P, J = 20.0 Hz), −11.9 (d, 1P, J = 20.0 Hz). LRMS (ESI−), m/z = 749.2 $(M-H)^-$; (ESI+), m/z = 773.1 $(M+Na)^+$, 795.1 $(M+2Na)^+$, 817.1 $(M+3Na)^+$, 839.1 $(M+4Na)^+$. HRMS (ESI−), m/z calcd for $C_{23}H_{35}N_4O_{20}P_2$ 749.1320 $(M-H)^-$, found 749.1335. HPLC: Column C_{18} Phenomenex Luna 5μ; mobile phase: 2% acetonitrile, 98% aqueous trifluoroacetic acid (0.01%), flow rate 1.5 mL/min; injection volume: 10 μL; retention time: 11.8 min (100% @ 254 nm, 99.2% @ 210 nm).

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