Synthetic strategies for non-hydrolyzable pyrophosphate analogues

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Chapter 6

Summary and Future Prospects

Introduction

Pyrophosphates play an important role in a variety of biological processes. The intrinsic chemical and enzymatic lability of the pyrophosphate moiety challenges the study of the processes, in which they are involved. The research described in this Thesis focuses on the development of non-hydrolyzable pyrophosphate analogues as inhibitors and/or structural probes to investigate the structure and mode of action of enzymes that employ them as substrates. **Chapter 1** introduces various pyrophosphate containing molecules and highlights the involvement of cytidine diphosphate glycerol (CDP-Gro) and cytidine diphosphate ribitol (CDP-Rbo) in Wall Teichoic Acid (WTA) biosynthesis and the role of nucleoside diphosphate (NDP) sugars as donor substrates for glycosyltransferases. The chapter then provides an overview of the strategies devised to generate pyrophosphate mimetics, focusing on the synthetic methodologies of bisphosphonate and oxysulfonyl carbamate analogues, thereby providing the background for the design and synthesis of the stable analogues described in the subsequent chapters.

Chapter 2 established a straightforward synthetic approach for the preparation of a series of CDP-Gro and CDP-Rbo mimics (**1**-**9**) (Figure 6.1) as potential inhibitors of wall teichoic acid polymerases. The sulfonyl carbamate and oxyphosphonyl carbamate groups were introduced as pyrophosphate bioisosteres, providing enhanced chemical stability and resistance to enzymatic hydrolysis. In the described synthesis routes, chlorosulfonyl isocyanate (CSI) and dichlorophosphoryl isocyanate (dCPI) reagents were used as versatile coupling agents. These reagents can be utilized in two synthetic directions—either by reacting first with the cytidine moiety and subsequently with the glycerol or ribitol component, or *vice versa*—providing flexible routes to different CDP-Gro and CDP-Rbo mimics. The inhibitory activities against the enzymes utilizing CDP-Gro and CDP-Rbo remains to be established.

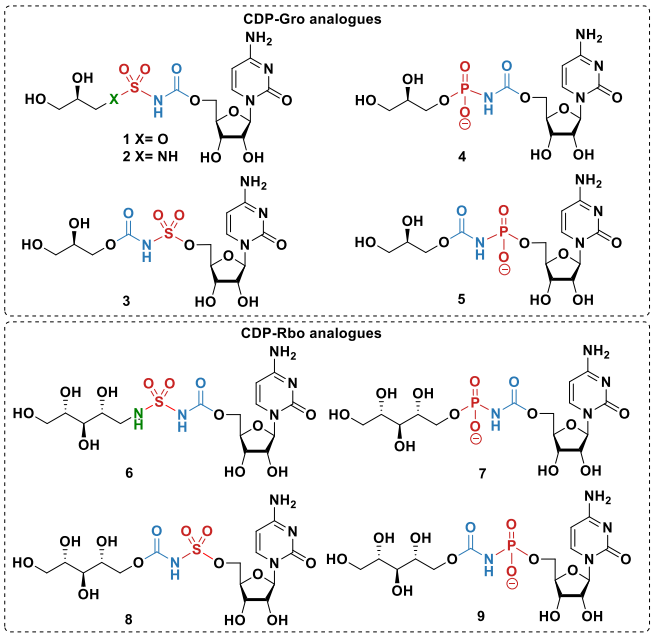


Figure 6.1. Various CDP-Gro and CDP-Rbo analogues described in Chapter 2.

Chapter 3 describes the design and synthesis of two uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) mimetics **10** and **11** (Figure 6.2). UDP-carbaGlcNAc **10** was designed with the GlcNAc moiety being replaced with non-hydrolyzable carbaGlcNAc, while the analogue **11** incorporates a carba-GlcNAc unit and a methylene bisphosphonate linkage, rendering both the glycosidic and pyrophosphate linkages non-hydrolyzable. The synthesis of carba-GlcNAc is based on routes originally developed by Ileana *et al.* towards cyclohexene glucose analogue **12**.[1] Subsequently, a Sharpless aminohydroxylation was used to stereoselectively install the α-1,2-cis-amino alcohol motif as a key transformation (Scheme 6.2A). The *N*-tosylated amino alcohol **14** was converted into the carbamate **15**, followed by deprotection and acetylation to afford building block **16**. Deacetylation of **16** provided the α-carbaGlcNAc **17**, which was used to synthesize UDP-carbaGlcNAc **10** via P^{III}-P^{V} chemistry, as well as a P–CH_2–P–linked UDP-carbaGlcNAc analogue **11** using a phosphoramidite-phosphodiester reagent.

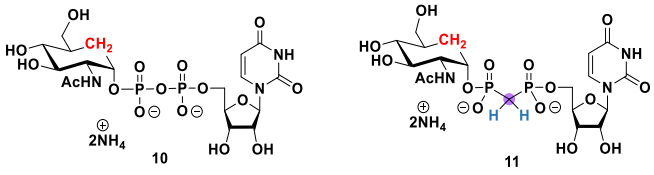
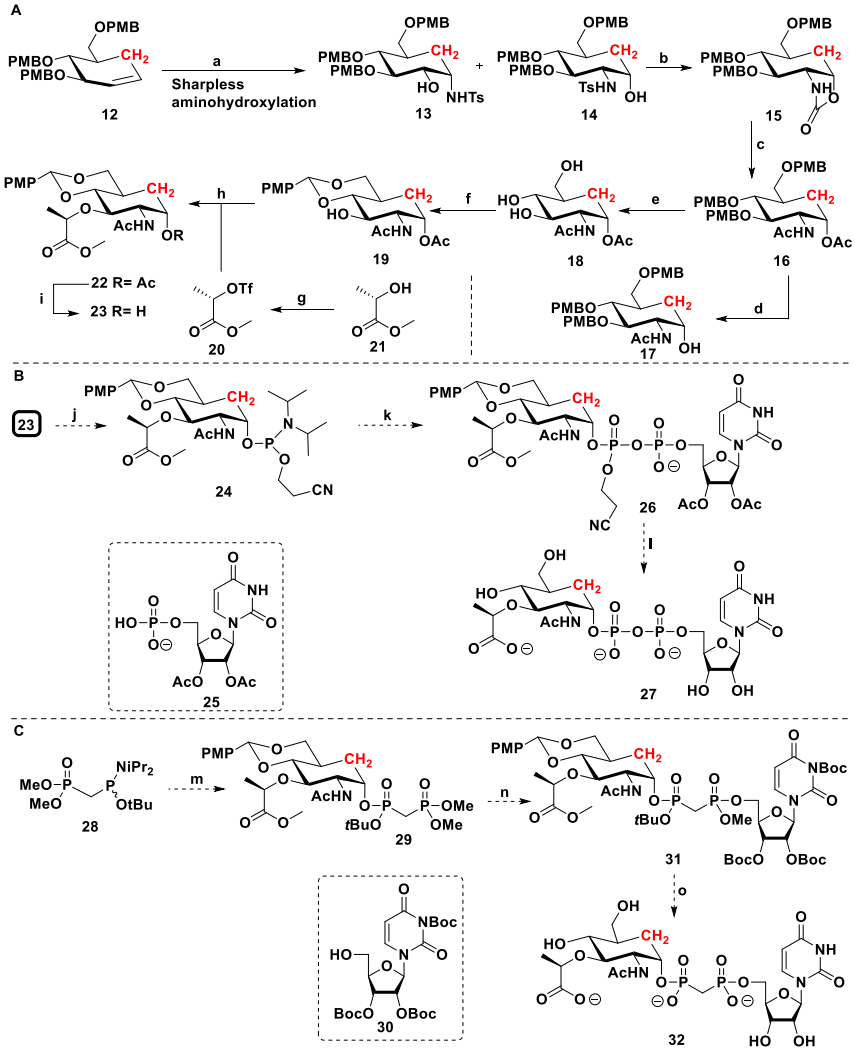


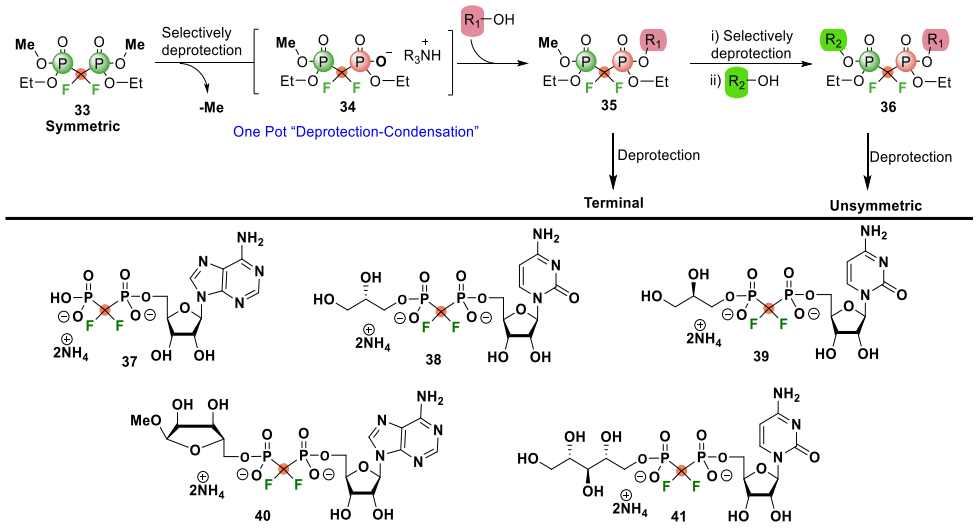
Figure 6.2. UDP-carbaGlcNAc analogues described in Chapter 3.

Building on the synthesis route established for UDP-GlcNAc inhibitors, this strategy can be further expanded to the development of UDP-MurNAc inhibitors. UDP-MurNAc (uridine diphosphate *N*-acetylmuramic acid) is the natural donor substrate for MurC in the biosynthesis of bacterial peptidoglycan.[2-4] Stabilized UDP-MurNAc mimetics may act as novel inhibitors by blocking MurC activity, thereby disrupting bacterial cell wall biosynthesis. Building block **16** not only served as a precursor for the synthesis of carbaGlcNAc through deacetylation but can also represent a key intermediate for the construction of α-carbaMurNAc. In some initial steps, compound **18** was obtained by removal of all the PMB protecting groups from **16** with 30% TFA in DCM (Scheme 6.1A). Subsequent regioselective protection of the 4,6-diol in compound **18** with a *p*-methoxybenzylidene afforded compound **19** in 68% yield. Alkylation of **19** with methyl (*S*)-lactate triflate **20**, which was prepared form methyl (*S*)-lactate **24**, afforded **22** in 70% yield. This building block could be transformed into the stabilized UDP-MurNAc analogue by deacetylation under sodium methoxide in methanol, which would provide α-carbaMurNAc **23**. Installation of the phosphoramidite on α-carbaMurNAc **23** would furnish the P^{III} building block **24** (Scheme 6.1B) and subsequent coupling with compound **25** using P^{III}-P^{V} chemistry as described in Chapter 3 would provide the fully protected UDP-carbaMurNAc **26**. Subsequent global deprotection would then yield the target UDP-carbaMurNAc **27**. In addition, a plausible synthetic route to P–CH_2–P linked UDP-carbaMurNAc analogue is outlined in Scheme 6.1C. The P–CH_2–P linkage can be introduced by coupling carbaMurNAc **23** with the phosphoramidite–phosphodiester reagent **28** to give intermediate **29**. Subsequent demethylation and condensation with compound **30** would afford the fully protected UDP-carbaMurNAc **31**, and global deprotection would provide the proposed UDP-carbaMurNAc **32**.



Scheme 6.1. Synthetic scheme towards carbaGlcNAc and proposed UDP-MurNAc analogues. (A) Synthetic scheme towards carbaGlcNAc **17** and carbaMurNAc **23**. (B) Proposed synthetic scheme towards UDP-carbaMurNAc mimic **27**. (C) Proposed synthetic scheme towards P-CH_2-P linked UDP-carbaMurNAc mimic **32**. Reagents and conditions: a) Chloramine-T, benzyl triethylammonium chloride, OsO_4 (5 mol %), $CHCl_3/H_2O$ (1:1 v/v), **13**, 46%; **14**, 30%. b) (i) triphosgene, pyridine, DCM, rt; (ii) Na, naphthalene, THF, -78 °C, 71% over two steps, c) (i) LiOH, THF/H_2O (ii) pyridine, Ac_2O. d) MeONa, MeOH, rt, 89% over 3 steps. e) TFA, DCM 0 °C, 75%. f) anisaldehyde dimethyl acetal, pyridinium *p*-toluene sulfonate, 68%. g) Tf_2O, pyridine, DCM, -10 °C. h) NaH, **20**, THF, 0 °C, 70%. i) $NaOCH_3$, CH_3OH. j) 2-Cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, DMF, DIPEA, rt. k) (i) DCI, **25**, MeCN; (ii) *t*BuOOH. l) (i) DBU; (ii) TFA, DCM, 0 °C to rt; (iii) LiOH, H_2O. m) (i) DCI, **23,** DCM; (ii) *t*BuOOH. n) (i) MeCN/Et_3N/PhSH (3:3:2), 35 °C; (ii) PyNTP, DIPEA, **30**, MeCN, rt. o) (i) TFA, TES, DCM; (ii) LiOH, H_2O.

Chapter 4 describes a new orthogonal desymmetrization strategy for the synthesis of unsymmetrical difluoromethylene bisphosphonates, which are stabilized analogues of natural pyrophosphates with a P–CF_2–P linkage. The key symmetrical diethyl(dimethyl)difluoromethylene bisphosphonate reagent **33** was synthesized on 18 gram scale. The approach exploits the differential reactivity between methyl and ethyl ester groups to enable a stepwise demethylation–condensation sequence in which one methyl can be selectively removed to generate intermediate **34**, which can be coupled to the first alcohol to provide tetraester **35** (Scheme 6.2). Global deprotection of **35** provides terminal difluoromethylene bisphosphonate molecules. Alternatively, selective removal of the remaining methyl group in **35** allows coupling with a second alcohol to produce a precursor for unsymmetrical difluoromethylene bisphosphonate. The applicability of the devised methodology was demonstrated in the synthesis of a series of ADP, ADPr, CDP-glycerol, and CDP-ribitol difluoromethylene bisphosphonate analogues (**37**-**41**) (Scheme 6.2).



Scheme 6.2. Overview of synthetic methodology of the difluoromethylene bisphosphonate analogue and the synthesized analogues described in Chapter 4.

To further expand on the unsymmetrical difluoromethylene bisphosphonates described in **Chapter 4**, **Chapter 5** describes the design and synthesis of (difluoro) methylene bisphosphonate UDP-carbagalactose analogues as non-hydrolyzable UDP-Gal mimetics. The analogues incorporate carbagalactose and P-CH_2-P and P-CF_2-P motifs, thereby enhancing chemical and enzymatic stability while retaining key structural features essential for biological recognition. In contrast to what was observed in **Chapter 4**, the use of Wada's reagent proved abortive when employed for the coupling of difluoromethylene bisphosphonate with secondary alcohols. A variety of approaches, including different condensating agents, nucleophilic substitution of tosylates, and Mitsunobu reactions, were investigated but proved unsuccessful. Ultimately, the

nucleophilic substitution of the carba-β-galactose triflate by the tetrabutylammonium salt of a difluoromethylene bisphosphonate led to the construction of fully protected P-CF_2-P linked UDP-Gal analogue.

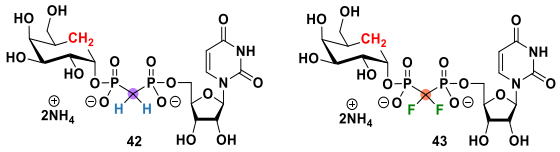
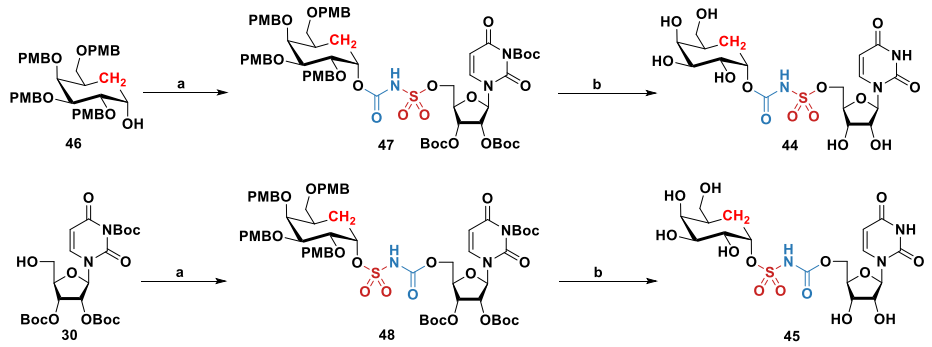


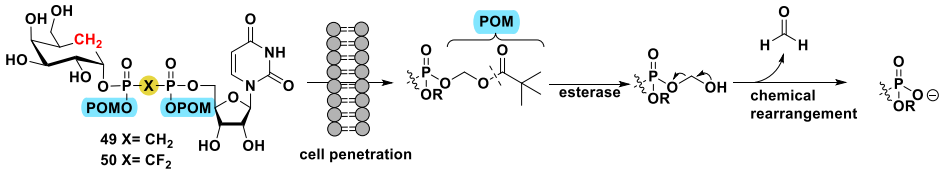
Figure 6.3. Synthesized UDP-carbaGal mimetics described in Chapter 5.

The P-CH_2-P and P-CF_2-P analogues **42** and **43** (Figure 6.3) were evaluated in an *in vitro* biochemical assay for the inhibition of recombinant human A4GALT, showing IC_{50} values of 26.0 ± 2.8 μM and 22.0 ± 1.2 μM, respectively, despite the relatively high concentration of UDP-Gal substrate (500 μM) used in the assay. These results suggest that the P-CH_2-P and P-CF_2-P analogues bind more strongly than natural UDP-Gal under these conditions. However, when evaluated in cell-based assays at concentrations up to 50 μM, no detectable inhibition was observed, likely due to the highly charged and polar nature of these analogues, which may limit their cellular uptake.[5] On this basis, two neutral analogues **44** and **45** (Scheme 6.3) were synthesized by replacing the pyrophosphate moiety with an uncharged oxysulfonyl carbamate moiety, following the strategy described in Chapter 2. The α-carbagalactose **46** was reacted with chlorosulfonyl isocyanate at 0 °C in dry dichloromethane, and after complete consumption of the starting compound **37**, the resulting intermediate was reacted in situ with the 5′-hydroxyl of **30** to give the protected analogue **47** in 48% yield. For the fully protected analogue **48**, the coupling order was reversed: compound **30** was first treated with chlorosulfonyl isocyanate to form the uridine sulfonyl chloride intermediate, which was then reacted with carba-galactose **46** to afford **48** in a yield comparable to that of analogue **47**. Global deprotection was accomplished by treating compound **47** and **48** with 30% TFA and triethylsilane, providing the analogues **44** and **45** in good yields. However, the oxysulfonyl carbamate linked analogues exhibited no inhibition at concentrations up to 100 μM in the *in vitro* assay. Preserving the pyrophosphate-like structure appears to be important for maintaining affinity toward the A4GALT active site, likely due to its role in metal ion coordination.[6]



Scheme 6.3. Synthesis of oxysulfonyl carbamate UDP-Gal analogues. Reagents and conditions: a) (i) chlorosulfonyl isocyanate, DCM, 0 °C to rt; (ii) pyridine, 0 °C to rt, **30**→**47**, 48%; **46**→**48**, 45%. b) 30% TFA/DCM, TES, 0 °C, **44**, 74%; **45**, 75%.

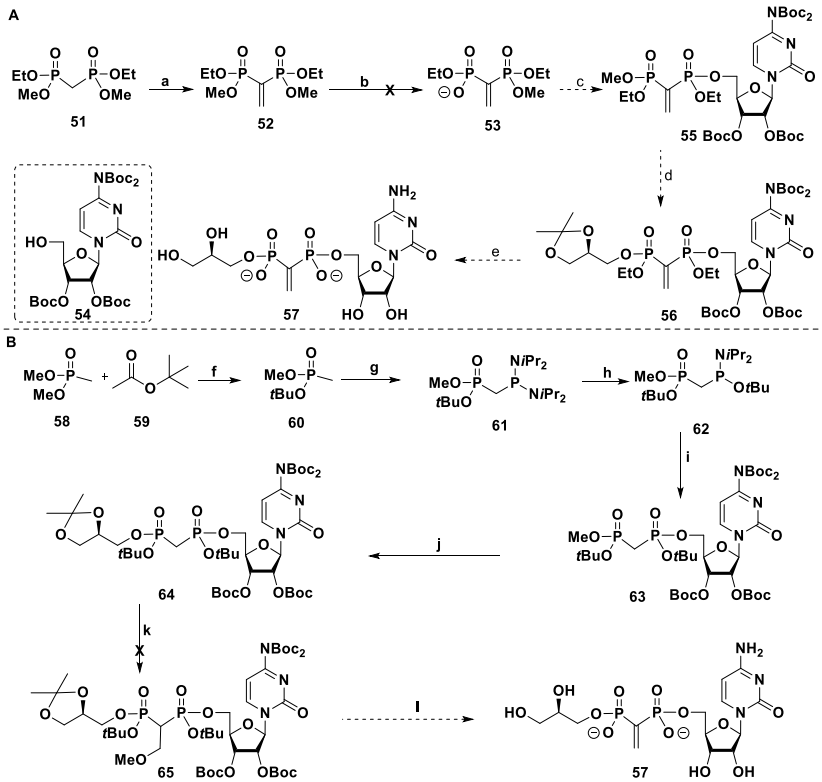
Although the bisanionic motif of bisphosphonates facilitates high binding affinities through ionic interactions, the high charge density significantly limits membrane permeability. A prodrug strategy would help to overcome this limitation by temporarily masking the charged groups and improving membrane permeability. Esterases can then liberate the active inhibitor.[5, 7] The inherent stability of the bisphosphonate analogues provides a solid foundation for this approach. Pivaloyloxymethyl (POM) esters have been used to mask bisphosphonates[8-9] and have also been applied in the FDA-approved prodrug adefovir dipivoxil.[7, 10] Accordingly, prodrugs **49** and **50** bearing POM groups are proposed (Scheme 6.4). The degradation of POM-prodrugs is triggered by esterase-mediated hydrolysis, yielding an unstable hydroxymethyl phosphonate intermediate that undergoes fragmentation to form formaldehyde and the active bisphosphonate inhibitor.[5, 11] These prodrugs would be interesting to evaluate the potency of the bisphosphonate analogues in cell-based assays. If the compounds **49** and **50** are still too polar to cross the membrane, the alcohols can be (partially) acetylated to enhance uptake.



Scheme 6.4. Proposed POM-protected UDP-Gal prodrugs and their mechanism of activation.

In addition to (difluoro) methylene bisphosphonate and sulfonyl- and phosphoryl-carbamate groups, the vinylidene bisphosphonate motif may also be explored as a pyrophosphate bioisoster. Notably, installation of this moiety on the pyrophosphate containing molecules was envisioned to enable covalent binding to the target enzyme, forming stable enzyme–inhibitor complexes and leading to irreversible inhibition. The first strategy explored to introduce this moiety onto CDP-Gro involved a selective deprotection–condensation sequence, as described in **Chapter 4**. Therefore, a symmetrical vinylidene bisphosphonate tetramethyl ester **52** was designed and targeted form

compound **51** (Scheme 6.5). Treatment of **51** with paraformaldehyde in the presence of methanol and diethylamine, followed by an elimination reaction catalyzed by *p*-toluenesulfonic acid produced reagent **52**. It was envisaged that selective demethylation with tetrabutylammonium benzoate could be followed by condensation with a suitably protected cytidine, after which a second demethylation- condensation sequence with (*S*)-solketal could give **56**. Global deprotection of **56** would then afford the vinylidene bisphosphonate linked CDP-Gro analogue **57**. However, selective demethylation of **52** using tetrabutylammonium benzoate (1 equiv) was unsuccessful, even upon extending the reaction time and increasing the temperature. Alternative deprotection conditions (*e.g.* using TMSBr) have not yet been explored.



Scheme 6.5. Attempted synthesis of a vinylidene bisphosphonate CDP-Gro analogue. Reagents and conditions: a) (i) paraformaldehyde, reflux; (ii) TsOH, reflux, 62% over two steps. b) (i) Bu_4NOBz, MeCN. c) cytidine **54**, PyNTP, DIPEA. d) (i) Bu_4NOBz, MeCN; (ii) (*S*)-solketal, PyNTP, DIPEA. e) (i) TMSBr, DCM, 0 °C; (ii) TFA. f) KO*t*Bu, THF, 48%. g) *n*-BuLi, diisopropylamine, THF, 55%. h) DCI, *t*BuOH, DCM, 80%. i) (i) DCI, **54**; (ii) *t*BuOOH, 79% over two steps. j) (i) MeCN/TEA/PhSH (3:3:2), 35 °C; (ii) PPh_3, DIAD, (*S*)-solketal, 31% over 2 steps. k) paraformaldehyde, MeOH, reflux. l) 30% TFA, DCM

Instead, an alternative strategy was investigated, in which the cytidine and (*S*)-solketal moieties were first installed onto the methylene bisphosphonate core, and the resulting intermediate was then subjected to olefination. Therefore, mixed phosphoramidite–phosphodiester reagent **62** was

used, which was synthesized following a procedure previously developed by Engelsma *et al*.[12] Acid-labile *tert*-butyl protecting groups were employed on the building block to allow their simultaneous removal during the final elimination reaction. First, dimethyl methylphosphonate (DMMP, **58**) was reacted with compound **59** in a KO*t*Bu catalyzed ester exchange reaction to afford *tert*-butyl methylphosphonate **60**.[13] *Tert*-butyl ester **60** was treated with 1.0 equiv of LDA, and then reacted with bis(diisopropylamino)chlorophosphine to afford intermediate **61** in 55% yield. Subsequent activation of compound **61** with 4,5-dicyanoimidazole (DCI, 0.6 equiv), followed by reaction with *tert*-butanol, afforded reagent **62** in 80% yield. Condensation of compound **54** with compound **62** was accomplished using DCI as an activator. Subsequent in situ oxidation of the phosphonite-phosphonate intermediate with *t*BuOOH yielded the methylene bisphosphonate derivative **63** in 80% yield over two steps. Next, removal of one of the methyl groups was achieved using thiophenol in a mixture of MeCN/Et_3N, and this was followed by a Mitsunobu reaction with (*S*)-solketal to give compound **64** in 31% yield over two steps. With compound **64** in hand, olefination of the methylene group was explored. To this end, compound **64** was treated with paraformaldehyde in the presence of methanol and diethylamine.[14] However, only a small amount of the desired product **65** was detected by LC–MS, with the starting material mainly being recovered. According to literature reports, this limited reactivity may depend on the steric bulk of the ester protecting groups.[14] This issue could potentially be addressed through the adoption of an alternative protecting group strategy in the near future, for example using methyl or cyclopropylmethyl esters.[14]

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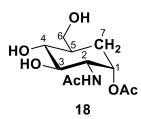
Experimental section

General information

All chemicals were commercial grade and used as received and all moisture sensitive reactions were performed under an argon or nitrogen atmosphere, at ambient temperature (21°C), unless stated otherwise. For TLC analysis were used aluminium sheets (Merck, TLC silica gel 60 F_{254}), sprayed with a solution of H_2SO_4 (20%) in EtOH or with a solution of $(NH_4)_6Mo_7O_{24}$•$4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4$•$2H_2O$ (10g/L) in 10% aqueous H_2SO_4 or with a solution of $KMnO_4$ (2%) and K_2CO_3 (1%) in H_2O and then heated at ~150°C. For column chromatography was used 60Å silica gel (40-63 μm, SD Screening Devices). NMR spectra (^1H, ^{13}C, and ^{31}P) were recorded with Bruker AV-400wb or Bruker AV-500. Chemical shifts (δ) are given in ppm relative to tetramethylsilane as internal standard (^1H NMR in $CDCl_3$) or the residual signal of the deuterated solvent. Chemical shifts for ^{31}P spectra are indirectly referenced to H_3PO_4 (0.00 ppm) according to the IUPAC method. ^{31}P NMR spectra measured to monitor reactions were made by charging an NMR tube with an aliquot of the reaction mixture and fitting the tube with an acetone-d_6 capillary. Coupling constants (*J*) are given in Hz. All ^{13}C NMR and ^{31}P NMR spectra are proton decoupled. NMR peak assignments were made using COSY and HSQC experiments. LC/MS analysis was performed on a Surveyor HPLC system (Thermo Finnigan) equipped with a C18 column (Gemini, 4.6 mm x 50 mm, 5 μm particle size, Phenomenex), coupled to a LCQ Adventage Max (Thermo Finnigan) ion-trap spectrometer (ESI^+). Solvent system: A: Milli-Q H_2O, B: MeCN, C: 1% TFA (aq). High resolution mass spectra were recorded by direct injection on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive ion mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 275°C) with resolution R = 60000 at m/z 400 (mass range m/z = 150-4000) and dioctyl phthalate (m/z = 391.28428) as a lock mass. Size-exclusion chromatography was performed on Sephadex™ LH-20 gel using CH_2Cl_2/MeOH (1:1, v/v) as the eluent.

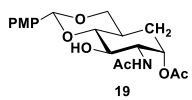
Carba-glucose triol 18



18

Compound **16** (1.0 g, 1.6 mmol) was dissolved in DCM (2.8 mL) and cooled to 0 °C, TFA (1.2 ml, 16 mmol) was added, and the reaction was stirred at rt for 1 h. The reaction was complete as indicated by TLC analysis. The reaction mixture was concentrated *in vacuo*. Purification by silica gel column chromatography (R_f = 0.2, acetone/DCM 8:2, v/v) gave compound **18** (318 mg, 1.2 mmol, 75 %) as a colorless oil. ^{1}H NMR (400 MHz, MeOD) δ 5.23 – 5.16 (m, 1H, H-1), 3.82 (dd, *J* = 10.9, 3.0 Hz, 1H, H-2), 3.72 (dd, *J* = 10.8, 3.9 Hz, 1H, H-5), 3.65 – 3.54 (m, 2H, H-3, H-5), 3.30 – 3.26 (m, 1H, H-4), 2.08 (s, 3H, OAc), 1.95 (s, 4H, NHAc, H-7), 1.85 – 1.75 (m, 1H, H-5), 1.54 – 1.45 (m, 1H, H-7). ^{13}C NMR (101 MHz, MeOD) δ 172.3 (C=O OAc), 170.7 (C=O NHAc), 74.4 (C-4), 72.8 (C-3), 70.7 (C-1), 62.5 (C-6), 54.2 (C-2), 39.0 (C-5), 28.9 (C-7), 21.1 (NHAc), 19.6 (OAc). HRMS (ESI) M/Z: $[M+Na]^+$: Calcd for $C_{11}H_{19}NO_6Na$ 284.1105; Found 284.1107.

Carba-glucose 19



19

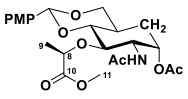
Compound **18** (50 mg, 0.19 mmol) was dissolved in anhydrous DMF (1.0 mL), anisaldehyde dimethyl acetal (42 µL, 0.25 mmol) and a catalytic amount of *p*-TsOH were added, and the reaction was stirred at 35 °C under reduced pressure for 3 h. The reaction was complete as indicated by TLC analysis. The reaction was quenched with sat. aq. $NaHCO_3$ and extracted with Et_2O. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by silica gel column chromatography (R_f = 0.2, acetone/DCM 10:1, v/v) gave compound **19** (49 mg, 0.13 mmol, 68%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.48 – 7.37 (m, 2H, CH_{arom}), 6.93 – 6.85 (m, 2H, CH_{arom}), 5.78 (d, *J* = 7.9 Hz, 1H, NH), 5.53 (s, 1H, CHPMP), 5.26 – 5.17 (m, 1H, H-1), 4.19 – 4.04 (m, 2H, H-2, H-6), 3.91 – 3.76 (m, 4H, H-4, OMe), 3.65 – 3.43 (m, 2H, H-3, H-6), 2.15 – 2.07 (m, 4H, H-5, OAc), 2.02 (s, 3H, NHAc), 1.86 (dt, *J* = 14.7, 3.5 Hz, 1H, H-7), 1.37 – 1.28 (m, 1H, H-7). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.8 (C=O NHAc), 160.2, 130.3 ($C_{q\text{-}arom}$), 127.6, 113.7 (CH_{arom}), 101.9 (CHPMP), 83.9 (C-3), 71.2 (C-1), 71.0 (C-4), 70.8 (C-6), 55.4 (OMe), 53.8 (C-2), 32.3 (C-5), 27.8 (C-7), 23.4 (NHAc), 21.2 (OAc). HRMS (ESI) M/Z $[M+Na]^+$: Calcd for $C_{19}H_{25}NO_7Na$ 402.1523; Found 402.1523.

Triflate 20

20

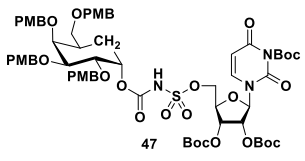
Trifluoromethanesulfonic anhydride (2.8 mL, 16.2 mmol) was added to a mixture of pyridine (1.42 mL, 17.6 mmol) and DCM (30 mL) at 0 °C. The mixture was stirred for 10 min. Methyl (*S*)-(-)-lactate (1.53 mL, 16.0 mmol) was then added dropwise, and the reaction was stirred at 0 °C for 30 min and then allowed to warm to room temperature. The reaction mixture was filtered, and the solid residue was washed with ether. The combined filtrate was concentrated under reduced pressure at <25 °C. The crude product was triturated with ether, filtered, and the residue was purified by passing through a short flash column (4 cm) using ether as eluent. Removal of the solvent yielded the desired product as a colorless oil (2.1 g, 11.4 mmol, 56%), the product was dried over molecular sieves (3 Å) and stored at –20 °C.

Protected carba-*N*-acetylmuramic acid 22



Compound **19** (11 mg, 29 µmol) was co-evaporated with toluene three times and dissolved in DMF. The reaction mixture was cooled to 0 °C, NaH (60% dispersion in mineral oil, 48 mg, 118 µmol) was added and stirred for 5 minutes. Compound **20** (21 mg, 87 µmol) was added, and the reaction was allowed to warm to room temperature and stirred overnight. The reaction mixture was cooled to 0 °C and quenched by the addition of MeOH. The solution was further diluted with water and subsequently extracted with Et_2O, dried with $MgSO_4$, filtered, and concentrated. Purification by flash column chromatography (DCM/acetone 4:1, v/v) gave compound **22** (9 mg, 20 µmol, 70%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (d, *J* = 4.1 Hz, 1H, NHAc), 7.44 – 7.35 (m, 2H, CH_{arom}), 6.96 – 6.89 (m, 2H, CH_{arom}), 5.58 (d, *J* = 3.1 Hz, 2H, H-1, CHPMP), 4.66 (q, *J* = 7.1 Hz, 1H, H-8), 4.13 (dd, *J* = 11.0, 4.4 Hz, 1H, H-6), 3.84 (s, 3H, OMe), 3.83 – 3.78 (m, 2H, H-2, H-3), 3.77 (s, 3H, H-11), 3.67 (dd, *J* = 10.3, 8.7 Hz, 1H, H-4), 3.65 – 3.60 (m, 1H, H-6), 2.19 – 2.12 (m, 1H, H-5), 2.09 (s, 3H, OAc), 2.04 (s, 3H, NHAc), 1.83 – 1.75 (m, 1H, H-7), 1.43 (d, *J* = 7.0 Hz, 3H, H-9), 1.35 – 1.30 (m, 1H, H-7). ^{13}C NMR (126 MHz, $CDCl_3$) δ 176.1 (C=O OAc), 171.1 (C=O NHAc), 169.8 (C-10), 159.9, 130.5, 127.8 ($C_{q\text{-}arom}$), 127.0, 113.7 (CH_{arom}), 101.2 (CH PMP), 85.1 (C-4), 76.1 (C-3), 75.2 (C-8), 70.9 (C-6), 69.5 (C-1), 55.3 (OMe), 54.4 (C-2), 52.2 (C-11), 33.3 (C-5), 27.2 (C-7), 23.2 (NHAc), 21.1 (OAc), 18.7 (C-9). HRMS (ESI) M/Z: $[M+Na]^+$: Calcd for $C_{23}H_{31}NO_9Na$ 488.1891; Found 488.1893.

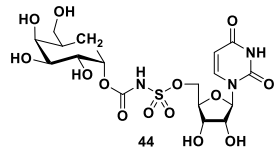
Protected UDP-carbagalactose analogue 47



The carbagalactose **46** (120 mg, 0.18 mmol) was dried by co-evaporation with anhydrous toluene three times, and then dissolved in dry DCM (1.8 mL, 0.1 M). Fresh flame-dried molecular sieves (3 Å) were added and the mixture was cooled to 0 °C. Chlorosulfonyl isocyanate (16 µL, 0.18 mmol) was added dropwise to the mixture. Afterwards, the mixture was allowed to warm to room temperature and stirred for 3 h. Upon full conversion, dry pyridine (44 µL, 0.54 mmol, 3.0 eq) and the protected uridine **30** (118 mg, 1.2 mmol) were added and the mixture was stirred at room temperature overnight. After the reaction was complete, the solvent was removed under reduced pressure. The crude product was purified by silica gel column chromatography (R_f = 0.2, pentane/EtOAc 1:1, v/v) to yield **47** as a colorless oil (113 mg, 86 µmol, 48%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 (s, 1H, H-6), 7.34 – 7.20 (m, 6H, CH_{arom}), 7.17 – 7.05 (m, 2H, CH_{arom}), 6.92 – 6.77 (m, 8H, CH_{arom}), 6.06 – 5.91 (m, 1H, H-1'), 5.85 – 5.71 (m, 1H, H-5), 5.40 – 5.31 (m, 1H, H-1''), 5.29 – 5.20 (m, 2H, H-2', H-3'), 4.89 – 4.78 (m, 1H, C*H*H PMB), 4.71 – 4.57 (m, 3H, C*H*H PMB, CH*H* PMB, CH*H* PMB), 4.57 – 4.41 (m, 3H, C*H*H PMB, CH*H* PMB, CH*H* PMB), 4.45 – 4.27 (m, 4H, C*H*H PMB, H-5', H-4'), 4.10 – 4.04 (m, 1H, H-4''), 3.96 – 3.89 (m, 1H, H-2''), 3.81 (dd, *J* = 5.6, 3.1 Hz, 13H, OMe PMB, OMe PMB, OMe PMB, OMe PMB, H-3'') 3.46 – 3.35 (m, 1H, H-6''), 3.23 (dd, *J* = 9.0, 5.5 Hz, 1H, H-6''), 2.12 – 1.98 (m, 1H, H-5''), 1.70 – 1.64 (m, 1H, H-7''), 1.61 (m, 9H, $C(CH_3)_3$), 1.48 (m, *J* = 8.7, 4.6 Hz, 19H, H-7'', $C(CH_3)_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ

160.3 (C=O uracil), 159.3, 159.3, 159.1 ($C_{q\text{-}arom}$), 152.1 (C=O carbamate), 152.0 (C=O uracil), 148.6, 147.6 (C=O carbonate), 139.6 (C-6), 131.5, 131.2, 130.3, 130.3 ($C_{q\text{-}arom}$), 129.8, 129.7, 129.5, 129.1, 113.9, 113.9, 113.8, 113.8, 113.7, 113.6, 113.6 (CH_{arom}), 103.1 (C-5), 88.0 (C-1'), 87.2, 84.0, 83.8 ($C(CH_3)_3$), 80.7 (C-3''), 79.4 (C-4'), 77.3 (C-2''), 75.2 (C-4''), 74.6 (C-2'), 74.4 (CH_2 PMB), 73.8 (C-1''), 73.0 (C-5'), 72.9, 72.7, 72.4 (CH_2 PMB), 72.0 (C-3'), 70.1 (C-6''), 55.4, 55.3 (OMe PMB), 35.9 (C-5''), 27.7, 27.7, 27.5 ($C(CH_3)_3$), 26.7 (C-7''). HRMS (ESI) M/Z: $[M+Na]^+$: Calcd for $C_{64}H_{81}N_3O_{24}Na$ 1330.4823; Found 1330.4834.

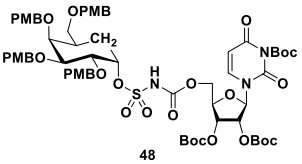
UDP-carbagalactose analogue 44



44

Compound **47** (100 mg, 76 µmol) was co-evaporated with dry toluene and dissolved in dry DCM (1.3 ml), TES (122 µL, 0.76 mmol) and trifluoroacetic acid (570 µL, 30% v/v, final concentration 0.04 M) were added at 0 °C. The reaction mixture was stirred for 24 h. The progress of the reaction was monitored by LC-MS until complete deprotection. The mixture was concentrated and the residue was purified by size-exclusion chromatography (HW40-column, 0.15 M aqueous NH_4OAc in water) followed by lyophilization to yield UDP-carbagalactose analogue **44** (29.6 mg, 56 µmol, 74%) as a white powder. ^{1}H NMR (400 MHz, D_2O) δ 7.84 (d, *J* = 8.1 Hz, 1H, H-6), 5.94 – 5.88 (m, 2H, H-5, H-1'), 5.00 – 4.93 (m, 1H, H-1''), 4.37 (dd, *J* = 12.0, 2.8 Hz, 1H, H-5'), 4.34 – 4.26 (m, 4H, H-2', H-3', H-4', H-5'), 4.11 – 4.05 (m, 1H, H-4''), 3.80 (dd, *J* = 10.4, 3.0 Hz, 1H, H-2''), 3.76 (dd, *J* = 10.3, 2.7 Hz, 1H, H-3''), 3.59 (dd, *J* = 11.0, 7.8 Hz, 1H, H-6''), 3.45 (dd, *J* = 11.0, 6.4 Hz, 1H, H-6''), 2.03 – 1.92 (m, 1H, H-5''), 1.76 – 1.69 (m, 1H, H-7''), 1.57 – 1.45 (m, 1H, H-7''). ^{13}C NMR (101 MHz, D_2O) δ 166.2 (C=O uracil), 159.7 (C=O carbamate), 151.7 (C=O uracil), 141.6 (C-6), 102.5 (C-5), 88.8 (C-1'), 81.9 (C-4'), 73.7 (C-2'), 73.6 (C-1''), 71.6 (C-3''), 69.8 (C-4''), 69.7 (C-2''), 69.5 (C-3'), 67.8 (C-5'), 62.4 (C-6''), 36.8 (C-5''), 25.7 (C-7''). HRMS (ESI) M/Z: $[M+Na]^+$: Calcd for $C_{17}H_{25}N_3O_{14}Na$ 500.0949; Found 500.0960.

Protected UDP-carbagalactose analogue 48

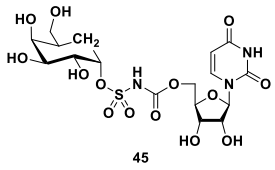


48

The protected uridine **30** (55 mg, 0.10 mmol) was dried by co-evaporation with anhydrous toluene three times, and then dissolved in dry DCM (1.0 mL, 0.1 M). Fresh flame-dried molecular sieves (3 Å) were added, and the mixture was cooled to 0 °C. Chlorosulfonyl isocyanate (9 µL, 0.10 mmol) was added dropwise to the mixture. Afterwards, the mixture was allowed to warm to room temperature and stirred for 3 h. Upon full conversion, dry pyridine (24 µL, 0.3 mmol, 3.0 eq) and carbagalactose **46** (66 mg, 1.0 mmol) were added, and the mixture was stirred at room temperature overnight. After the reaction was complete, the solvent was removed under reduced pressure. The crude product was purified by silica gel column chromatography (R_f = 0.2, pentane/EtOAc 1:1, v/v) yielded **48** as a colorless oil (59 mg, 45 µmol, 45%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61 – 7.44 (m, 1H, H-6), 7.32 – 7.18 (m, 6H, CH_{arom}), 7.16 – 7.07 (m, 2H, CH_{arom}), 6.91 – 6.75 (m, 8H, CH_{arom}), 6.09 (d, *J* = 5.6 Hz, 1H, H-1'), 5.81 (d, *J* = 8.2 Hz, 1H, H-5), 5.26 – 5.18 (m, 2H, H-2',

H-3'), 5.03 (bs, 1H, NH), 4.84 – 4.58 (m, 5H, C*H*H PMB, C*H*H PMB, CH*H* PMB, C*H*H PMB, C*H*H PMB), 4.43 – 4.26 (m, 6H, C*H*H PMB, C*H*H PMB, C*H*H PMB, H-4', H-5'), 4.16 – 4.09 (m, 2H, H-1'', H-4''), 3.97 (dd, *J* = 7.0, 4.7 Hz, 1H, H-3''), 3.79 (dd, *J* = 5.8, 3.7 Hz, 12H, OMe, OMe, OMe, OMe), 3.77 – 3.70 (m, 1H, H-2''), 3.39 (t, *J* = 9.0 Hz, 1H, H-6''), 3.22 (dd, *J* = 9.0, 5.2 Hz, 1H, H-6''), 2.22 – 2.10 (m, 1H, H-5''), 1.58 (m, 10H, H-7'', $C(CH_3)_3$), 1.53 – 1.40 (m, 19H, H-7'', $C(CH_3)_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.3 (C=O uracil), 159.6, 159.4, 159.2, 159.2 ($C_{q\text{-}arom}$), 152.2 (C=O carbamate), 152.1 (C=O uracil), 148.7, 147.7 (C=O carbonate), 139.6 (C-6), 131.3, 130.7 ($C_{q\text{-}arom}$), 130.4 (CH_{arom}), 130.3 ($C_{q\text{-}arom}$), 129.7, 129.6, 129.5, 129.2, 129.1, 129.0, 114.0, 113.9, 113.9, 113.7, 113.7, 113.6 (CH_{arom}), 103.2 (C-5), 87.0 ($C(CH_3)_3$), 86.7 (C-1'), 83.8, 83.7 ($C(CH_3)_3$), 80.9 (C-2''), 79.7 (C-4'), 76.5 (C-3''), 74.6 (C-1''), 74.5 (CH_2 PMB), 74.5 (C-4''), 72.9, 72.5 (CH_2 PMB), 69.7 (C-6''), 65.0 (C-5'), 55.4, 55.4, 55.3 (OMe PMB), 35.5 (C-5''), 29.8 (C-7''), 27.7, 27.7, 27.5 ($C(CH_3)_3$). HRMS (ESI) M/Z: $[M+Na]^+$: Calcd for $C_{64}H_{81}N_3O_{24}Na$ 1330.4823; Found 1330.4837.

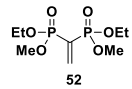
UDP-carbagalactose analogue 45



45

Compound **48** (30 mg, 23 µmol) was co-evaporated with dry toluene and dissolved in dry DCM (0.4 mL), TES (37 µL, 0.23 mmol) and trifluoroacetic acid (173 µL, 30% v/v, final concentration 0.04 M) were added at 0 °C. The reaction mixture was stirred for 24 h. The progress of the reaction was monitored by LC-MS until complete deprotection. The mixture was concentrated and the residue was purified by size-exclusion chromatography (HW40-column, 0.15 M aqueous NH_4OAc in water) followed by lyophilization to yield UDP-carbagalactose analogue **45** (9.1 mg, 17 µmol, 75%) as a white powder. ^{1}H NMR (400 MHz, D_2O) δ 7.79 (d, *J* = 8.1 Hz, 1H, H-6), 5.92 (d, *J* = 4.8 Hz, 1H, H-1'), 5.89 (d, *J* = 8.1 Hz, 1H, H-5), 4.84 – 4.80 (m, 1H, H-1''), 4.39 – 4.31 (m, 2H, H-2', H-3'), 4.29 – 4.17 (m, 3H, H-4', H-5'), 4.06 (d, *J* = 2.8 Hz, 1H, H-4''), 3.76 (dd, *J* = 10.4, 3.2 Hz, 1H, H-2''), 3.67 (dd, *J* = 10.4, 3.0 Hz, 1H, H-3''), 3.56 (dd, *J* = 11.0, 8.1 Hz, 1H, H-6''), 3.42 (dd, *J* = 11.0, 6.2 Hz, 1H, H-6''), 2.07 – 1.96 (m, 1H, H-5''), 1.95 – 1.86 (m, 1H, H-7''), 1.57 – 1.47 (m, 1H, H-7''). ^{13}C NMR (101 MHz, D_2O) δ 166.3 (C=O uracil), 160.0 (C=O carbamate), 151.8 (C=O uracil), 141.6 (C-6), 102.6 (C-5), 88.8 (C-1'), 82.1 (C-4'), 80.4 (C-1''), 73.4 (C-2'), 71.4 (C-3''), 69.8 (C-2''), 69.6 (C-4'), 69.4 (C-4''), 63.8 (C-5'), 62.1 (C-6''), 36.6 (C-5''), 25.7 (C-7''). HRMS (ESI) M/Z: $[M+Na]^+$: Calcd for $C_{17}H_{25}N_3O_{14}Na$ 500.0949; Found 500.0959.

Vinylidenebisphosphonate tetraester 52



52

Paraformaldehyde (1.0 g, 33.3 mmol) and diethylamine (499 mg, 6.8 mmol) were dissolved in methanol (20 mL), and the mixture was heated until a clear solution was obtained. After cooling to room temperature, diethyl(dimethyl)methylene bisphosphonate (1.7 g, 6.5 mmol) was added, and the reaction mixture was heated at reflux for 48 h. Additional methanol (20 mL) was added, and the solution was concentrated *in vacuo*. Toluene (20 mL) was added, and the solution was concentrated again to afford the intermediate as a clear liquid. The intermediate was diluted in dry toluene (20 mL) and a catalytic

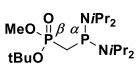
amount of *p*-toluenesulfonic acid was added. The reaction mixture was refluxed in a Dean-Stark trap for 12 h. The solvent was removed under reduced pressure. The crude product was purified by silica gel column chromatography (R_f = 0.2, DCM/acetone 2:1, v/v) to yield **52** as a colorless oil (1.1 g, 4.0 mmol, 62%).^{1}H NMR (500 MHz, $CDCl_3$) δ 7.11 – 6.94 (m, 2H, =CH_2), 4.23 – 4.10 (m, 4H, CH_2 Et), 3.83 – 3.76 (m, 6H, P(O)OMe), 1.37 (t, J = 7.1 Hz, 6H, CH_3 Et). ^{13}C NMR (126 MHz, $CDCl_3$) δ 150.0, 150.0 (=CH_2), 131.0 (t, $J_{P\text{-}C}$ = 166.9 Hz, P-C-P), 63.0 (t, $J_{P\text{-}C}$ = 2.9 Hz, P(O)O$CH_2$$CH_3$), 53.1 (t, $J_{P\text{-}C}$ = 2.9 Hz, P(O)OMe), 16.4 (t, $J_{P\text{-}C}$ = 3.3 Hz, CH_3 Et). ^{31}P NMR (202 MHz, $CDCl_3$) δ 14.34 (s). HRMS (ESI) M/Z: $[M+Na]^+$: Calcd for $C_8H_{19}O_6P_2$ 273.0651; Found 273.0657.

Reagent 60

60

In a flame-dried 250 mL flask under nitrogen, dimethyl methylphosphonate (3.1 g, 25 mmol) was dissolved in hexanes (100 mL). Tert-butyl acetate (50 mmol, 6.7 mL) and a solution of KO*t*-Bu (140 mg, 5 mol %) in THF (10 mL) were added dropwise over ~5 min. After stirring for an additional 10 min, the reaction mixture was turned deep golden yellow, and small crystals formed on the flask walls. The solution was decanted, solvent was removed *in vacuo*, and the product was purified by column chromatography (R_f = 0.3, pentane/acetone 7:3, v/v), to yield **60** as a yellow oil (2.0 g, 12 mmol, 48%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.67 (d, J = 11.2 Hz, 3H, OMe), 1.49 (s, 9H, C(CH_3)$_3$), 1.42 (d, J = 17.5 Hz, 3H, Me). ^{13}C NMR (101 MHz, $CDCl_3$) δ 82.3 (d, $J_{P\text{-}C}$ = 8.4 Hz, C(CH_3)$_3$), 51.8 (d, $J_{P\text{-}C}$ = 6.2 Hz, P(O)OMe), 30.4 (d, $J_{P\text{-}C}$ = 4.0 Hz, C(CH_3)$_3$), 12.9 (d, $J_{P\text{-}C}$ = 147.4 Hz, Me). ^{31}P NMR (162 MHz, $CDCl_3$) δ 27.85 (s). HRMS (ESI) M/Z: $[M+Na]^+$: Calcd for $C_6H_{15}PO_3Na$ 189.0651; Found 189.0651.

Reagent 61

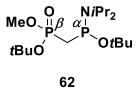


61

n-BuLi (2.5M in *n*-hexane, 10 mL, 25.2 mmol) was carefully added to a cooled solution (-78 ˚C) of diisopropylamine (3.5 mL, 25.2 mmol) in dry THF (10 mL), under argon atmosphere. The reaction mixture was stirred for 30 minutes. A solution of **60** (2.0 g, 12 mmol) in THF (10 mL) was added dropwise over 5 minutes, and the reaction mixture was stirred for an additional 30 minutes. Next, a solution of bis(diisopropylamino)chlorophosphoamidite (3.9 g, 14.4 mmol) in n-hexane/THF (2:1 v/v, 12 mL) was prepared and added dropwise through a syringe fitted with a silica filter to remove insoluble diisopropylamine hydrochloride originating from the commercial P^{III} reagent. After addition, the reaction mixture was allowed to warm to room temperature and stirred overnight. The reaction mixture was poured into sat. aq. $NaHCO_3$ and extracted with EtOAc. The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by silica gel column chromatography (pentane/EtOAc 9:1 + 1% Et_3N) yielded the compound **61** as a colorless oil (3.2 g, 6.6 mmol, 55%). *A mixture of diastereoisomers.* ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.68 (d, J = 11.2, 3H, P(O)OMe), 3.68 (d, J = 11.2, 3H, P(O)OMe*), 3.58 – 3.39 (m, 4H, CH(CH_3)$_2$, CH(CH_3)$_2$*), 2.41 – 1.70 (m, 4H, P-CH_2-P, P-CH_2-P*), 1.33 – 1.27 (m, 18H, C(CH_3)$_3$*, C(CH_3)$_3$*), 1.22 – 1.14 (m, 12H, CH(CH_3)$_2$, CH(CH_3)$_2$*), 1.13 – 1.07 (m, 12H, CH(CH_3)$_2$, CH(CH_3)$_2$*). ^{13}C NMR (75 MHz, $CDCl_3$) δ 51.6 (m, P(O)OMe), 44.8 (d, $J_{P\text{-}C}$ = 12.0 Hz), 44.7 (d, $J_{P\text{-}C}$ = 12.0 Hz),

34.8 (dd, $J_{P\text{-}C}$ = 133.8, 134.4 Hz), 34.3 (dd, $J_{P\text{-}C}$ = 134.0, 134.7 Hz), 30.7, 30.6, 30.6, 30.5 (P(O)O*C*H(CH$_3$)$_3$, P(O)O*C*H(CH$_3$)$_3$*), 24.7, 24.7, 23.5, 23.5 (CH(C*H*$_3$)$_2$, C*H*(C*H*$_3$)$_2$*). ^{31}P NMR (121 MHz, CDCl$_3$) δ 101.57 (d, J = 48.2 Hz, α-P), 101.17 (d, J = 50.4 Hz, α-P*), 26.97 (d, J = 48.2 Hz, β-P), 26.53 (d, J = 50.4 Hz, β-P*). HRMS (ESI) M/Z: [M+Na]$^+$: Calcd for $C_6H_{15}PO_3Na$ 189.0651; Found 189.0651.

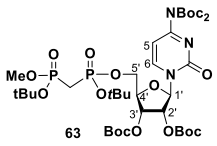
Reagent 62



62

Reagent **61** (2.5 g, 5.1 mmol) was co-evaporated with toluene, dissolved in MeCN and put under argon atmosphere. Dry *t*BuOH (381 mg, 5.1 mmol) and DCI (361 mg, 3.1 mmol, 0.6 eq) were added. The reaction progression was followed by ^{31}P NMR, which indicated completion after 6 hours. The reaction mixture was concentrated in vacuo and purified by silica gel column chromatography (DCM/acetone 5:1 + 2% Et$_3$N) to yield compound **62** as a colorless oil (1.7 g, 4.1 mmol, 80%). *A mixture of diastereoisomers.* ^{1}H NMR (400 MHz, CD$_3$CN) δ 3.60 (dd, J = 11.2, 1.0 Hz, 4H), 3.57 – 3.45 (m, 2H), 2.22 – 2.07 (m, 1H), 1.90 – 1.74 (m, 1H), 1.46 (s, 9H), 1.29 (d, J = 0.7 Hz, 9H), 1.18 (s, 3H), 1.17 (s, 3H), 1.11 (d, J = 1.5 Hz, 3H), 1.09 (d, J = 1.6 Hz, 3H). ^{13}C NMR (101 MHz, CD$_3$CN) δ 51.9 (d, $J_{P\text{-}C}$ = 26.7, P(O)OMe), 51.8 (d, $J_{P\text{-}C}$ = 26.1, P(O)OMe*), 45.5 (d, $J_{P\text{-}C}$ = 12.1 Hz, *C*H(CH$_3$)$_2$), 45.5 (d, $J_{P\text{-}C}$ = 11.8 Hz, *C*H(CH$_3$)$_2$*), 35.6 (t, $J_{P\text{-}C}$ = 34.6 Hz, P-CH$_2$-P), 34.3 (t, $J_{P\text{-}C}$ = 34.7 Hz, P-CH$_2$-P*), 30.8 (d, $J_{P\text{-}C}$ = 8.4 Hz, POC(*C*H$_3$)$_3$), 34.5 (d, $J_{P\text{-}C}$ = 3.7 Hz, P(O)OC(*C*H$_3$)$_3$), 30.5 (d, $J_{P\text{-}C}$ = 3.8 Hz, P(O)OC(*C*H$_3$)$_3$*), 24.8 (CH(*C*H$_3$)$_2$), 23.8 (CH(*C*H$_3$)$_2$*). ^{31}P NMR (162 MHz, CD$_3$CN) δ 100.61 – 99.95 (m, α-P), 25.74 – 24.35 (m, β-P). HRMS (ESI) M/Z: [M+Na]$^+$: Calcd for $C_{16}H_{37}NO_4P_2Na$ 392.2090; Found 392.2099.

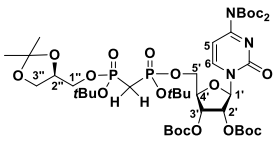
Cytidine 63



Reagent **62** (1.0 g, 2.4 mmol) and protected cytidine **17** (1.9 g, 2.9 mmol, 1.2 eq) were co-evaporated with toluene, dissolved in MeCN (8 ml, 0.3M), and put under argon atmosphere. DCI (425 mg, 3.6 mmol, 1.5 eq) was added and the reaction mixture was stirred for 30 min at room temperature. The mixture was cooled to 0 °C, tert-butyl hydroperoxide (5.0-6.0 M in decane, 1.3 ml, 7.2 mmol, 3 eq) was added. The reaction mixture was stirred for an additional 30 minutes. The solution was poured into DCM and washed with sat. aq. NaHCO$_3$ and brine. The organic layer was dried over anhydrous Na$_2$SO$_4$ and concentrated in vacuo. Purification by silica gel column chromatography (R_f = 0.5, pentane:EtOAc 1:5, v/v) yielded **63** as a white solid (2.0 g, 1.9 mmol, 79%). *A mixture of diastereoisomers.* ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.19, 8.16, 8.10 and 8.08 (4 x d, J = 7.6 Hz, 1H, H-6), 7.13 and 7.11 (2 x d, 1H, H-5), 6.09 – 6.03 (m, 1H, H-1'), 5.32 (m, 1H, H-2'), 5.24 – 5.11 (m, 1H, H-3'), 4.55 – 4.25 (m, 3H, H-4', H-5'), 3.82 – 3.70 (m, 3H, OMe), 2.50 – 2.33 (m, 2H, P-CH$_2$-P), 1.56 – 1.48 (m, 36H, C(CH$_3$)$_3$), 1.45 (d, J = 2.5 Hz, 18H, C(CH$_3$)$_3$). ^{13}C NMR (101 MHz, CDCl$_3$) δ 162.8, 162.8 (C=O, cytosine), 154.0, 154.0 ($C_{q\text{-}arom}$, cytosine), 152.0, 151.9, 151.9, 149.5, 149.5 (C=O Boc), 144.4, 144.3, 144.2 (C-6), 96.7, 96.6, 96.6, 96.5 (C-5), 89.8, 89.6, 89.5, 89.3 (C-1'), 85.0, 85.0, 83.5, 83.3, 83.3, 83.3 (C(CH$_3$)$_3$), 79.9, 79.8, 79.8, 79.7, 79.6, 79.6, 79.5 (m, C-4'), 76.0, 75.9, 75.8, 75.8 (C-2'), 71.5, 71.4, 71.2, 71.1

(C-3'), 64.2, 64.1, 64.0, 64.0, 63.9 (C-5'), 53.1, 53.1, 53.1, 53.0, 52.9, 52.9 (m, OMe), 30.5, 30.4, 30.4 ($C(CH_3)_3$), 29.8, 29.6, 29.6, 28.7, 28.4, 28.2, 28.2 (m, P-CH_2-P), 27.8, 27.7, 27.7, 27.7 ($C(CH_3)_3$), 27.3, 27.1, 26.9, 26.8, 26.6 (m, P-CH_2-P). ^{31}P NMR (162 MHz, $CDCl_3$) δ 16.92 (d, *J* = 6.4 Hz), 16.73 (d, *J* = 8.1 Hz), 16.46 – 16.39 (m), 16.35 (d, *J* = 6.3 Hz), 16.23 (d, *J* = 8.2 Hz), 16.16 – 16.08 (m). HRMS (ESI) M/Z: $[M+Na]^+$: Calcd for $C_{39}H_{67}N_3O_{18}P_2Na$ 950.3787; Found 950.3790.

Protected CDP-Gro analogue 64



Compound **63** (500 mg, 0.54 mmol) was dissolved in MeCN/Et_3N/PhSH (3:3:2, 1.6 mL) and stirred for 6 hours at 35 °C. After complete consumption of the starting material, the solution was concentrated *in vacuo*. The residue was quickly purified by flash silica gel column chromatography (100% DCM to 100% MeOH). The product was co-evaporated with toluene, dissolved in dry THF (1.8 ml, 0.3 M). (*R*)-solketal (107 mg, 0.81 mmol) and triphenylphosphine (213 mg, 0.81 mmol) were added. The mixture was cooled to 0 °C, DIAD (164 mg, 0.81 mmol) was added dropwise, and the mixture was allowed to warm to room temperature and was stirred overnight. The reaction mixture was concentrated under reduced pressure. Purification by silica gel column chromatography (R_f= 0.4, pentane:EtOAc 5:1, v/v) yielded compound **64** (174 mg, 0.16 mmol, 31%) as white foam. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22, 8.15, 8.14 and 8.07 (4 x d, *J* = 7.6 Hz, 1H, H-6), 7.16 – 7.12 (m, 1H, H-5, H-5), 6.14 – 6.03 (m, 1H, H-1'), 5.36 – 5.32 (m, 1H, H-2'), 5.26 – 5.14 (m, 1H, H-3'), 4.57 – 4.28 (m, 4H, H-4', H-5'', H-2''), 4.25 – 4.02 (m, 3H, H-1'', H-3''), 3.87 – 3.78 (m, 1H, H-3''), 2.55 – 2.37 (m, 2H, P-CH_2-P), 1.59 – 1.50 (m, 36H, $C(CH_3)_3$), 1.49 – 1.44 (m, 18H, $C(CH_3)_3$), 1.44 – 1.41 (m, 3H, Me), 1.35 (s, 3H, Me). ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.8 (C=O, cytosine), 154.1 ($C_{q\text{-}arom}$, cytosine), 152.0, 149.5 (C=O Boc), 144.6, 144.3 (C-6), 96.7, 96.5 (C-5), 90.0, 89.6, 89.5, 89.4 (C-1'), 85.1, 85.0, 83.5, 83.5, 83.3 ($C(CH_3)_3$), 79.9, 79.9, 79.6, 79.5 (C-4'), 76.0, 75.9, 75.8 (C-2'), 74.7, 74.6, 74.5, 74.5 (C-2''), 71.6, 71.2 (C-3'), 66.8, 66.8, 66.7 (C-1''), 66.5, 66.3, 66.2 (C-3''), 64.5, 64.5, 64.4 (C-5'), 30.5, 30.5 ($C(CH_3)_3$), 27.8, 27.8, 27.8, 27.7 ($C(CH_3)_3$), 26.9, 26.9, 25.4 (Me). ^{31}P NMR (162 MHz, $CDCl_3$) δ 16.91 – 16.82 (m), 16.66 – 16.59 (m), 16.44 – 16.37 (m), 16.01 (d, *J* = 6.7 Hz), 15.83 – 15.75 (m), 15.61 – 15.51 (m), 15.26 (d, *J* = 6.7 Hz). HRMS (ESI) M/Z: $[M+Na]^+$: Calcd for $C_{44}H_{75}N_3O_{20}P_2Na$ 1050.4311; Found 1050.4315.

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