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# Syntheses of inhibitors for the enzyme thymidine 5′-diphosphate-glucose-4,6-dehydratase (EC 4.2.1.46)

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**Abstract**

Inhibitors of the enzyme thymidine 5′-diphosphate-glucose-4,6-dehydratase (EC 4.2.1.46) were synthesised starting from thymidine, 2-deoxy-uridine and -cytidine. The diphosphate moieties have either been replaced by methylene diphosphonate or phosphoryl hydroxyacetic acid to yield inhibitors in a similar range to thymidine 5′-diphosphate itself. Spacers were attached to various positions, of which those at C-5 of the nucleobase were suitable analogues and showed a mixed type of inhibition characteristics, whereas attachment at other sites led to marginally active or inactive inhibitors. These investigations will prepare the ground for developing a purification procedure based on affinity chromatography. 

*Keywords:* dTDP-Glucose-4,6-dehydratase (EC 4.2.1.46); Nucleoside diphosphate; Diphosphate analogues; Spacer-modified nucleobases

## 1. Introduction

The enzymatic synthesis of activated deoxy sugars is still underdeveloped if compared with the vast number of reports on mammalian oligosaccharides. One of the obstacles is the low availability of glycosyl donors, i.e., the nucleoside diphosphosugars and the respective enzymes to produce them. In the past few years cloning techniques have made those gene clusters available, which encode for the biosynthesis of bacterial deoxysaccharide structures [1,2]. We embarked on a programme to develop the synthesis of the nucleoside-diphosphate activated and deoxygenated glycosyl donors starting from relatively easily accessible thymidine 5′-diphosphate-(TDP) glucose derivatives. Preparative syntheses of various intermediates along such enzymatic routes have been previously reported by us and others [2–6].

Early reports on the inhibition of TDP-glucose-4,6-dehydratase by TDP have prompted us to forward the idea of developing analogues of the latter suitable for affinity chromatography. This paper, therefore, will report on the synthesis of such analogues and their suitability to interfere with the substrate, TDP-glucose, in the dehydratase reaction.

## 2. Discussion

As outlined in Fig. 1, five distinct positions can be envisaged for the attachment of a spacer by which the nucleoside can be linked

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onto a solid support. The programme towards the development of suitable affinity materials therefore consists of three goals: (i) the replacement of the diphosphate by a more stable isosteric group, (ii) the attachment of bifunctional spacers, and (iii) the measurement of kinetic parameters of the synthesised N diphosphate analogues in the dehydratase reaction.

Substitution of the bridging oxygen atom in the pyrophosphate by a methylene group yielded a more stable phosphonate (**2**). This was obtained from tosylate **1** by direct nucleophilic replacement with methylene diphosphonate according literature procedures [8,21].

The diphosphate mimic, compound **5**, has been addressed based on the notion that in TDP-glucose only two negative charges are presented to the enzyme's active site. For TDP as an inhibitor, it is reasonable to assume that a third negative charge will only have minor impact in the binding. For the synthesis of **5**, 3′-*O*-benzoyl-thymidine was converted to give phosphoamidite **2**. By acid catalysis, the amidite was substituted by methyl glycolate and subsequently oxidised to yield the corresponding triester **4**. Since both phoshoamidite **2** and triester **4** were found to be prone to hydrolysis, all respective chromatographic steps had to be carried out using anhydrous solvents and dried silica gel. Controlled hydrolysis to give the target compound, phosphoglycolate **5**, was achieved by automatic addition of aqueous LiOH at pH 12.5 and the cleavage was carefully checked for completion by TLC.

After having developed the chemistry for the diphosphate mimics, the attachment of spacers having a chain length of 6–8 carbon atoms to the different positions of the nucleotide (Fig. 1) was next on the agenda. Coupling of Z-protected amino hexanoic acid to 5′-*O*-TBDMS protected thymidine was carried out in analogy to the procedure described by Safarti and co-workers [7] for 3′-*O*-(6-aminohexanoyl)-dTTP. Compound **7** was phosphorylated after O-desilylation and subjected to pyrophosphorylation according to the procedure reported by Davisson et al. [8] to give the target compound, TDP-3′-hexanoate **8** in 53% yield, which could be unequivocally characterised by $^{1}H$ and $^{13}C$ NMR spectroscopy, and FABMS.

It was conceivable that compound **8** or its immobilised counterpart would be sensitive to

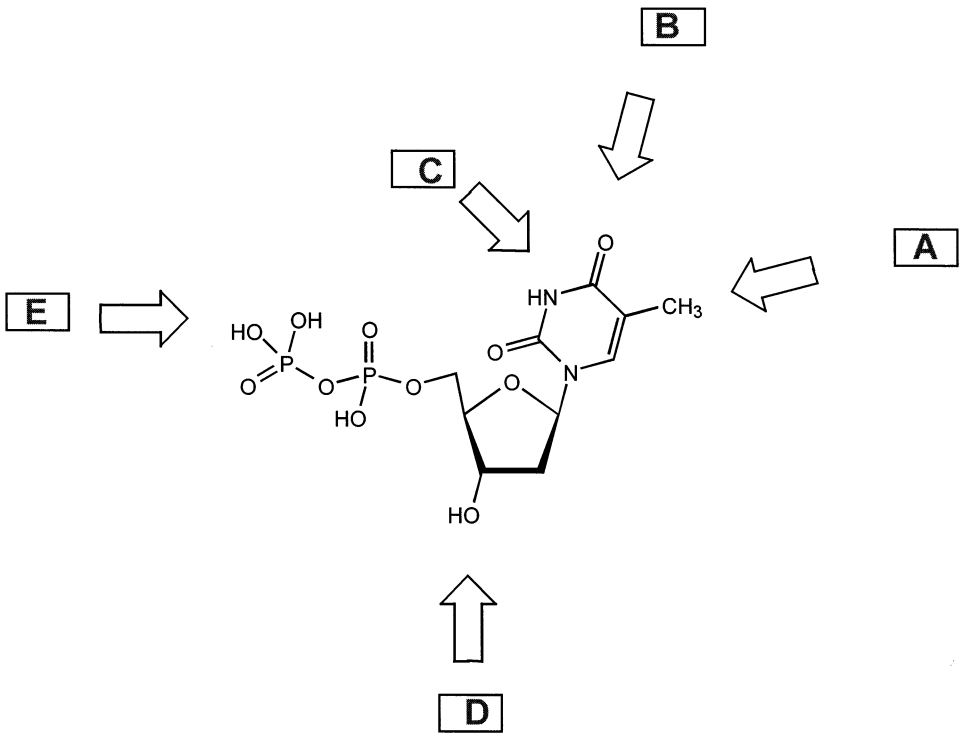
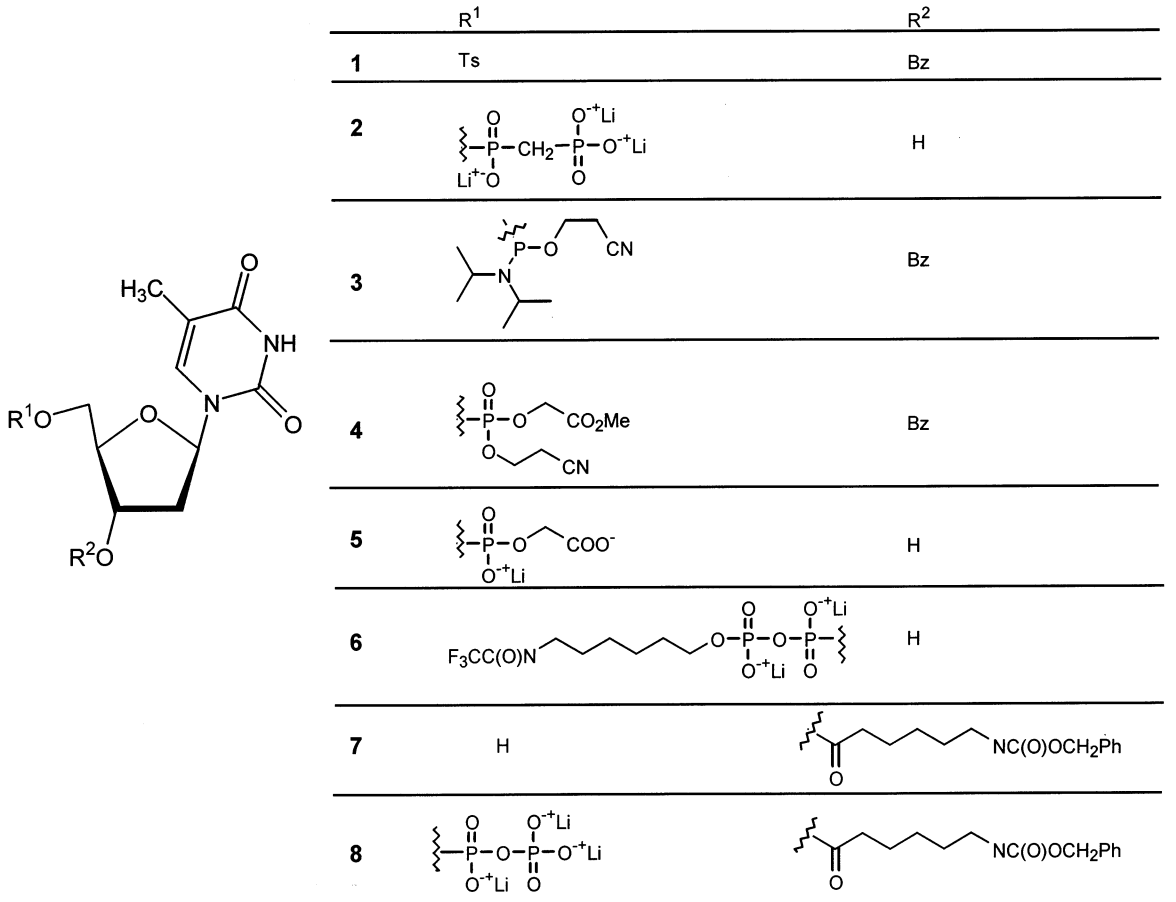



Fig. 1. Nucleotide positions suitable for spacer attachment.




Scheme 1.

saponification due to any residual esterase activity in the protein extracts. A preliminary testing with commercially available lipases (PPL, CCL) indeed confirmed this assumption. Derivative **8** decomposed within 24 h in a 50 mM phosphate buffer (pH 7.0) at 37 °C and therefore showed no advantage over the previously tested TDP-hexanolamine **6** [22] carrying a pyrophosphate linker sensitive to hydrolysis (Scheme 1).

For the stable linkage (type 'A', see Fig. 1), 5-iodouridine was subjected to a Heck reaction. This was carried out in anhydrous triethylamine with 1-trifluoroacetamido-oct-7-in and **11** in the presence of tris(triphenylphosphine) palladium(II) chloride/copper(I) iodide to yield **12** in 85% yield. Upon pyrophosphorylation and de-esterification, the 5′-diphosphate **18** could be isolated in 51% yield.

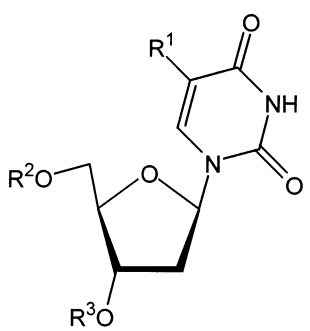
Compound **12** also served as a starting point for the synthesis of diphosphate analogue **16**. Phosphoamidation of **12** was followed by substitution with methyl glycolate and oxidation to furnish triester **15**. This, in turn, was treated similarly to the non-spacer modified compound **4**. Simultaneous cleavage of all three blocking groups, 3-benzoate, cyanoethyl ester, and methyl ester, delivered the deblocked 5-octinyl spacered glycolate mimic **16** in 91% yield with a dU content of 72% (Scheme 2). The structure was fully assigned by NMR spectroscopy, with a single $^{31}P$ NMR signal at $+1.23$ ppm decisive for phophodiesters. The intactness of the ester was underlined by a [M − Li] ESPMS peak at $m/z$ 488.1.

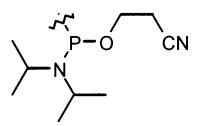
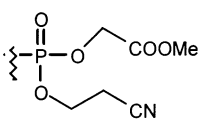
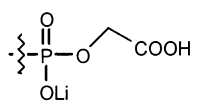
Trifluoroacetamido-1-iodo-hexane [9,10] proved very suitable for the envisaged derivatisation at $N^3$. N-Alkylation of 3′-*O*-benzoylthymidine in *N*,*N*-dimethylformamide, under carefully optimised Purdie conditions to avoid benzoyl migration, gave rise to the spacer-bridged derivative **17**. The site of alkylation was indicated by $^{13}C$ NMR: whereas a derivatisation at positions different from $N^3$ would be indicated by a shift at C-5, the

carbon signals for the nucleobase in **17** remained indistinguishable from literature data [11,12]. However, a value of 40.1 ppm for C-1 of the hexyl spacer confirmed the expected N-alkylation. Diphosphate **18** was obtained after treatment with the tributylammonium pyrophosphate and ester cleavage under controlled conditions in aqueous LiOH at pH 12 in 72% yield (Scheme 3).

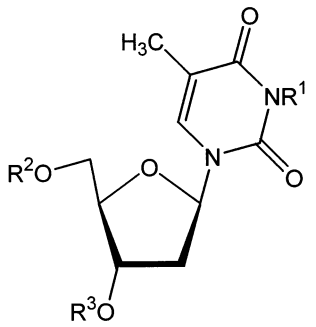
The last derivative that has been the focus of our interest was the attachment of a linker at position 'B' (cf. Fig. 1). As a starting point for the synthesis, 2′-deoxycytidine was chosen. Silylation gave the 5′-*O*-TBDMS ether, which was impossible to purify, but was instead reacted further to yield the bis(silyl) ether according to the 'transient protection method' [13]. The crude compound was, after Mukaiyama activation of the acid and following aqueous work-up, isolated as amide **19**. Benzoylation proceeded smoothly, but purification on silica gel was problematic in that it resulted in ester cleavage. It was conceived that the observed unexpected side reactions for acyl groups at 3′-OH are based on a conformational change in the furanoid ring, which is exerted by the base on the sugar [14]. Compared to dU and dT derivatives, the dC-ribose showed a different coupling pattern for H-1′ and H-3′. The O-desilylated derivative, however, was again stable, and benzoate **20** could be purified after previous cleavage of the 5-*O*-silyl ether. Conversion of the alcohol to give diphosphate **21** went along the standard pyrophosphorylation procedure subsequently followed by hydrazinolysis of the 3-benzoate [15] (Scheme 4).

Synthesised TDP analogues were evaluated in a 50% inhibition test at constant enzyme concentration and under substrate saturation (approx $3–4 \times K_M$). The respective concentrations of TDP analogues were varied between 100 and 2000 μM, and from a plot of $\Delta E$ versus [I] the 50% inhibition value was estimated. From this first set of experiments it became evident that compounds **8**, **18**, and **21** were not accepted at all by the enzyme and therefore the substitution at neither 3′-OH of the ribose nor the attachment of any substituent at a position of the nucleobase other



| | $R^1$ | $R^2$ | $R^3$ |
|---|---|---|---|
| **9:** | I | TBDMS | H |
| **10:** | I | TBDMS | Bz |
| **11:** | H | H | Bz |
| **12:** | $-C\equiv C-(CH_2)_6-NHC(O)CF_3$ | H | Bz |
| **13:** | $-C\equiv C-(CH_2)_6-NH_2$ | $-P(O)(OLi)OP(O)(OLi)_2$ | H |
| **14:** | $-C\equiv C-(CH_2)_6-NHC(O)CF_3$ |  | Bz |
| **15:** | $-C\equiv C-(CH_2)_6-NHC(O)CF_3$ |  | Bz |
| **16:** | $-C\equiv C-(CH_2)_6-NH_2$ |  | H |

Scheme 2.



| | $R^1$ | $R^2$ | $R^3$ |
|---|---|---|---|
| **17:** | $C(O)(CH_2)_5NHC(O)CF_3$ | H | Bz |
| **18:** | $C(O)(CH_2)_5NH_2$ | $-P(O)(OLi)OP(O)(OLi)_2$ | H |

Scheme 3.

than C-5 would yield effective inhibitors (**2**, **5**, **6**, **13**, **16**).

However, all other derivatives were subjected to further evaluation following the standard assaying protocol described earlier [16]. Apart from pyrophosphate, which was a clear uncompetitive inhibitor, all other derivatives showed a mixed type of inhibition. From observed apparent $V_{max}$ and $K_M$ values, $K_i$ and $K_i'$ are defined by the following two equations:

$$(V_{max}^{app}/K_M^{app}) = (V_{max}/K_M)/(1 + [I]/K_i)$$

and

$$V_{max}^{app} = V_{max}/(1 + [I]/K_i')$$

After rearrangement, the values for $K_i$ and $K_i'$ were defined as

$$K_i = [I]/\{(V_{max}^{app}/K_M^{app})/(V_{max}/K_M) - 1\}$$

and

$$K_i' = [I]/(V_{max}^{app}/V_{max}) - 1$$

where $K_i' \to \infty$ for competitive inhibition, $K_i \to \infty$ for uncompetitive inhibition, and $K_i = K_i'$ for non-competitive inhibition.

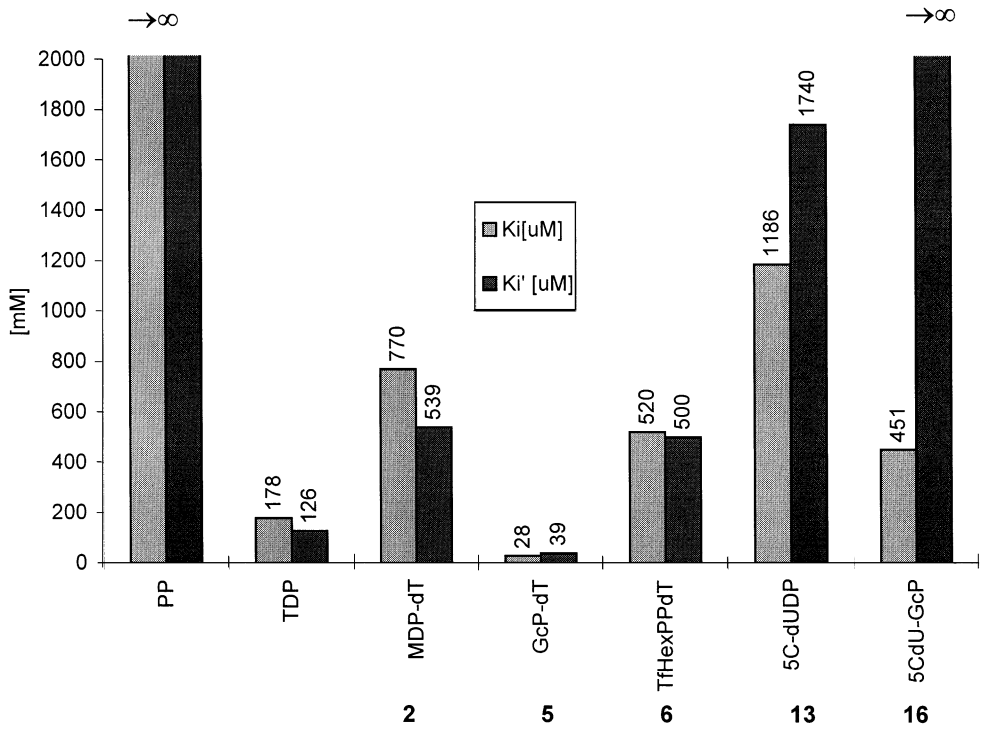
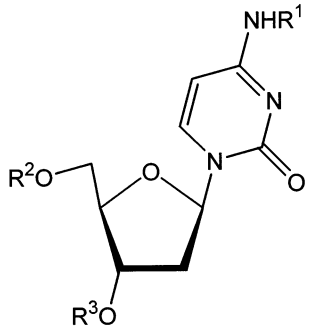



Fig. 2. Competitive ($K_i$) and uncompetitive ($K_i'$) contributions to the mixed inhibition observed for compounds **2**, **5**, **6**, **13**, and **16** (assayed according to Ref. [16]).




| | $R^1$ | $R^2$ | $R^3$ |
|---|---|---|---|
| **19:** | $C(O)(CH_2)_5NHC(O)OBn$ | TBDMS | H |
| **20:** | $C(O)(CH_2)_5NHC(O)OBn$ | H | Bz |
| **21:** | $C(O)(CH_2)_5NHC(O)OBn$ | $-P(O)(OLi)OP(O)(OLi)_2$ | H |

Scheme 4.

Fig. 2 depicts all resulting data for $K_i$ and $K_i'$; bargraphs in grey represent the uncompetitive and those in black the competitive contributions to the mixed inhibition. Values tending to $\infty$ indicate that the dissociation of the enzyme–inhibitor complex prevails and therefore the association is not within the measurable data range.

As explained above, the tested derivatives were not displaying a simple inhibition pattern, but instead they were showing a complex pattern of mixed inhibition. This can be taken as evidence for a sequential, ordered mechanism [17]. Given this case, $K_i$ represents the competition between substrate and inhibitor for the enzyme in the same state, whereas $K_i'$ represents the competition of substrate and inhibitor for the enzyme in different states. As a consequence of the postulated ordered mechanism, the enzyme is yielded in different activation levels after recombination of the apo-enzyme with $NAD^+$ or NADH [18]. A final proof, however, would be the observation of an uncompetitive substrate inhibition, not yet described for the enzyme under investigation. The experiments did underpin such behaviour, since the reaction rate was leveling off after the optimum substrate concentration of 600 μM was exceeded.

Some inhibitors showed at low substrate concentrations between 25 and 100 μM an overestimated reaction rate, which was inversely proportional to the duration of preincubation of enzyme and inhibitor (Table 1). This was taken as a clear indication for a slow formation of the enzyme–inhibitor complex compared with the enzyme–substrate complex.

Conclusively, it turned out that derivatisation at C-5 of the nucleobase was yielding good analogues with values in the same order of magnitude as that of TDP. Furthermore, an interesting avenue has been pursued with the 2-phosphoacetic acid derivatives, which showed even better inhibition properties than the parent compound. On the basis of these results, experiments are underway to develop an affinity chromatography protocol for the facile purification of the title enzyme.

## 3. Experimental

*General procedures*

*5′-Silylation of nucleosides*. *tert*-Butyldimethylchlorosilane (760 mg, 5 mmol, 1.0 equiv) or *tert*-butyldiphenylchlorosilane (1.30 mL, 5 mmol, 1.0 equiv) and imidazole (680 mg, 10 mmol, 2 equiv) were dissolved in anhyd DMF (20 mL), and to the stirred solution the nucleoside (5 mmol) was added. After 3 h at room temperature (rt), the solvent was evaporated in vacuo. Derivatives of thymidine and uridine were isolated after column chromatography, nucleosides furnished with an exocyclic amino group were further protected according to the described transient protection method [13].

*Benzoylation*. The alcohol (5 mmol) was dissolved in anhyd pyridine (10 mL), and stirred with benzoylchloride (0.7 mL, 6 mmol, 1.2 equiv) at rt. Upon completion of the reac-

tion, controlled by TLC to prevent aminoacylation of thymidine and uridine derivatives, the reaction was stopped by evaporation of the solvent in vacuo, the mixture was diluted with $CH_2Cl_2$ (200 mL), and washed three times with a satd aq soln of $NaHCO_3$. The organic layer was dried over $MgSO_4$, filtered and concentrated to leave a raw product, which was purified over $SiO_2$ using the given eluents.

*Desilylation*. The silylated compound (5 mmol) was dissolved in anhyd THF (10 mL) to which TBAF·$3H_2O$ (1.74 g, 5.5 mmol, 1.1 equiv) was added. Depending on the amount of water, reaction times ranged from 1 to 5 h. The solution was evaporated and purified on $SiO_2$ using the specified eluents.

*5′-Tosylation of nucleosides*. The 5′-unblocked nucleoside (1 mmol) was dissolved in $CH_2Cl_2$ (20 mL) together with DMAP (160 mg, 1.3 mmol, 1.3 equiv), and *p*-toluenesulfonyl chloride (230 mg, 1.3 mmol, 1.2 equiv) and stirred for 15 h at rt, before passed over a short $SiO_2$ column (20:1 $CH_2Cl_2$–MeOH). The product fractions were pooled and concentrated, the product was precipitated from $CH_2Cl_2$ with *n*-hexane.

*Nucleoside diphosphates*. Tetrasodium pyrophosphate (5.0 g) was dissolved in warm distilled water (50 mL), and applied to an ion-exchanger column Dowex 50W (pyridinium-form, $4 \times 10$ cm). The eluate was lyophilised and applied to a second ion-exchange step (Dowex 50W, tributylammonium form, equilibrated with MeOH, $2 \times 8$ cm). The eluate was concentrated in vacuo and dried over $P_2O_5$.

The 5′-unblocked nucleoside (1 mmol), previously prepared and dried pyrophosphate (3.0 g, approx 3 equiv), and *N*,*N*′-dicyclohexylcarbodiimide (2.06 g, 10 mmol, 10 equiv) were dissolved in anhyd pyridine (20 mL) and stirred for 5 days at rt. After evaporation of the solvent, the residue was taken up at 0 °C in aq LiOH (0.1 N, 10 mL) and the suspension was quickly adjusted to pH 8, before filtered over a Büchner funnel. The precipitate was washed with 1:1 water–MeOH, and the filtrate was concentrated and finally lyophilised.

N-Detrifluoroacetylation and 3′-*O*-debenzoylation were achieved quantitatively by saponification with aq LiOH at pH 12 in 2 h. The resulting solutions were directly fractionated over Sephadex G10.

The $N^4$-acylated deoxycytidine derivative **20** was dissolved in anhyd DMF (20 mL) to which hydrazine hydrate (150 μL, 3 mmol, 3 equiv) was added. After 15 h, the solvent was evaporated ($T < 30$ °C) and the residue was subjected to Sephadex G10 chromatography.

Phosphates were purified by chromatography over Dowex $2 \times 8$ ($Cl^-$-form) and eluted by a linear gradient $0 \rightarrow 0.8$ M LiCl. Product-containing fractions are pooled, lyophilised and desalted by passing twice over Sephadex G10. In cases where the counterion has not been exchanged completely, the TDP derivatives were passed additionally over Dowex 50W ($Li^+$-form).

*Estimation of 50% inhibition*. According to Ref. [18], TDP-4-ketoglucose is measured photometrically at constant enzyme concentration under substrate saturation ($3–4 \times K_M$). The respective TDP analogue was added in concentrations between 100 and 2000 μM. Buffer: Tris–HCl (50 mM, pH 7.6), EDTA (2 mM). Substrate: TDP-glucose (Sigma, 14.6 mg) dissolved in bidistilled water (1 mL), resulting in an assay concentration of 0.4 mM, equivalent to $4K_M$. Enzyme: 4,6-dehydratase (3–5 mU). Inhibitor: the respective TDP analogue (15 mg) was dissolved in bidistilled water (1 mL) and the thymidine content was estimated photometrically by comparison with a TMP standard solution.

The pipetting scheme is shown in Table 2.

The concentration at which the enzyme activity had dropped to 50% was read directly from a $\Delta E$ versus [I] plot.

*Inhibition constants*. At given enzyme and inhibitor concentrations, the turnover to give TDP-6-deoxy-4-keto-glucose was estimated for substrate concentrations between 25 and

Table 1
Reaction rates of inhibitors as a function of preincubation time

| Preincubation (min) | blank | 0 | 5 | 15 |
|---|---|---|---|---|
| μmol product/min mL | 0.003 | 0.0018 | 0.0014 | 0.0008 |

Table 2
Pipetting scheme for estimating 50% inhibition

| Step | Assay soln (μL) | Blind soln |
|---|---|---|
| Tris–buffer | 480 − X | 480 − X |
| Inhibitor | X | X |
| TDP-Glc soln | 10 | 10 |
| Incubate for 1 min at 37 °C | | |
| NaOH (0.2 N) | | 500 |
| Enzyme soln | 10 | 10 |
| Incubate for 15 min at 37 °C | | |
| NaOH (0.2 N) | 500 | |
| Incubate for 15 min at 37 °C; measure extinction against blind control | | |

Table 3
Pipetting scheme for determining inhibition constants

| Step | Assay soln (μL) | Sample aliquot |
|---|---|---|
| Tris–buffer | 1990–I–S | |
| Inhibitor | I | |
| TDP-Glc soln | S | |
| Incubate for 1 min at 37 °C | | |
| Enzyme soln | 10 | |
| Mix, take sample for control and incubate at 37 °C | | |
| Take aliquot | | 200 |
| NaOH (0.1 N) | | 800 |
| Incubate for 15 min at 37 °C; measure extinction against blind control at 318 nm | | |

1000 μM (approx 0.5–10 × $K_M$). Buffer: Tris–HCl (50 mM, pH 7.6), EDTA (2 mM). Substrate: TDP-glucose (Sigma, 14.6 mg) dissolved in bidistilled water (1 mL); per assay (2 mL) were used 100 μL (1000 μM), 40 μL (400 μM), 20 μL (200 μM), 10 μL (100 μM), 5 μL (50 μM), 2.5 μL (25 μM). Enzyme: 4,6-dehydratase (10–15 mU each, similar batches). Inhibitor: the respective TDP analogue (15 mg) was dissolved in bidistilled water (1 mL) and the thymidin content was estimated photometrically at 267 nm (or 270 and 290 nm, respectively) by comparison with a TMP standard solution. Inhibitor concentrations were: pyrophosphate (2000 μM), TDP (250 μM), **3** (1000 μM), **5** (100 μM), **6** (500 μM), **13** (1000 μM), **16** (500 μM).

The pipetting scheme is shown in Table 3.

Control: subsequently upon addition of enzyme solution, the control is quenched by addition of NaOH.

Molar extinction for enolised ulose $\varepsilon_{318} = 4800$ cm$^2$/mmol. Product concentration in assay solution (200 μL aliquot) was $c_{prod} = (\Delta E \times 1.042)$ μmol/mL. From a [prod] versus $t$ plot, initial rates were determined and the respective constants were estimated by hyperbolic regression analysis according to the Marquart algorithm [19].

*Thymidine 5′-dilithiummethylenediphosphonate* (**2**).—Methylene diphosphonic acid (1.0 g, 5.6 mmol) was dissolved in 1:1 pyridine–water (3 mL), and passed over a Dowex 50W ion-exchange column (tributylammonium form, 2 × 8 cm, previously equilibrated over MeOH). The filtrate was concentrated, the residue dried over $P_2O_5$ in vacuo. 3′-*O*-Benzoyl-5′-*O*-*p*-toluenesulfonyl-thymidine (**1**, 500 mg, 0.5 mmol) [20] and the previously prepared phosphonate (1.50 g, approx 1.5 equiv) were suspended in anhyd DMF (5 mL), and stirred for 36 h at 80 °C under an Ar atmosphere. After concentration in vacuo, the residue was dissolved in ice-cold aq LiOH (0.5 N, 10 mL), and further LiOH was added at pH 12 to saponify the benzoate completely. The resulting solution was directly fractionated on a Sephadex G10 column, the product-containing fractions were further purified by anion-exchange chromatography, as described for nucleoside diphosphates under the general procedures (vide supra). After final lyophilisation, **2** (460 mg, 57%, photometrically determined dT content: 52%) was obtained as its dilithium salt. $^1$H NMR and $R_f$ were in agreement with the specifications given in Ref. [21]. $^{13}$C NMR (100 MHz, $D_2O$): $\delta$ 162.7 (C-4), 154.2 (C-2), 139.9 (C-6), 114.2 (C-5), 87.8 (d, C-4′), 87.4 (C-1′), 73.4 (C-3′), 66.2 (d, C-5′), 40.8 (C-2′), 31.3 (t, P–$CH_2$–P), 14.1 ($CH_3$); $J_{P,C,P}$ 119.0, $J_{4',P}$ 8.6, $J_{5',P}$ 5.7 Hz. FABMS$^-$: $m/z$ 399.2 $[M^{3-} + 2H^+]^-$.

*3′-O-Benzoyl-thymidine 5′-[(2-cyanoethyl)-N,N-diisopropyl] phosphoramidite* (**3**).—Compound **1** (1.18 g, 3.25 mmol) was dissolved in anhyd MeCN (20 mL); under an Ar atmosphere *N*,*N*-diisopropylethylamine (1.08 mL, 6.34 mmol, 1.95 equiv) and 2-cyanoethoxy-*N*,*N*-diisopropylchlorophosphoamidite (1.0 g, 4.22 mmol, 1.3 equiv) were added. After stirring for 30 min, the solvent was removed under reduced pressure and the resulting

residue was purified over previously dried $SiO_2$ (3:1 anhyd toluene–anhyd acetone). The diastereomeric phosphites **3** were obtained in equal amounts (1.43 g, 81%); $^1$H NMR (400 MHz, acetone-$d_6$): $\delta$ 8.09 (d, 2 H, *o*-Bz), 7.79 (s, H-6), 7.67 (m, *p*-Bz), 7.55 (m, 2 H, *m*-Bz), 6.48 and 6.41 (dd, each 1/2 H, H-1′), 5.65 and 5.62 (m, each 1/2 H, H-3′), 4.43 and 4.41 (m, each 1/2 H, H-4′), 4.13–3.90 (m, 4 H, H-5a′, 5b′, P(O)C$H_2$), 3.77–3.68 (m, 2 H, 2 isopropyl-C*H*), 2.83–2.79 (m, 2 H, C$H_2$CN), 2.63–2.43 (m, 2 H, H-2a′, 2b′), 1.91 and 1.90 (s, each 3/2 H, C$H_3$), 1.27–1.22 (m, 12 H, 4 isopropyl-C$H_3$); $^{13}$C NMR (100 MHz, acetone-$d_6$): $\delta$ 166.0 (*C*=O), 163.7 (C-4), 150.8 (C-2), 135.8/135.6 (C-6), 13.8 (*p*-Bz), 130.3 (Bz), 129.9 (2 C, *o*-Bz), 129.0 (2 C, *m*-Bz), 118.4 (*C*N), 110.8 and 110.6 (each C-5), 85.2 and 84.9 (C-1′), 84.4 and 84.3 (each d, each C-4′), 76.4 and 76.1 (each C-3′), 64.6 and 64.4 (each d, each C-5′), 59.7 and 59.5 (each d, each PO*C*$H_2$), 43.5 (d, PN*C*H), 37.8 and 37.5 (each C-2′), 24.5 and 24.4 (2 s, NCH(*C*$H_3$)$_2$), 20.3 (*C*$H_2$CN), 12.2 (*C*$H_3$); $J_{4',P}$ 9.6, $J_{5',P}$ 4.3, $^2J_{POCH}$ 20.9, $^3J_{CHCN,P}$ 6.0, $^2J_{PNCH}$ 12.4 Hz.

*3′-O-Benzoyl-thymidine 5′-(2-cyanoethyl)-methoxycarbonylmethyl phosphate* (**4**).—Compound **3** (1.32 g, 2.41 mmol) and methyl glycolate (600 μL, 7.8 mmol, 3 equiv) were dissolved in anhyd MeCN (20 mL) under an Ar atmosphere. After the addition of 1-H tetrazole (340 mg, 4.83 mmol, 2 equiv) the mixture was stirred for 30 min, before *tert*-butylyhydroperoxide (70% in water, 850 μL, 3 equiv) was added. After a further 60 min, the solution was concentrated under reduced pressure ($T < 30$ °C) and the residue was purified by chromatography over previously dried $SiO_2$ (1:1 anhyd toluene–acetone) to yield **4** (1.06 g, 80%); m.p. 60 °C; $^1$H NMR (400 MHz, acetone-$d_6$, diastereoisomers are indistinguishable): $\delta$ 8.09 (d, 2 H, *o*-Bz), 7.70–7.65 (m, 3 H, H-6, *p*-Bz), 7.54 (m, 2 H, *m*-Bz), 6.46 (dd, H-1′), 5.64 (m, H-3′), 4.75 (dd, 2 H, C$H_2$-glycolate), 4.54 (m, 2 H, H-5a′, 5b′), 4.49 (m, H-4′), 4.46–4.39 (m, 2 H, PO*C*$H_2$), 3.75 (d, 3 H, $H_3$COC(O)), 3.01–2.96 (m, 2 H, C$H_2$CN), 2.66–2.53 (m, 2 H, H-2a′, 2b′), 1.88 (s, 3 H, C$H_3$); $^{13}$C NMR (100 MHz, acetone-$d_6$): $\delta$ 168.8 (d, C(O)OMe), 165.9 (C=O), 163.6 (C-4), 150.8 (C-2), 135.8 (C-6), 133.8 (*p*-Bz), 130.2 (Bz), 130.0 (2 C, *o*-Bz), 129.0 (2 C, *m*-Bz), 117.6 (*C*N), 111.0 (C-5), 85.1 (C-1′), 82.9 (d, C-4′), 75.3 (C-3′), 68.0 (d, C-5′), 64.0 (d, $H_2CC$(O)O$CH_3$), 63.3 (d, PO*C*$H_2$), 52.1 (C(O)O*C*$H_3$), 37.0 (C-2′), 19.5 (d, *C*$H_2$CN), 12.0 (*C*$H_3$); $J_{4',P}$ 7.6, $J_{5',P}$ 5.6, $^2J_{CH_2COOMe,P}$ 5.4, $^3J_{COOMe,\ P}$ 4.8, $^2J_{POCH}$ 4.8, $^3J_{CH_2CN,P}$ 8.2 Hz; $^{31}$P NMR (81 MHz, acetone-$d_6$): $\delta$ +0.05, +0.01. Anal. Calcd for $C_{23}H_{26}N_3O_{11}P$ (515.46): C, 50.10; H, 4.75; N, 7.62; P, 5.62. Found C, 49.72; H, 4.84; N, 7.89; P, 5.51.

*Thymidine 5′-monolithium carboxymethylphosphate* (**5**).—Compound **4** (0.95 g, 1.72 mmol) [dissolved in 3:1 EtOH–water (10 mL) and aq LiOH (0.5 N)] was constantly added at rt by means of an automatic titrator (pH 12.5). The reaction was controlled by TLC (5:3:2 2-propanol–EtOH–water, 5% triethylamine, 2% HOAc). The resulting solution was directly fractionated by Sephadex G10 chromatography; product-containing fractions were additionally desalted over Sephadex G10 or Biogel P2 to yield **5** (870 mg, 93%, photometrically determined dT content: 72%); $^1$H NMR (400 MHz, $D_2O$): $\delta$ 7.90 (s, H-6), 6.52 (dd, H-1′), 4.74 (m, H-3′), 4.42 (d, 2 H, *C*$H_2$COOH), 4.34 (m, H-4′), 4.28 (m, 2 H, H-5a′, 5b′), 2.56–2.52 (m, 2 H, H-2a′, 2b′), 2.10 (s, 3 H, $CH_3$); $^{13}$C NMR (100 MHz, $D_2O$): $\delta$ 176.5 (d, *C*OOH), 167.0 (C-4), 152.2 (C-2), 137.7 (C-6), 112.2 (C-5), 85.8 (d, C-4′), 85.4 (C-1′), 71.4 (C-3′), 65.5 (d, C-5′), 64.2 (d, O*C*$H_2$COOH), 39.1 (C-2′), 12.0 ($CH_3$); $^3J_{COOH,P}$ 7.7, $^2J_{POCH_2C(O)}$ 5.7, $J_{4',P}$ 9.2, $J_{5',P}$ 5.2 Hz. ESIMS$^-$ (4.5 kV): $m/z$ 379.2 $[M^{2-} + H^+]^-$.

*Thymidine 5′-dilithium-(6-*N*-trifluoroacetamidohexyl) diphosphate* (**6**).—6-Trifluoroacetamido-hexyl-1-phosphate [22] (1.0 g, approx 1.5 mmol, approx 2 equiv) was co-evaporated with anhyd pyridine to remove traces of water, then dissolved in anhyd MeCN (10 mL) and stirred for 2 days at rt with thymidine-5′-monophosphomorpholidate (commercially available dicyclohexylurea salt, 500 mg, 0.73 mmol). The solvent was distilled off under reduced pressure, the residue was then dissolved in aq LiOH (0.1 N, 10 mL) at 0 °C and quickly adjusted with further LiOH to pH 8.0. The phosphates were separated by anion-exchange chromatography (Dowex 2×

8, $Cl^-$-form) and eluted by a linear LiCl-gradient (0 → 0.8 M). Product-containing fractions were pooled, lyophilised and desalted by passing twice over Sephadex G10. Product **6** (360 mg, 55%, photometrically determined dT content: 68%) was obtained after final lyophilisation; $^1$H NMR (400 MHz, $D_2O$): $\delta$ 7.94 (s, H-6), 6.52 (dd, H-1′), 4.78 (m, H-3′), 4.37–4.32 (m, 3 H, H-4′, H-5a′, 5b′), 4.08 (dt, H-1″), 3.46 (t, H-6″), 2.56–2.50 (m, 2 H, H-2a′, 2b′), 2.10 (s, 3 H, $CH_3$), 1.77 (m, 2 H, H-2″), 1.71 (m, 2 H, H-5″), 1.53–1.46 (m, 4 H, H-3″, H-4″); $^{13}$C NMR (100 MHz, $D_2O$): $\delta$ 166.9 (C-4), 151.1 (C-2), 137.9 (C-6), 112.2 (C-5), 85.9 (d, C-4′), 85.3 (C-1′), 71.3 (C-3′), 67.0 (d, C-1″), 65.7 (d, C-5′), 40.2 (C-6″), 39.1 (C-2′), 30.1 (d, C-2″), 28.0 (C-5″), 26.0 (C-4″), 24.9 (C-3″), 12.1 (C-6″); $J_{1'',P}$ 6.1, $J_{2'',P}$ 7.1, $J_{5',P}$ 6.1, $J_{4',P}$ 10.2 Hz. ESPMS$^-$ (4.5 kV): $m/z$ 596.0 $[M^{2-} + H^+]^-$.

*3′-O-(6″-N-Benzyloxycarbonylamino)-hexanoyl)-thymidine 5′-trilithiumdiphosphate* (**8**).—Compound **7** [7] (200 mg, 0.40 mmol) was pyrophosphorylated according to the general procedures to yield **8** (250 mg, 53%, photometrically determined dT content: 56%); $^1$H NMR (400 MHz, $D_2O$): $\delta$ 7.66 (s, H-6), 7.38–7.29 (m, 5 H, Bn), 6.26 (dd, H-1′), 5.40 (m, H-3′), 5.02 (s, 2 H, Bn–C$H_2$), 4.34 (m, H-4′), 4.18 (m, 2 H, H-5a′, 5b′), 2.93 (m, H-6″), 2.42–2.31 (m, 4 H, H-2a′, 2b′, H-2″), 1.87 (s, 3 H, C$H_3$), 1.47 (m, 2 H, H-3″), 1.27 (m, 2 H, H-5″), 1.10 (m, 2 H, H-4″); $^{13}$C NMR (100 MHz, $D_2O$): $\delta$ 176.4 (*C*(O)-1″), 164.8 (C-4), 155.2 (N*C*(O)O), 152.1 (C-2), 137.5 (C-6), 136.8, 129.1, and 127.8 (Bn), 112.4 (C-5), 85.2 (C-1′), 83.5 (C-4′), 76.2 (C-3′), 67.1 (Bn–*C*$H_2$), 66.5 (d, C-5′), 40.6 (C-6″), 36.9 (C-2′), 34.1 (C-2″), 28.8 (C-5″), 26.6 (C-4″), 24.3 (C-3″), 12.1 ($CH_3$); $J_{4',P}$ 8.6, $J_{5',P}$ 4.8 Hz. FABMS$^-$: $m/z$ 647.3 $[M^{3-} + 2H^+]^-$.

*3′-O-Benzoyl-5′-O*-tert-*butyldimethylsilyl-2′-deoxy-5-iodouridine* (**10**).—5-Iodouridine **9** [23] (920 mg, 1.96 mmol) was benzoylated according to the general procedure and purified by $SiO_2$ chromatography (2:1 toluene–EtOAc) to give **10** (880 mg, 78%); m.p. 223 °C; $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 8.81 (br s, NH), 8.17 (s, H-6), 8.03, 7.58, 7.45 (d, m and m, 2 H, 1 H and 2 H, Bz), 6.39 (dd, H-1′), 5.49 (m, H-3′), 4.29 (m, H-4′), 4.02 (dd, H-5a′), 3.97 (dd, H-5b′), 2.65 (ddd, H-2a′), 2.21 (H-2b′), 0.95 (s, 9 H, SiC(C$H_3$)$_3$), 0.20 and 0.19 (each s, 6 H, Si(C$H_3$)$_2$); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ 166.2 (C=O), 159.8 (C-4), 149.9 (C-2), 144.1 (C-6), 133.6, 129.7, 129.2 and 128.6 (Bz), 86.2 (C-4′), 85.7 (C-1′), 76.2 (C-3′), 68.9 (C-5), 63.7 (C-5′), 38.8 (C-2′), 26.2 ((H$_3$C)$_3$)*C*Si), 18.5 ((H$_3$*C*)$_3$)CSi), −5.0 and −5.2 ((H$_3$*C*)$_2$Si). Anal. Calcd for $C_{22}H_{29}IN_2O_6Si$ (572.48): C, 46.16; H, 5.11; I, 22.17; N, 4.89. Found C, 46.03; H, 5.18; I, 21.99; N, 4.88.

*3′-O-Benzoyl-2′-deoxy-5-iodouridine* (**11**).—Compound **10** (720 mg, 1.26 mmol) was desilylated according to the described procedure (vide supra) and purified by $SiO_2$ chromatography (1:1 toluene–EtOAc) to give **11** (540 mg, 94%); m.p. 195 °C, Ref. [24] 183–185 °C; $^1$H NMR data were in accordance with Ref. [24]; $^{13}$C NMR (100 MHz, acetone-$d_6$): $\delta$ 165.9 (CO–Bz), 160.1 (C-4), 150.5 (C-2), 145.7 (C-6), 133.7 (*p*-Bz), 130.3 (q-Bz), 129.9 (2 C, *o*-Bz), 129.0 (2 C, *m*-Bz), 86.1 (C-4′), 85.9 (C-3′), 68.4 (C-5), 62.4 (C-5′), 38.4 (C-2′).

*3′-O-Benzoyl-2′-deoxy-5-(8-*N*-trifluoroacetamido-oct-1-ynyl)uridine* (**12**).—Previously prepared 5-iodouridine **11** (540 mg, 1.18 mmol), 1-trifluoroacetamido-oct-7-yn (340 mg, 1.53 mmol, 1.3 equiv), tris(triphenylphosphine)-palladium(II) chloride (21 mg, 0.03 mmol, 0.025 equiv), and anhyd copper(I) iodide (23 mg, 0.12 mmol, 0.1 equiv, 4:1 Cu–Pd) were dissolved in anhyd triethylamine (20 mL). The mixture was stirred under an Ar atmosphere for 2 h at 50 °C, then concentrated under reduced pressure and co-distilled with toluene. The residue was quenched with satd aq $NaHCO_3$ (10 mL), and extracted twice with $CH_2Cl_2$ (50 mL each). The organic layer was dried over $MgSO_4$, concentrated and the residue was purified by $SiO_2$ chromatography (1:1 petroleum ether–acetone). Product containing fractions were pooled, concentrated and crystallised from THF–*n*-hexane to yield **12** (550 mg, 85%); m.p. 63 °C; $^1$H NMR (400 MHz, acetone-$d_6$): $\delta$ 10.22 (br s, NH), 8.41 (br s NH), 8.25 (s, H-6), 8.09, 7.67, and 7.55 (d, m, and m, 5 H, Bz), 6.42 (dd, H-1′), 5.64 (m, H-3′), 4.55 (br s, HO-5′), 4.33 (m, H-4′), 3.99 (dd, H-5a′), 3.92 (dd, H-5b′), 3.35 (m, 2 H, H-8″), 2.65–2.47 (m, 2 H, H-2a′, 2b′), 2.38 (m,

2 H, H-3″), 1.63 (m, 2 H, H-7″), 1.56 (m, 2 H, H-4″), 1.49 (m, 2 H, H-5″), 1.41 (m, 2 H, H-6″); $^{13}$C NMR (100 MHz, acetone-$d_6$): $\delta$ 166.4 (C=O), 62.2 (C-4), 150.5 (C-2), 43.2 (C-6), 134.3, 130.9, 130.4, and 129.5 (Bz), 101.2 (C-5), 94.0 (C-2″), 86.5 (C-4′), 86.3 (C-1′), 76.6 (C-3′), 73.4 (C-1″), 62.9 (C-5′), 40.4 (C-8″), 38.8 (C-2′), 29.4 (C-7″), 29.2 (C-4″), 29.0 (C-5″), 26.9 (C-6″), 19.8 (C-3″). Anal. Calcd for $C_{26}H_{28}F_3N_3O_7$ (551.52): C, 56.62; H, 5.12; F, 10.33; N, 7.62. Found C, 56.91; H, 5.12; F, 10.74; N, 7.63.

*2′-Deoxy-5-(8-aminooct-1-ynyl)uridine 5′-trilithiumdiphosphate* (**13**).—According to the general procedures, compound **12** was pyrophosphorylated to yield **13** (290 mg, 51%, photometrically specified dU content: 40%); UV (200–400 nm): 217 (min), 232 (max), 259 (min), 291 (max); $^1$H NMR (400 MHz, $D_2O$): $\delta$ 8.14 (s, H-6), 6.42 (dd, H-1′), 4.79 (m, H-3′), 4.39–4.28 (m, 3 H, H-4′, H-5a′, 5b′), 3.14 (m, 2 H, H-8″), 2.60–2.45 (m, 4 H, H-3″, H-2a′, 2b′), 1.86 (m, 2 H, H-7″), 1.73 (m, 2 H, H-4″), 1.66 (m, 2 H, H-5″), 1.58 (m, 2 H, H-6″); $^{13}$C NMR (100 MHz, $D_2O$): $\delta$ 160.5 (C-4), 156.7 (C-2), 143.8 (C-6), 103.3 (C-5), 97.3 (C-2″), 85.9 (d, C-4′), 85.8 (C-1′), 74.5 (C-1″), 70.8 (C-3′), 65.2 (C-5′), 40.2 (C-8″), 39.8 (C-2′), 27.7 (C-5″), 27.6 (C-4″), 26.9 (C-7″), 25.3 (C-6″), 18.5 (C-3″); $J_{4',P}$ 8.5, $J_{5',P}$ 4.8 Hz. FABMS$^-$: $m/z$ 510.3 $[M^{3-} + 2H^+]^-$.

*3′-O-Benzoyl-2′-deoxy-5-(8-trifluoracetamidooct-1-ynyl)uridine 5′-[(2-cyanoethyl)*-N,N-*diisopropyl] phosphoramidite* (**14**).—Compound **12** (500 mg, 0.91 mmol) was dissolved in anhyd MeCN (10 mL), and *N*,*N*-diisopropylethylamine (310 μL, 1.77 mmol, 1.95 equiv) and 2-cyanoethoxy-*N*,*N*-diisopropylchlorophosphoamidite (300 mg, 1.18 mmol, 1.3 equiv) were added under an Ar atmosphere. After stirring for 30 min, the solvent was removed under reduced pressure and the resulting residue was purified over previously dried $SiO_2$ (4:1 anhyd toluene–anhyd acetone). The diastereomeric phosphites **14** (590 mg, 86%) were obtained as a clear syrup. Pairs of diastereomeric signals had an integration ratio of 2:3 in $^1$H NMR; $^1$H NMR (400 MHz, acetone-$d_6$): $\delta$ 10.11 (br s, NH), 8.39 (br s, NH), 8.09, 7.68, and 7.55 ($C_6H_5$), 8.06 and 7.99 (each s, H-6), 6.43 and 6.37 (each dd, H-1′), 5.66 and 5.61 (m, H-3′), 4.48 and 4.45 (m, H-4′), 4.18-3.92 (m, 4 H, POC$H_2$, H-5a′, 5b′), 3.78-3.69 (m, 2 H, N(C$H$(C$H_3$)$_2$)$_2$), 3.35 (m, 2 H, H-8″), 2.85–2.76 (m, 2 H, C$H_2$CN), 2.65 (dd, H-2a′), 2.50 (ddd, H-2b′), 2.41 (m, 2 H, H-3″), 1.65–1.56 (m, 4 H, H-7″, H-4″), 1.51 (m, 2 H, H-5″), 1.42 (m, 2 H, H-6″), 1.26–1.24 (m, 12 H, N(CH(C$H_3$)$_2$)$_2$); $^{13}$C NMR (100 MHz, acetone-$d_6$): $\delta$ 166.4 ($C$(O)$C_6H_5$), 162.6 (C-4), 151.3 (C-2), 142.2 (C-6), 134.2, 130.6, 130.4, and 129.4 ($C_6H_5$), 118.7 ($C$N), 101.2 (C-5), 94.4 (C-2″), 85.6 and 85.5 (C-1′), 84.9 and 84.8 (each d, C-4′), 76.2 (C-3′), 73.4 (C-1″), 64.3 and 64.0 (each d, C-5′), 59.7 (d, PO$C$H$_2$), 43.9 (d, PN($C$H(CH$_3$)$_2$)$_2$), 39.7 (C-8″), 38.4 and 38.1 (C-2′), 28.9 (C-7″), 28.8 (C-4″), 28.6 (C-5″), 26.4 (C-6″), 25.0 and 24.9 (N($C$H($C$H$_3$)$_2$)$_2$), 20.3 (d, $C$H$_2$CN), 19.4 (C-3″); $J_{4',P}$ 8.4, $J_{5',P}$ 5.0, $^2J_{POCH}$ 20.2, $^3J_{CHCN,P}$ 7.9, $^2J_{PNCH}$ 12.2 Hz; $^{31}$P NMR (81 MHz, acetone-$d_6$): $\delta$ +149.74, +149.59. Anal. Calcd for $C_{35}H_{45}F_3N_5O_8P$ (751.75): C, 55.92; H, 6.03; F, 7.58; N, 9.32; P, 4.12. Found C, 55.78; H, 6.22; N, 9.11.

*3′-O-Benzoyl-2′-deoxy-5-(8-trifluoroacetamidooct-1-ynyl)uridine 5′-(2-cyanoethoxy)-methoxycarbonylmethyl phosphate* (**15**).—Compound **14** (420 mg, 0.56 mmol) and methyl glycolate (150 μL, 1.95 mmol, >3 equiv) were dissolved in anhyd MeCN (20 mL) under an Ar atmosphere. After the addition of 1-H tetrazole (80 mg, 1.14 mmol, 2 equiv), the mixture was stirred for 30 min, before *tert*-butylhydroperoxide (70% in water, 200 μL, >3 equiv) were added. After a further 60 min, the solution was concentrated under reduced pressure ($T < 30$ °C) and the residue was purified by chromatography over previously dried $SiO_2$ (2:1 anhyd toluene–acetone) to yield **15** (350 mg, 83%); m.p. 143 °C; $^1$H NMR (400 MHz, acetone-$d_6$, diastereoisomers were indistinguishable): $\delta$ 8.45 (br s, NH), 8.09, 7.68, and 7.55 ($C_6H_5$), 7.96 (s, H-6), 6.41 (dd, H-1′), 5.66 (m, H-3′), 4.78 (dd, 2 H, C$H_2$-glycolate), 4.57 (m, 2 H, H-5a′, 5b′), 4.48–4.40 (m, 4 H, H-4′, POC$H_2$), 3.77 (d, 3 H, $H_3$COC(O)), 3.35 (m, 2 H, H-8″), 3.03–2.93 (m, 2 H, C$H_2$CN), 2.71–2.54 (m, 2 H, H-2a′, 2b′), 2.39 (m, 2 H, H-3″), 1.66–1.54 (m, 4 H, H-7″, H-4″), 1.49 (m, 2 H, H-5″), 1.38

(m, 2 H, H-6″); $^{13}$C NMR (100 MHz, acetone-$d_6$): $\delta$ 168.7 (d, $C$(O)OCH$_3$), 165.9 ($C$(O)C$_5$H$_6$), 161.7 (C-4), 149.9 (C-2), 142.2 (C-6), 133.9, 130.1, 130.0, and 129.0 ($C_6$H$_5$), 117.6 ($C$N), 100.9 (C-5), 94.0 (C-2″), 85.9 (C-1′), 83.1 (d, C-4′), 75.2 (C-3′), 72.6 (C-1″), 67.9 (d, C-5′), 64.1 (d, H$_2C$C(O)OCH$_3$), 63.4 (d, PO$C$H$_2$), 52.5 (C(O)O$C$H$_3$), 39.8 (C-8″), 37.4 (C-2′), 28.9 (C-7″), 28.7 (C-4″), 28.5 (C-5″), 26.4 (C-6″), 19.4 (d, $C$H$_2$CN), 19.3 (C-3″); $J_{4',P}$ 7.3, $J_{5',P}$ 5.1, $^2J_{CH_2COOMe,P}$ 5.5, $^3J_{COOMe,P}$ 6.5, $^2J_{POCH}$ 5.3, $^3J_{CH_2CN,P}$ 6.9 Hz; $^{31}$P NMR (81 MHz, acetone-$d_6$): $\delta$ 0.05, 0.16. Anal. Calcd for $C_{32}H_{36}F_3N_4O_{12}P$ (756.63): C, 50.80; H, 4.80; F, 7.53; N, 7.40; P, 4.09. Found C, 50.45; H, 4.95; N, 7.76.

*2′-Deoxyuridine 5′-monolithium-methoxycarbonylmethyl phosphate* (**16**).—Compound **15** (300 mg, 0.40 mmol) was dissolved in 3:1 2-propanol–water (10 mL) and aq LiOH (0.5 N) was constantly added at rt by means of an automatic titrator (pH 12.5). The reaction was controlled by TLC (5:3:2 2-propanol–EtOH–water, 5% triethylamine, 2% HOAc). The resulting solution was directly fractionated by Sephadex G10 chromatography, product containing fractions were additionally desalted over Sephadex G10 or Biogel P2 to yield **16** (250 mg, 91%, photometrically determined dU content: 72%); UV (200–400 nm): 217 (min), 233 (max), 259 (min), 291 (max); $^1$H NMR (400 MHz, D$_2$O): $\delta$ 7.91 (s, H-6), 6.29 (dd, H-1′), 4.49 (m, H-3′), 4.22 (dd, 2 H, $C$H$_2$COOH), 4.12 (m, H-4′), 4.05 (m, 2 H, H-5a′, 5b′), 2.96 (m, 2 H, H-8″), 2.38 (m, 2 H, H-3″), 2.30–2.21 (m, 2 H, H-2a′, 2b′), 1.64 (m, 2 H, H-7″), 1.53 (m, 2 H, H-4″), 1.46–1.34 (m, 4 H, H-5″, H-6″); $^{13}$C NMR (100 MHz, D$_2$O): $\delta$ 176.5 (d, $C$OOH), 161.6 (C-4), 156.5 (C-2), 142.7 (C-6), 100.7 (C-5), 95.7 (C-2″), 85.9 (d, C-1′), 85.6 (C-4′), 73.2 (C-1″), 71.2 (C-3′), 65.3 (d, C-5′), 64.3 (d, O$C$H$_2$COOH), 39.7 (C-8″), 39.1 (C-2′), 27.7 (C-5″), 26.8 (C-7″), 25.3 (C-6″), 18.9 (C-3″); $^3J_{COOH,P}$ 9.5, $^2J_{POCH_2C(O)}$ 5.9, $J_{4',P}$ 8.6, $J_{5',P}$ 4.8 Hz; $^{31}$P NMR (81 MHz, D$_2$O): $\delta$ + 1.23; ESPMS$^-$ (4.5 kV): $m/z$ 488.1 [M − Li]$^-$.

*3′*-O-*Benzoyl*-N$^3$-*(6*-N-*trifluoroacetamido-1-hexyl)thymidine* (**17**).—3′-*O*-Benzoyl-thymidine (800 mg, 2.31 mmol, Sigma) and 6-*N*-trifluoroacetamido-1-iodohexane (2.24 g, 6.93 mmol, 3 equiv) were dissolved in anhyd DMF (20 mL). To the solution, kept at 80 °C under an Ar atmosphere, anhyd silver oxide (330 mg, 1.39 mmol, 0.6 equiv) was added. The resulting mixture was stirred for 3 h before being concentrated under reduced pressure and passed over a SiO$_2$ column (2:1 petroleum ether–acetone). The product containing fractions were collected, concentrated and recrystallised from CH$_2$Cl$_2$–*n*-hexane to yield **17** (980 mg, 78%); m.p. 109 °C; $^1$H NMR (400 MHz, acetone-$d_6$): $\delta$ 8.09, 7.67, and 7.55 (Bz), 7.89 (s, H-6), 6.48 (dd, H-1′), 5.62 (m, H-3′), 4.46 (br s, HO-5′), 4.20 (m, H-4′), 3.98–3.86 (m, 4 H, H-5a′, 5b′, H-1″), 3.34 (m, 2 H, H-6″), 2.58–2.50 (m, 2 H, H-2a′, 2b′), 1.86 (s, 3 H, CH$_3$), 1.63–1.55 (m, 4 H, H-2″, H-5″), 1.43–1.32 (m, 4 H, H-3″, H-4″); $^{13}$C NMR (100 MHz, acetone-$d_6$): $\delta$ 166.3 ($C$=O), 163.6 (C-4), 151.7 (C-2), 151.7 (C-2), 135.2 (C-6), 134.2, 130.8, 130.3, 129.4 (Bz), 110.2 (C-5), 86.4 (C-1′), 86.1 (C-4′), 76.7 (C-3′), 62.9 (C-5′), 41.3 (C-1″), 40.1 (C-6″), 38.3 (C-2′), 29.3 (C-2″), 28.1 (C-5″), 27.0 (C-4′), 26.9 (C-3″), 13.3 (CH$_3$). Anal. Calcd for $C_{25}H_{30}F_3N_3O_7$ (541.53): C, 55.45; H, 5.58; F, 10.52; N, 7.76. Found C, 55.31; H, 5.63; F, 10.41; N, 7.61.

N$^3$*-(6-Amino-1-hexyl)-thymidine 5′-trilithiumdiphosphate* (**18**).—Compound **17** (270 mg, 0.5 mmol) was pyrophosphorylated according to the general procedures to yield **18** (400 mg, 72%, photometrically determined dT content: 46%); UV (200–400 nm): 207 (max), 238 (min), 267 (max); $^1$H NMR (400 MHz, D$_2$O): $\delta$ 7.82 (s, H-6), 6.43 (dd, H-1′), 4.76 (m, H-3′), 4.30 (m, 2 H, H-5a′, 5b′), 4.09 (m, H-4′), 4.01 (m, H-1″), 3.10 (m, H-6″), 2.50–2.42 (m, 2 H, H-2a′, 2b′), 2.04 (s, CH$_3$), 1.79–1.66 (m, 4 H, H-2″, H-5″), 1.52 (m, 2 H, H-4″), 1.47 (m, 2 H, H-3″); $^{13}$C NMR (100 MHz, D$_2$O): $\delta$ 166.2 (C-4), 152.2 (C-2), 136.0 (C-6), 111.4 (C-5), 86.4 (C-1′), 85.8 (d, C-4′), 70.9 (C-3′), 65.7 (C-5′), 41.9 (C-1″), 39.7 (C-6″), 39.0 (C-2′), 26.9 (C-5″), 26.8 (C-2″), 25.8 (C-4″), 25.6 (C-3″), 12.8 ($C$H$_3$); $J_{4',P}$ 8.6, $J_{5',P}$ 4.8 Hz. FABMS$^-$: $m/z$ 500.2 [M$^{3-}$ + 2H$^+$]$^-$.

N$^4$*-(6-Benzyloxycarbonylaminohexanoyl)-5′*-O-tert-*butyldimethylsilyl-2′-deoxycytidine* (**19**).—A solution of *tert*-butyldimethylchlorosilane (580 mg, 3.79 mmol, 1.0 equiv) in anhyd

DMF (20 mL) was stirred with imidazole (520 mg, 7.58 mmol, 2 equiv) for 20 min at rt, then 2′-deoxycytidine hydrochloride (1.0 g, 3.79 mmol) was added. After 3 h, the reaction mixture was concentrated under reduced pressure, the residue then suspended in HMDSA (6.4 mL, 30 mmol, 8 equiv) and stirred for 12 h. After concentration and co-evaporation with toluene, the residue was dissolved in anhyd dioxane (50 mL) with 6-benzyloxycarbonylamino hexanoic acid [7] (4.63 g, 17.4 mmol, 4.6 equiv), 2-chloro-*N*-methylpyridinium iodide (4.85 g, 19.0 mmol, 5 equiv), and *N*,*N*-diisopropylethylamine (6.5 mL, 38 mmol, 10 equiv). The solution was stirred at 50 °C under an Ar atmosphere for 40 h, before being concentrated under reduced pressure, co-evaporated with toluene and quenched by the addition of satd aq $NaHCO_3$ (50 mL). The product was extracted with $CH_2Cl_2$ (2 × 200 mL), the combined organic layers were dried over $MgSO_4$ and concentrated. Purification over $SiO_2$ (1:2 petroleum ether–acetone) yielded **19** (1.50 g, 67%) as a clear syrup; $^1$H NMR (400 MHz, acetone-$d_6$): $\delta$ 9.72 (br s, NH), 8.27 (d, H-6), 7.35–7.26 (m, 6 H, H-5, $C_6H_5$–$CH_2$), 6.28 (br s, NH), 6.18 (dd, H-1′), 5.03 (s, 2 H, $C_6H_5$–$CH_2$), 4.51 (m, H-3′), 3.96 (m, H-4′), 3.97 (dd, H-5a′), 3.93 (dd, H-5b′), 3.14 (m, 2 H, H-6″), 2.53 (m, 2 H, H-2″), 2.42 (d, H-2a′), 2.19 (ddd, H-2b′), 1.67 (m, 2 H, H-3″), 1.53 (m, 2 H, H-5″), 1.38 (m, 2 H, H-4″), 0.94 (s, 9 H, $SiC(CH_3)_3$), 0.13 and 0.12 (each s, 6 H, $Si(CH_3)_2$); $^{13}$C NMR (100 MHz, acetone-$d_6$): $\delta$ 174.2 (C-1″), 163.4 (C-4), 157.1 (N*C*(O)OBn), 155.3 (C-2), 145.1 (C-6), 138.5, 129.1, 128.5, and 127.4 ($C_6H_5$), 95.9 (C-5), 88.5 (C-4′), 87.3 (C-1′), 71.6 (C-3′), 66.2 ($C_6H_5CH_2$), 63.0 (C-5′), 42.2 (C-2′), 41.3 (C-6″), 37.6 (C-2″), 30.3 (C-5″), 26.8 (C-4″), 26.2 ($(H_3C)_3CSi$), 25.2 (C-3″), 18.8 ($(H_3C)_3CSi$), −5.5, −5.6 ($(H_3C)_2Si$). Anal. Calcd for $C_{29}H_{44}N_4O_7Si$ (588.78): C, 59.16; H, 7.53; N, 9.52. Found C, 59.14; H, 7.66; N, 9.33.

$N^4$ - *(6 - Benzyloxycarbonylaminohexanoyl)-3′*-O-*benzoyl-2′-deoxycytidine* (**20**).—Previously prepared compound **19** (500 mg, 0.85 mmol) was first benzoylated and thereafter O-desilylated according to the general procedures (vide supra) to give a raw product, which was purified by $SiO_2$ column chromatography (2:3 petroleum ether–acetone). **20**: 370 mg, 76%; m.p. 157 °C; $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 8.45 (d, H-6), 8.06, 7.54, and 7.42 ($C(O)C_6H_5$), 7.48 (d, H-5), 7.34–7.27 ($CH_2C_6H_5$), 6.37 (dd, H-1′), 5.55 (m, H-3′), 5.05 (s, 2 H, $CH_2C_6H_5$), 4.35 (m, H-4′), 3.91 (m, 2 H, H-5a′, 5b′), 3.14 (m, 2 H, H-6″), 2.81 (dd, H-2a′), 2.44–2.32 (m, 3 H, H-2″, H-2b′), 1.69 (m, 2 H, H-3″), 1.53 (m, 2 H, H-5″), 1.37 (m, 2 H, H-4″); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ 173.9 (C-1″), 166.0 ($C(O)C_6H_5$), 162.4 (C-4), 161.0 ($NC(O)OCH_2C_6H_5$), 156.0 (C-2), 144.5 (C-6), 136.4, 132.4, 129.3, 129.2, 128.9, 128.1, 128.0, 127.8, 127.5, and 127.3 ($CH_2C_6H_5$ and $C(O)C_6H_5$), 96.9 (C-5), 87.1 (C-1′), 85.9 (C-4′), 75.4 (C-3′), 66.0 ($CH_2C_6H_5$), 61.3 (C-5′), 40.1 (C-6″), 38.8 (C-2′), 36.7 (C-2″), 29.0 (C-5″), 25.7 (C-4″), 23.9 (C-3″). Anal. Calcd for $C_{30}H_{34}N_4O_8$ (578.63): C, 62.27; H, 5.92; N, 9.68. Found C, 62.24; H, 5.63, N, 9.53.

*(6″ - Benzyloxycarbonylamino - hexanoyl) - 2′-deoxy* - $N^4$-*cytidine 5′-trilithiumdiphosphate* (**21**).—Compound **20** was pyrophosphorylated and purified as described under the general procedures to give **21** (420 mg, 60%, photometrically estimated dC content: 48%); UV (200–400 nm): 224 (min), 231 (max), 251 (min), 270 (max); $^1$H NMR (400 MHz, $D_2O$): $\delta$ 7.98 (d, H-6), 7.41–7.31 (m, 5 H, $C_6H_5$), 6.37 (dd, H-1′), 6.19 (d, H-5), 5.43 (m, H-3′), 5.03 (s, 2 H, $CH_2C_6H_5$), 4.41 (m, H-4′), 4.28–4.19 (m, 2 H, H-5a′, 5b′), 3.37 (m, 2 H, H-6″), 2.53–2.35 (m, 4 H, H-2″, H-2a′, 2b′), 1.69 (m, 2 H, H-3″), 1.61 (m, 2 H, H-5″), 1.39 (m, 2 H, H-4″); $^{13}$C NMR (100 MHz, $D_2O$): $\delta$ 176.4 (C-1″), 166.5 (C-4), 159.2 ($NC(O)OCH_2C_6H_5$), 157.8 (C-2), 141.9 (C-6), 137.6, 129.1, and 128.0 ($C_6H_5$), 97.3 (C-5), 86.5 (C-1′), 83.8 (d, C-4′), 76.2 (C-3′), 68.2 ($CH_2C_6H_5$), 66.2 (d, C-5′), 40.0 (C-6″), 37.8 (C-2′), 34.1 (C-2″), 27.8 (C-5″), 25.8 (C-4″), 24.1 (C-3″); $J_{4',P}$ 7.0, $J_{5',P}$ 5.7 Hz. FABMS$^-$: $m/z$ 595.2 $[M^{3-} + 2H^+]^-$.

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