2′-Deoxyimmunosine: stereoselective synthesis, base pairing and duplex stability of oligonucleotides containing 8-oxo-7-thiaguanine†

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Oligonucleotides containing 7-thia-8-oxoguanine represent a new class of molecules in which sulfur replaces the 7-nitrogen of a purine base. The monomeric 7-thia-8-oxoguanine 2′-deoxyribonucleoside (2′-deoxyimmunosine, **4**) was prepared by nucleobase anion glycosylation in a regio- and stereoselective way employing 5-{[(di-*n*-butylamino)methylidene]amino}thiazolo[4,5-*d*]pyrimidine-2,7(3*H*,6*H*)-dione (**18**) and 1-chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-*erythro*-pentofuranose (**6**). The nucleoside was converted into the phosphoramidite **22** and oligonucleotides were prepared by solid-phase synthesis. Oligonucleotide duplexes containing the **4**–dC base pair show a similar stability as those containing the dG–dC motif. Thus the sterically demanding sulfur and the additional 8-oxo group are well accommodated in the major groove of DNA. As expected, compound **4** does not form a Hoogsteen pair, as reported for 8-oxo-2′-deoxyguanosine. Compared to 2′-deoxyguanosine, 2′-deoxyimmunosine shows a better mismatch discrimination in Watson–Crick base pairs.

Introduction

The development of clinically useful agents that restore and enhance the ability of the human immune system to ward off infections or other invasion challenges has become a major objective of current pharmaceutical research efforts. The AIDS epidemic and the need for adjuvant therapy to boost the immune system of the elderly and cancer patients has brought the area of immunopotentiation into focus.[1–4] Many different types of compounds have been demonstrated to possess immune stimulatory activity. Among them are the class of nucleosides[5–9] as well as oligonucleotides with particular sequence motifs.[10,11]

Among the monomeric nucleosides, guanosine analogues such as 7-thia-8-oxoguanosine (**1**, immunosine, TOG, isatoribine) and its analogues,[12–15] 7-allyl-8-oxoguanosine (**2b**, loxoribine)[16] or 7-deazaguanosine (**3a**)[17] (purine numbering is used throughout this paper) and its 2′-deoxy derivative (**3b**)[11] (Fig. 1) possess *in vivo* activity against a variety of DNA and RNA viruses. Immunosine exhibits a stimulatory effect on both cellular and humoral components of the immune response; the observed antiviral effect has been attributed primarily to the induction of α-interferon.[15] Recently, it was found that immunosine and other guanosine analogues activate immune cells *via* Toll-like receptor 7 (TLR7).[18] Also, oligonucleotides can activate the immune system, and the CpG motif was found to generate such an activity in nucleic acids.[10] Oligonucleotides incorporating 7,8-dihydro-8-oxo-2′-deoxyguanosine (OxodG),[19,20] 7-methyl-8-oxo-2′-deoxyguanosine[21] and 7-deaza-2′-deoxyguanosine (**3b**)[11,22,23] have been described. However, very little is known about the synthesis of 2′-deoxyimmunosine (**4**) as well as of oligonucleotides incorporating the 8-oxo-7-thiaguanine as nucleobase. Herein, we wish to report on the stereoselective synthesis of 2′-deoxyimmunosine, its protection and conversion into a phosphoramidite building block as well as its incorporation into oligo-2′-deoxyribonucleotides. The base pairing properties of 2′-deoxyimmunosine will be studied. This is the first report on oligonucleotides containing a sulfur atom in the five-membered ring of the nucleobase.

Fig. 1 Structures of nucleosides **1–4**.

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Results and discussion

1. Synthesis of 2′-deoxyimmunosine (4)

Two synthetic routes were taken into consideration for the 2′-deoxyimmunosine (**4**) synthesis. The first one is a convergent approach which involves the nucleobase **5** and the halogenose **6** as starting materials, while the other route requires deoxygenation of the ribonucleoside immunosine (**1**). The Barton deoxygenation is the more lengthy protocol used for the ribonucleoside immunosine (**1**). Reports for the synthesis of **4**

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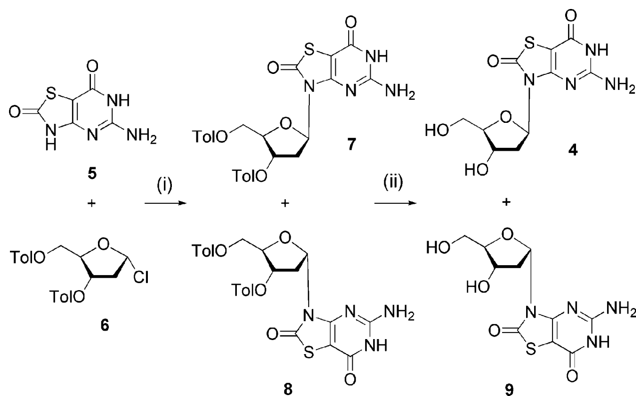
already exist. In 1991 Cottam and Robins reported that 2′-deoxyimmunosine can be synthesized by the fusion procedure. 5-Aminothiazolo[4,5-*d*]pyrimidin-2,7(3*H*,6*H*)-dione (**5**)[24] was silylated with hexamethyldisilazane, and the silylated intermediate was fused with 1-chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-*erythro*-pentofuranose (**6**) to yield an anomeric mixture of the protected 2′-deoxyribonucleosides in 14% yield.[25] After deprotection, the anomeric mixture was separated by crystallization from EtOH to give nucleoside **4**. However, no proof of the anomeric configuration was given. The separation of the anomers is tedious and the unambiguous configurational assignment on the basis of ^{1}H-NMR data is difficult. In a second route the McCombie–Barton deoxygenation of the ribonucleoside was employed (Z. Sözen, Thesis, Ulm, 2001). However, the overall yield is low (3.6%).

Next, we initiated studies to use the nucleobase anion glycosylation, paying particular attention to the stereoselectivity of the glycosylation reaction. Normally, this protocol forms 2′-deoxyribonucleosides in a stereoselective way but shows drawbacks when the nucleobase is not fully soluble in the reaction mixture. Our first synthetic attempt towards the 2′-deoxyimmunosine synthesis made use of the non-protected nucleobase. The 5-aminothiazolo[4,5-*d*]pyrimidin-2,7(3*H*,6*H*)-dione (**5**) was prepared in 5 steps from 2,4-diamino-5-hydroxypyrimidine according to Baker and Chatfield.[24] Treatment of the nucleobase with halogenose **6** in DMF in the presence of sodium hydride (room temperature) afforded a mixture (29% yield) of the protected nucleosides. Although compound **5** contains two lactam functions, the reaction mixture was tentatively assigned to the anomeric compounds **7/8**. Deblocking with 0.2 M $NaOCH_3$ in MeOH gave an anomeric mixture of the nucleosides **4/9** in a 2 : 1 ratio, which could not be separated by flash column (FC) chromatography. Nevertheless, from the ^{13}C NMR spectrum it was apparent that two nucleosides are formed, which was confirmed by the HPLC profile resulting in two peaks in a ratio of 2 : 1 (63% : 30%). The almost identical ^{13}C NMR chemical shifts observed for the nucleobase moieties made the formation of anomers with nitrogen-9 as the glycosylation site more likely over that of regioisomers with sugar moieties in the six-membered and the five-membered rings (Scheme 1).



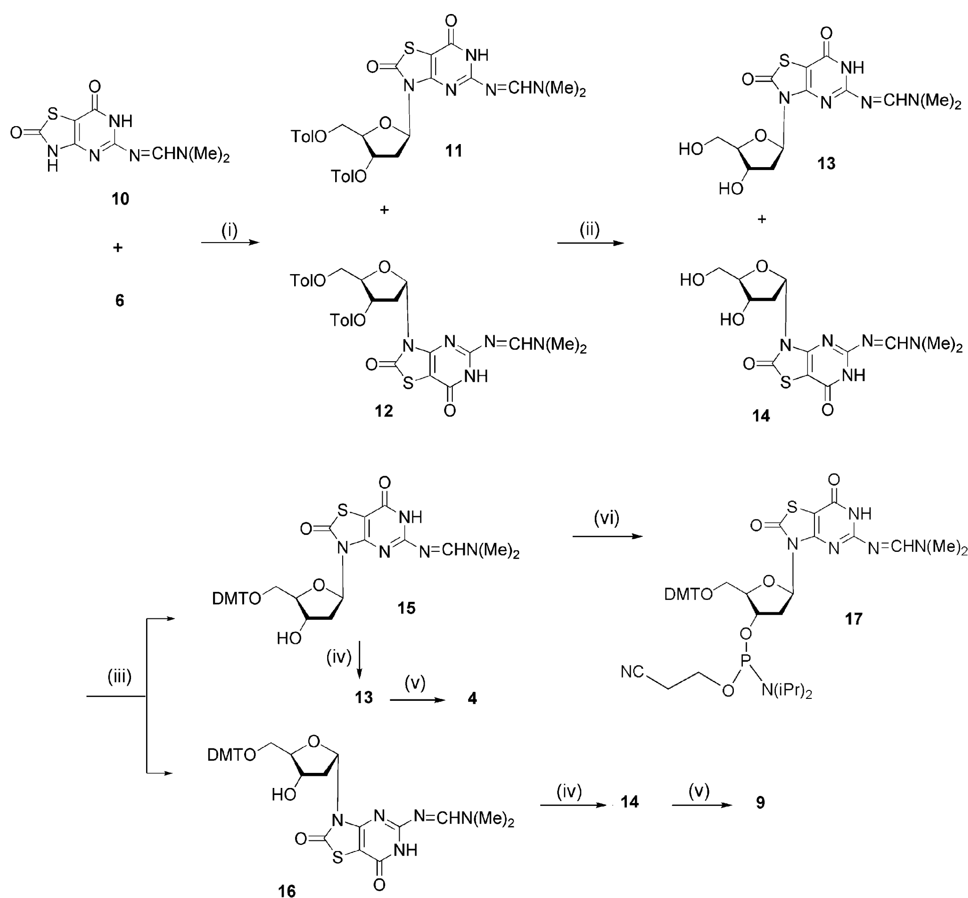
Scheme 1 *Reagents and conditions*: (i) NaH, DMF, r.t.; (ii) 0.2 M $NaOCH_3$, 2 h, r.t.

As the formation of anomeric glycosylation mixtures had already been observed when the anion glycosylation was performed in DMF[26,27] but not in MeCN, this solvent was used next for the glycosylation of **5** with halogenose **6**. Unfortunately, the nucleobase **5** is poorly soluble in this solvent, and so we increased the nucleobase lipophilicity by protecting the amino group of **5** with the lipophilic (*N*,*N*-dimethylamino)methylidene residue. For this, compound **5** was treated with *N*,*N*-dimethylformamide dimethyl acetal to yield the derivative **10**. Glycosylation of the protected nucleobase **10** with halogenose **6** in MeCN with sodium hydride as base (room temperature) resulted in a 55% yield of protected reaction products still forming a mixture of anomers **11/12**. Deprotection of the sugar moiety was performed with the mixture of **11/12** (in 0.1 M NaOMe/MeOH), yielding the nucleosides **13/14**. Neither the sugar-protected intermediates **11/12** nor the amidine-protected nucleosides **13/14** could be separated on preparative scale. However, as it was found that the 5′-DMT derivatives are separable by column chromatography, the **13/14** mixture was treated with DMT-Cl, affording a mixture of **15/16** from which the single anomers **15** and **16** were isolated in a pure form by flash chromatography. Detritylation of **15** yielded **13**, while under the same conditions **16** gave **14**. The free nucleosides **4** and **9** were isolated after treatment of compounds **13** and **14** with saturated NH_3/CH_3OH for 3 days at room temperature. Reaction of compound **15** with chloro(2-cyanoethoxy)-*N*,*N*-diisopropylaminophosphine afforded the phosphoramidite **17** (Scheme 2).

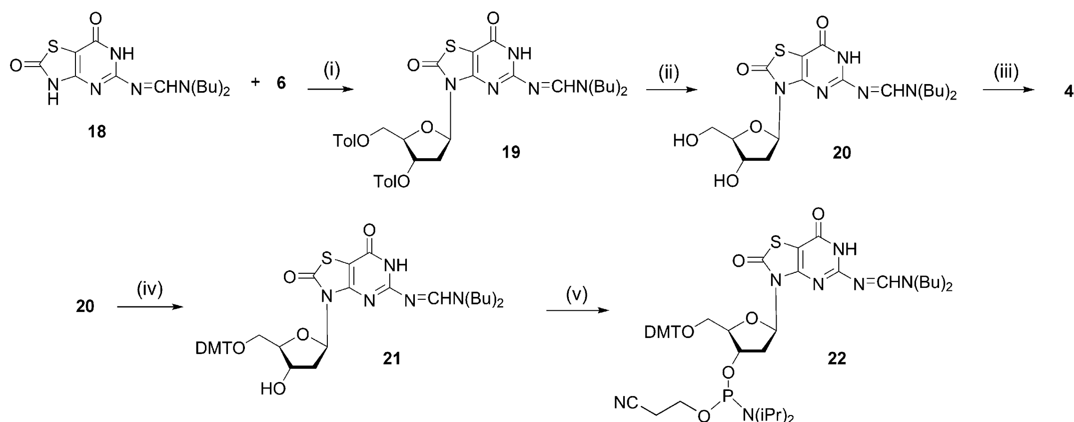
Despite the improved glycosylation yield and the successful separation of anomers it was difficult to run the glycosylation reaction to completion. The yield could not be improved by using an excess of more than 1.3 equivalents of the chloro sugar **6**, as side products were formed. A further increase of the sugar halide led to the introduction of two sugar residues (one at each lactam moiety). This results from the two immunosine lactam deprotonation sites with pK_a values of 7.2 and 10.0 (ESI†). As the separation of the DMT compounds was tedious—the material became partially deprotected during the chromatographic work-up process—we choose the more lipophilic *N*,*N*-dibutylaminomethylidene protecting group instead. This group has already been successfully employed for the protection of 7-deaza-2′-deoxyisoguanosine[28] and 5-methylisocytosine.[29] The reaction of **5** with *N*,*N*-dibutylaminoformamide dimethyl acetal[30] gave the amidine in 75% yield. Glycosylation of **18** with the chloro sugar **6** in MeCN at room temperature in the presence of powdered KOH and TDA-1 tris(3,6-dioxaheptyl)amine resulted in a 65% yield of the protected nucleosides as an anomeric mixture (in a ratio of 5 : 1), which was separable by FC to yield compound **19**. This was deprotected under mildly alkaline conditions (0.1 M NaOMe/MeOH) to afford the amidine **20**, which upon treatment with concentrated ammonium hydroxide gave 2′-deoxyimmunosine (**4**). Compound **20** was converted into the 4,4′-dimethoxytrityl derivative **21**. Reaction with chloro(2-cyanoethoxy)-*N*,*N*-diisopropylaminophosphine afforded the phosphoramidite **22** (Scheme 3).

2. Physical characterization of 2′-deoxyimmunosine and its derivatives

All compounds were characterized by ^{1}H- and ^{13}C-NMR spectra as well as by elemental analysis. ^{31}P-NMR spectra were taken for the phosphoramidites **17** and **22** (see Tables 2–4 and Experimental section). ^{1}H NMR data are displayed in Table 2, and ^{13}C NMR



Scheme 2 *Reagents and conditions*: (i) NaH, MeCN, r.t.; (ii) 0.1 M $NaOCH_3$, 2 h, r.t.; (iii) DMT-Cl, pyridine, 1 h, r.t.; (iv) 1% dichloroacetic acid, 20 min, r.t.; (v) saturated NH_3/CH_3OH, 72 h, r.t.; (vi) 2-(cyanoethyl)diisopropylphosphoramido chloridite, 15 min, r.t.



Scheme 3 *Reagents and conditions*: (i) KOH, TDA-1 tris(3,6-dioxaheptyl)amine, MeCN, 30 min, r.t.; (ii) 0.1 M $NaOCH_3$, 2 h, r.t.; (iii) 25% aq. NH_3, 72 h, r.t.; (iv) DMT-Cl, pyridine, 1 h, r.t.; (v) 2-(cyanoethyl)diisopropylphosphoramido chloridite, 15 min, r.t.

chemical shifts are compiled in Table 3. Although the anomeric 2′-deoxyimmunosines showed noticeable changes of the ^{13}C NMR chemical shifts of the sugar moieties, no unambiguous assignment was possible from these data. Also, NOE difference spectra could not be used for identification as no proton is present in the 8-position, and thus a single-crystal X-ray analysis was performed.[31] The crystal structure of compound **4** is shown in Fig. 2. In the solid state, compound **4** adopts the *syn* conformation with a torsion angle of $\chi = 61.0°$. This is different from most of the other 2′-deoxyribonucleosides, which adopt an *anti* conformation. The sugar ring has an *N*-conformation (3′-*endo*-4′-*exo*, $^{3}T_{4}$). The conformation around the C4′–C5′ bond is in the +*ap* (*trans*) orientation. The *syn* conformation is typical for most of the 8-substituted guanine ribonucleosides, with a C2′-*endo* pucker and a (+) gauche conformation (+*sc*) around the C4′–C5′ bond.[32–35] Examples are 8-bromo-,[33] 8-chloro-,[34] and 7-methyl-8-thioxoguanosines.[36] Some compounds such as 7-deaza-8-methyl-2′-deoxyguanosine[37] display an *anti* conformation in the solid state,

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Table 1 $^3J_{H,H}$ coupling constants of the sugar moiety and conformer population of nucleoside **4**

H positions	$^3J_{H,H}$/Hz	Pseudorotational parameters	
1′,2′	7.06	%*N*	27
1′,2″	7.42	%*S*	73
2′,3′	7.30	P_S/deg	154
2″,3′	3.92	Ψ_S/deg	23
3′,4′	3.19	Rms	0.416

with the sugar in the *S*-conformation and a *trans* orientation of the C4′–C5′ bond.

An *N*-conformation of the nucleoside would lead to unfavourable effects during base pairing; the conformation was also studied in aqueous solution with the aid of the PSEUROT program (version 6.3).[38] A minimization of the differences between the experimental and calculation coupling is accomplished by a nonlinear Newton–Raphson minimization, the quality of the fit being expressed by the root-mean-square (rms) difference. This procedure presupposes the existence of a two state *N*/*S* equilibrium. The input contained the following coupling constants: ^3J(H1′,H2′), ^3J(H1′,H2″), ^3J(H2′,H3′), ^3J(H2″,H3′), ^3J(H3′,H4′). During the interactions either the puckering parameters (*P* and Ψ) of the minor conformer (*N*) or the puckering amplitudes of both conformers were constrained. The coupling constants and the pseudo-rotational parameters are shown in Table 1. The sugar moiety of compound **4** shows an *S*-confomer population of 73%, which is in line with that of other 2′-deoxyribonucleosides.

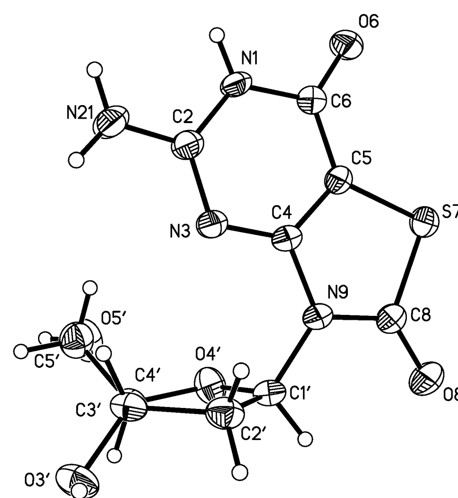

Fig. 2 Single crystal X-ray structure of 2′-deoxyimmunosine.

As nothing is known about the ^{13}C-NMR chemical shift assignment of 7-thia-8-oxoguanine nucleosides, we assigned all signals on the basis of gated-decoupled ^{1}H/^{13}C-NMR spectra as well as DEPT-135 spectra. The gated-decoupled spectrum of C8 and C4 of 2′-deoxyimmunosine (**4**) shows three bond couplings with the anomeric proton of C1′ with ^3J(C8,H1′) = 6.9 Hz and ^3J(C4,H1′) = 2.6 Hz. The anomeric compound

Table 2 ^{1}H-NMR chemical shifts (δ) of 5-aminothiazolo[4,5-*d*]pyrimidine derivatives[a]

Cpd	NH	NH_2	N=CH	H1′	5′-OH	3′-OH	H3′	H4′	H5′	H2′β	H2′α	N-CH_3
4 (β)	11.22	6.92	—	6.24	5.17	4.66	4.31	3.70	3.53	2.90	1.98	
9 (α)	11.24	6.89	—	6.11	5.17	4.68	3.58	3.36	4.07	2.74	2.40	
13 (β)	11.93	—	8.65	6.36	5.23	4.68	4.31	3.69	3.58	2.88	2.06	
14 (α)	11.92	—	8.92	6.21	5.47	4.69	3.61	3.40	4.15	2.85	2.50	
15 (β)	11.90	—	8.64	6.46	—	5.27	4.32	3.86	3.72	2.78	2.15	3.16, 3.05
16 (α)	11.84	—	8.78	6.33	—	5.48	4.32	4.15	3.22	2.72	2.56	3.03, 2.90
19 (β)	11.97	—	8.68	6.50	—	—	5.78	4.50	4.50	3.40	2.57	2.38, 2.34
20 (β)	11.89	—	8.62	6.34	5.21	4.67	4.33	3.69	3.48	2.91	2.06	
21 (β)	11.82	—	8.60	6.44	—	5.22	4.30	3.86	3.85	2.81	2.16	

[a] Measured in DMSO-d_6 at 25 °C.

Table 3 ^{13}C-NMR chemical shifts (δ) of 5-aminothiazolo[4,5-*d*]pyrimidine derivatives[a]

Cpd	C2[b] C5[c]	C4[b] C3a[c]	C5[b] C7a[c]	C6[b] C7[c]	C8[b] C2[c]	C1′	C2′	C3′	C4′	C5′	N=CH
1	155.5	154.3	85.8	156.5	169.2	88.8	70.4	69.6	84.8	62.1	—
4 (β)	155.6	154.3	85.8	156.7	169.1	83.0	35.2	71.1	87.5	62.2	—
9 (α)	155.4	154.1	85.8	156.5	169.4	81.9	35.6	70.1	85.6	61.0	—
5	155.7	156.5	87.6	157.2	171.2	—	—	—	—	—	—
10	157.2	154.1	92.1	159.6	169.9	—	—	—	—	—	—
13 (β)	159.4	152.9	90.6	157.3	168.7	82.5	36.0	70.6	87.3	61.9	158.9
14 (α)	159.4	153.1	90.6	157.4	169.4	82.2	35.9	70.3	86.0	61.4	159.5
15 (β)	159.3	152.9	90.6	157.4	168.7	82.5	36.8	70.8	85.3	64.5	—
16 (α)	159.3	152.9	90.6	157.4	169.3	82.5	35.9	71.2	85.4	64.6	—
18	159.6	154.1	92.1	157.2	169.8	—	—	—	—	—	—
19 (β)	159.4	152.7	90.9	157.4	169.2	82.1	33.8	74.0	82.1	63.5	158.5
20 (β)	159.4	152.9	90.7	157.4	168.9	82.4	36.1	70.6	87.3	61.9	—
21 (β)	159.5	152.9	90.8	157.4	168.9	82.5	36.9	70.9	85.3	64.5	—

[a] Measured in DMSO-d_6 at 25 °C. [b] Purine numbering. [c] Systematic numbering.

Table 4 $J_{C,H}$ values (in Hz) of 5-aminothiazolo[4,5-*d*]pyrimidine derivatives[a]

	4	9	13	14
C8,H1′	6.9	6.1	6.5	4.4
C4,H1′	2.6	2.8	3.2	4.4
C1′,H1′	165.5	165.6	165.7	166.4
C1′,H2′	—	6.8	—	6.8
C2′,H2′	132.8	133.4	132.7	135.3
C3′,H3′	149.9	143.7	147.8	142.7
C4′,H4′	146.2	146.6	145.2	147.1
C5′,H5′	139.5	138.5	140.0	138.6

[a] Measured in DMSO-d_6 at 25 °C; purine numbering used.

Table 5 UV maxima and extinction coefficients of compound **4** and related derivatives[a]

Compound	λ_{max}/nm	ε
4	247	8300
	302	9100
9	248	8300
	302	9200
dG[43]	255	12 900
3b (c^{7}dG)[44]	259	13 400
8-oxodG[42]	246	12 600
	293	9700

[a] Measured in H_2O.

9 shows similar values (Table 4). The C5 signal appears as a singlet with $\delta = 85.8$ ppm. The signals C2 and C6 could not be assigned unambiguously. Thus, the deuterium isotope upfield shift approach was used[39,40] to assign the carbon-2 signal, which is directly connected with the amino group. In DMSO solution in the presence of a H_2O–D_2O mixture it shows two singlets, the original one and one which is shifted 50 ppb upfield. Consequently, C2 was assigned to the 155.6 ppm signal and C6 to the 156.7 ppm signal. For the sugar portion of compound **4**, the following coupling constants were observed: ^1J(C1′,H1′) = 165.5 Hz, ^1J(C3′,H3′) = 149.9 Hz and ^1J(C4′,H4′) = 146.2 Hz. The large coupling constant of C1′ is indicative of the anomeric carbon.[41] The coupling constants of ^1J(C1′,H1′), ^1J(C2′,H2′), ^1J(C4′,H4′) and ^1J(C5′,H5′) of the α-D compound **9** are very similar to the β-D compound **4**, except for ^1J(C3′,H3′), which is 6 Hz less than that of compound **4** (Table 4). The largest difference between the α and β compounds is that the α-D compounds **9** and **14** show ^3J(C1′,H2′) = 6.8 Hz, while the β-D compounds **4** and **13** do not show this coupling (Table 4). This is indicative of the anomeric assignment. The signals of C5′ and C2′ are triplets. For compounds **15**, **16** and **21** with DMT-residues on the 5′-OH, the chemical shifts show characteristic values from about 62.0 ppm to 64.5 ppm, while the C4′ shift varies from ~87.3 ppm to 85.3 ppm.

The UV data of compound **4** and other dG derivatives are summarized in Table 5. The replacement of nitrogen-7 by sulfur changes the UV-spectrum significantly (Table 5 and Fig. 3). The UV maxima of compound **4** show a strong red shift compared to that with a nitrogen at the identical position: 8-oxo-2′-deoxyguanosine (oxodG) (λ_{max} = 246 nm, 293 nm)[42] *vs.* **4** (λ_{max} = 246 nm, 302 nm). Compound **4** also shows a lower pK_a value of deprotonation compared to 2′-deoxyguanosine or its derivatives: **4** (pK_a = 8.2), oxodG (pK_a = 8.6),[42] dG (pK_a = 9.4)[43] and c^{7}dG (pK_a = 10.2).[44]

3. Synthesis and base pairing of oligonucleotides containing 2′-deoxyimmunosine (4)

3.1 Oligonucleotide synthesis and characterization. Solid-phase oligonucleotide synthesis was performed employing the phosphoramidites **17** or **22** protected with a dialkylaminoalkylidene protecting group on an ABI 392-08 automated DNA synthesizer with controlled pored glass (CPG-500) serving as solid phase. Base labile (*tert*-butylphenoxy)acetyl (tac) groups were chosen for amino protection in the cases of dA, dG and dC. The syntheses followed the standard protocol.[45] The coupling time for the phosphoramidite **17** or **22** was 10 minutes and the coupling yields of the phosphoramidites were always higher than 95%. The oligonucleotides were deprotected by incubation with a 25% aqueous NH_3 solution at room temperature for 24 h. The oligonucleotides were purified before and after detritylation by reverse-phase HPLC.

As the thiazole system is prone to degradation, harsh alkaline condition had to be avoided. Ammonia treatment at 60 °C led to the formation of side products and gave very little of the target oligonucleotide. Thus, the stability of the nucleoside **4** was studied in aqueous ammonia. As shown in Fig. 4a, compound **4** is partially degraded under standard oligonucleotide deprotection conditions (25% aqueous NH_3, 60 °C, 16 h). However, at room

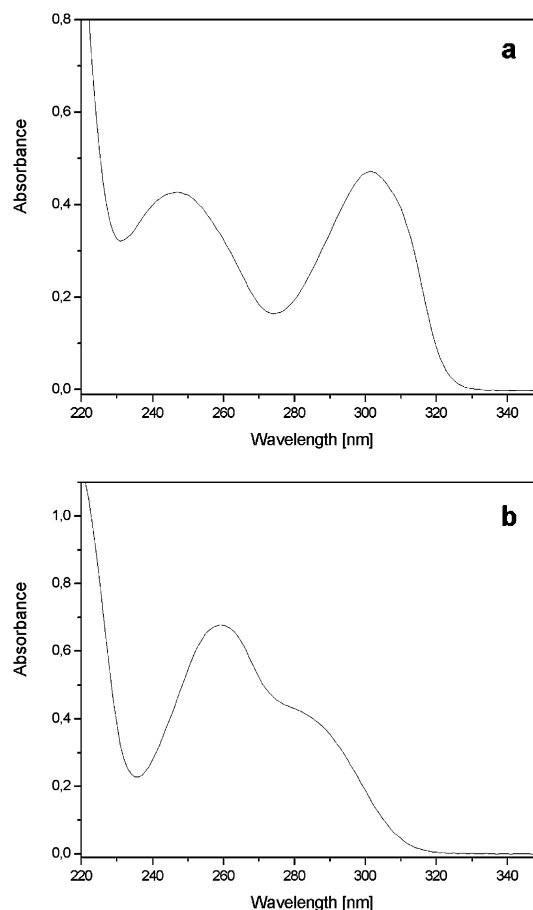

Fig. 3 UV spectra of (a) 2′-deoxyimmunosine (**4**) and (b) 7-deaza-2′-deoxyguanosine (**3b**) in water.

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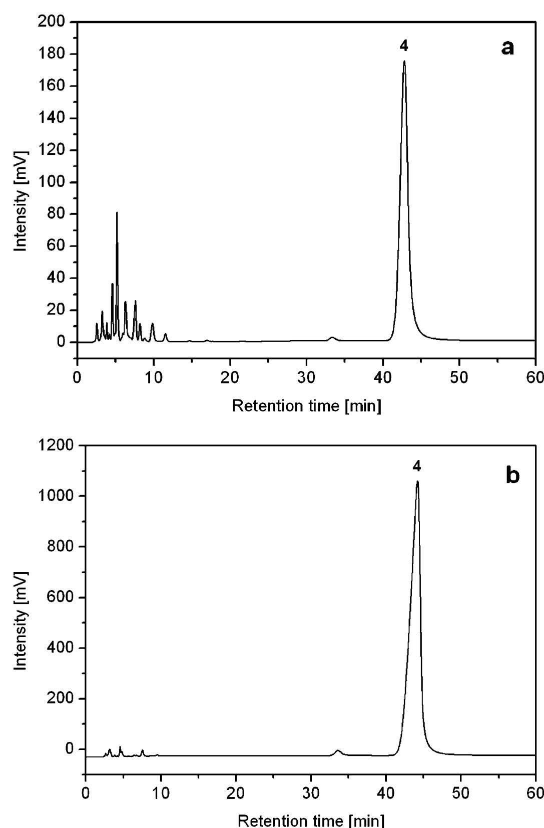

Fig. 4 Reverse-phase HPLC profiles (column 250 × 4 mm, RP-18) of **4** after treatment with 25% aqueous NH_3: (a) at 60 °C for 16 h, (b) at room temperature for 24 h. HPLC elution buffer: 5% MeCN in 0.1 M $(Et_3NH)OAc$, pH = 7.0, 1.0 mL min^{-1}. The profile was measured at 302 nm.

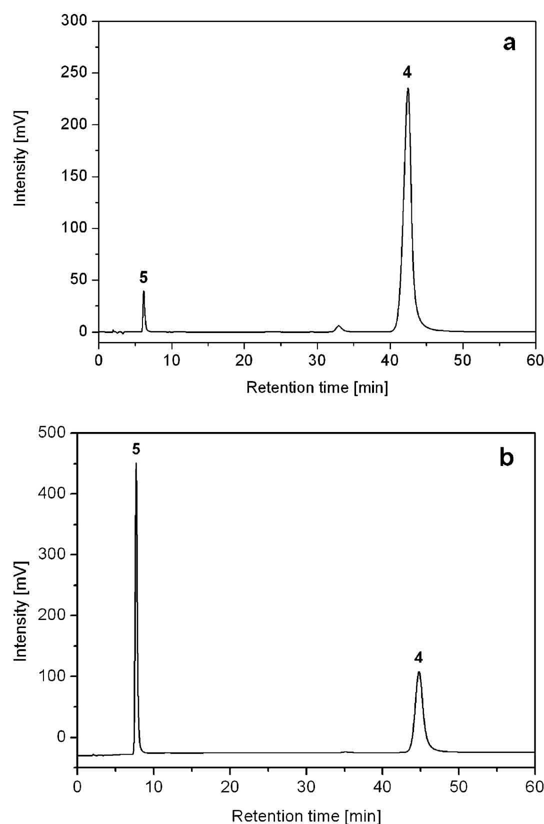

Fig. 5 Reverse-phase HPLC profiles (column 250 × 4 mm, RP-18) of the hydrolysis mixture of **4** in 0.5 M HCl at room temperature after (a) 50 min, (b) 19 h. Buffer: 5% MeCN in 0.1 M $(Et_3NH)OAc$, pH = 7.0, 1.0 mL min^{-1}. The profile was measured at 302 nm.

temperature (24 h) 2′-deoxyimmunosine is rather stable, leading to only very little degradation (Fig. 4b). In contrast, the deprotection of monomeric amidine-protected nucleosides **13** and **20** in 25% aqueous NH_3 solution at room temperature required 72 h for completion (see Experimental section). For oligonucleotide deprotection in 25% aqueous NH_3 at room temperature a reaction time of 24 h was sufficient.

In order to circumvent possible problems occurring during the acid-catalyzed detritylation in the oligonucleotide synthesis, the stability of compound **4** was also studied under acidic conditions. Nucleoside **4** was treated with 0.1 M HCl at room temperature for 10 h and the reaction was followed by RP18-HPLC. After 10 h treatment, almost no change was observed (data not shown). Then, the reaction was performed under stronger acidic conditions (0.5 M HCl) at room temperature. While compound **4** was not significantly hydrolyzed within 50 min (Fig. 5a), half of the educt was degraded after 19 h by glycosylic bond hydrolysis, to form the immunosine base **5** (Fig. 5b). For comparison, the glycosylic bond stability was also studied for dG and 7-deaza-2′-deoxyguanosine (c^7G_d). According to the HPLC profiles (ESI†), the glycosylic bond of 2′-deoxyimmunosine (**4**) is more stable than that of dG but less stable than that of 7-deaza-2′-deoxyguanosine (c^7G_d). Thus, no degradation of 2′-deoxyimmunosine is expected during oligonucleotide synthesis.

3.2 Base-pairing and duplex stability of oligonucleotides containing 2′-deoxyimmunosine (4).

Next, the base pairing of oligonucleotides containing 2′-deoxyimmunosine **4** was studied. Two oligonucleotides, 5′-d(TAG GTC AAT ACT) (**36**) and 3′-d(ATC CAG TTA TGA) (**27**) were used as reference compounds.[46] Nucleoside **4** was used to replace dG residues to form the new oligonucleotides **43**, **44**, **46** and **47**. The self-complementary oligonucleotide **45** leading to six modifications in the duplex structure was also synthesized. The oligonucleotides were characterized by MALDI-TOF mass spectra; the masses were in good agreement with the calculated values (Table 7 in the experimental part). The oligonucleotides **45** and **47** were hydrolyzed with snake-venom phosphodiesterase followed by alkaline phosphatase[47] to yield the free nucleosides, and the digest was separated by RP-18 HPLC (Fig. 6). According to the HPLC profiles of the enzymatic degradation, the composition of oligonucleotides was proved.

In the first series of experiments, the nucleoside **4** was used as an analogue of dG. Table 6 shows the T_m values when nucleoside **4** was placed opposite to dC. The incorporation of one **4**–dC base pair resulted in no change of the T_m values (ΔT_m = 0 °C) compared to the standard duplex **36·27**. Incorporation of two or three **4**–dC base pairs also resulted in no significant changes in the stability (ΔT_m = 1–2 °C). Duplexes containing central **4**–dC pairs (**47·27**) or separated central **4**–dC pairs (**36·46**) exhibited similar duplex stabilities, with ΔT_m = 0.5 °C per modification. The complete replacement of all dG residues was performed on the self-complementary duplex d(**4**–C)$_3$, and the stability was compared to the standard duplex d(G–C)$_3$. Here the T_m value of duplex melting also decreased by only 0.5 °C per modification. Thus, the

Table 6 T_m values and thermodynamic data of duplex melting of oligonucleotides with regular and base-modified nucleosides (**4** = 2′-deoxyimmunosine).[a]

Duplex	No.	T_m/°C	ΔT_m/°C [b]	ΔG_{310}/kcal mol^{-1}
5′-d(TAG GTC AAT ACT)[49] 3′-d(ATC CAG TTA TGA)	**36** **27**	50	—	−11.6
5′-d(TAG GTC AAT ACT) 3′-d(ATC CA**4** TTA TGA)	**36** **43**	50	0	−11.3
5′-d(TAG GTC AAT ACT) 3′-d(ATC CAG TTA T**4**A)	**36** **44**	50	0	−11.7
5′-d(TAG GTC AAT ACT) 3′-d(ATC CA**4** TTA T**4**A)	**36** **46**	49	−0.5	−11.1
5′-d(TA**4** **4**TC AAT ACT) 3′-d(ATC CAG TTA TGA)	**47** **27**	49	−0.5	−11.0
5′-d(TA**4** **4**TC AAT ACT) 3′-d(ATC CA**4** TTA TGA)	**47** **43**	48	−1	−10.7
5′-d(TA**4** **4**TC AAT ACT) 3′-d(ATC CAG TTA T**4**A)	**47** **44**	49	−0.5	−11.0
5′-d(TA**4** **4**TC AAT ACT) 3′-d(ATC CA**4** TTA T**4**A)	**47** **46**	47	−1	−10.2
5′-d(GCGCGC)[50] 3′-d(CGCGCG)		46	—	−8.2
5′-d(**4**C**4**C**4**C) 3′-d(C**4**C**4**C**4**)	**45** **45**	42	−0.5	−8.1
5′-d(TAG GTA AAT ACT) 3′-d(ATC CAG TTA TGA)	**37** **27**	35	−15	−7.3
5′-d(TAG GTA AAT ACT) 3′-d(ATC CA**4** TTA TGA)	**37** **43**	31	−19	−6.8
5′-d(TAG GTT AAT ACT) 3′-d(ATC CAG TTA TGA)	**38** **27**	36	−14	−7.7
5′-d(TAG GTT AAT ACT) 3′-d(ATC CA**4** TTA TGA)	**38** **43**	36	−14	−7.7
5′-d(TAG GTG AAT ACT) 3′-d(ATC CAG TTA TGA)	**39** **27**	33	−17	−7.1
5′-d(TAG GTGAAT ACT) 3′-d(ATC CA**4** TTA TGA)	**39** **43**	27	−23	−6.1

[a] Measured in 1 M NaCl, 100 mM $MgCl_2$ and 60 mM Na-cacodylate (pH 7.0) with 5 µM single-strand concentration. [b] Refers to the contribution of the modified residues divided by the number of replacements.

nucleoside **4** forms a strong base pair with dC, which has a similar stability to that of dG–dC. The sulfur-containing compound thioguanine (thioG) decreases the DNA stability significantly when incorporated as a 2′-deoxynucleoside, resulting from spatial requirement of the sulfur and its low H-bonding character. The T_m of one thioG–C base pair in a non-self-complementary 13-mer duplex is 6 °C lower than that of the G–C base pair.[48]

It is reported that 8-oxodG can pair with dA in the *syn* conformation thereby forming a Hoogsteen base pair.[20] Thus, it was of interest to investigate the base-pairing properties of 2′-deoxyimmunosine with the other canonical nucleosides (dA, dT and dG). Hybridization experiments were performed; the duplex melting data are shown in Table 6. As expected, compound **4** forms a weaker interaction with adenine, which is different from oxodG.[19,20] One incorporation opposite to dA (**37**·**43**) reduces the T_m by −19 °C, opposite to dT (**38**·**43**) by −14 °C, and opposite to dG (**39**·**43**) by −23 °C. Regarding these results, 2′-deoxyimmunosine shows an excellent discrimination against all canonical nucleosides. It does not form a Hoogsteen pair due to the absence of a nitrogen at the 7-position (as observed for 7-deaza-2′-deoxyguanosine[23,51]), and is different to 8-oxo-2′-deoxyguanosine, which forms a rather stable base pair with dA.[19,20] Compound **4** shows a better discrimination than dG when incorporated opposite to dA. Although compound **4** forms a *syn* conformation in the solid state, the conformation within the B-DNA duplex has to be *anti*. From the high stability of the **4**–dC base pair, it can be concluded that due to solid-state forces, no conclusion can be drawn regarding the base pairing from the single crystal X-ray structure of a nucleoside, as has been discussed for 8-substituted purine nucleosides such as 8-bromo-2′-deoxyguanosine.[52]

Fig. 8 displays the three-dimensional models of 8-oxo-7-thiaguanine, the nucleobase of 2′-deoxyimmunosine (**4**), the related 7-deazaguanine as well as 8-oxoguanine and guanine. From Fig. 8, it is obvious that the spatial requirements of the nucleobases

Table 7 Molecular masses of oligonucleotides determined by MALDI-TOF mass spectrometry[a]

Oligonucleotides	$[M]^+$ (calc.)	$[M]^+$ (found)
5′-d(AGT ATT **4**AC CTA) (**43**)	3677	3677
5′-d(A**4**T ATT GAC CTA) (**44**)	3677	3677
5′-d(**4**C**4**C**4**C) (**45**)	1892	1892
5′-d(A**4**T ATT **4**AC CTA) (**46**)	3709	3709
5′-d(TA**4** **4**TC AAT ACT) (**47**)	3709	3709

[a] **4** = 2′-deoxyimmunosine.

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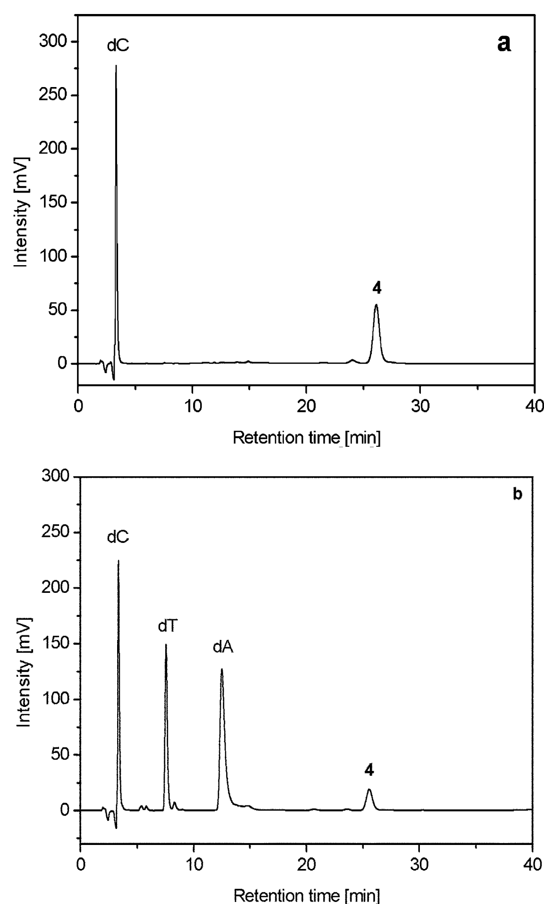

Fig. 6 RP-18 HPLC-elution profile of the enzymatic digests of the oligonucleotides (a) 5′-d(**4**C**4** C**4**C) (**45**) and (b) 5′-d(TA**4** **4**TC AAT ACT) (**47**). Buffer: 5% MeCN in 0.1 M $(Et_3NH)OAc$, pH = 7.0, 1.0 mL min^{-1}. The profile was measured at 247 nm.

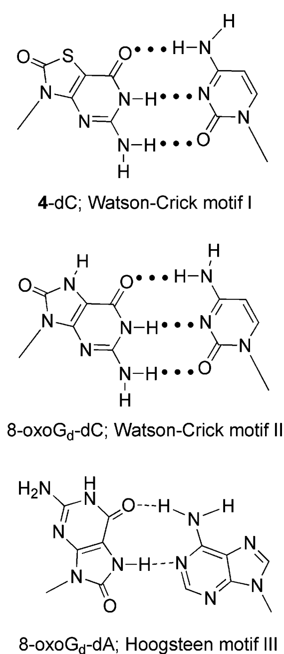

Fig. 7 Base pair motifs of 2′-deoxyimmunosine (**4**) and 8-oxoG_d with dC or dA.

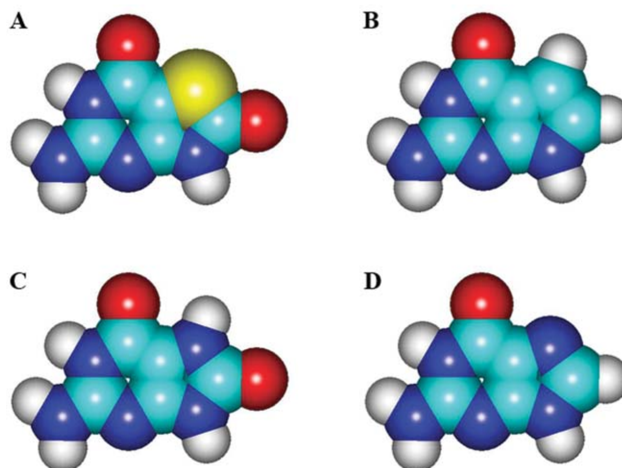

Fig. 8 Molecular models of (A) the immunosine base, (B) 7-deazaguanine, (C) 8-oxoguanine and (D) guanine.

are different. The space-demanding sulfur atom within the five-membered ring of **4** (Fig. 8A) shows a similar size to the hydrogen-bearing 7-carbon in 7-deazaguanine (Fig. 8B). The latter fits nicely in the B-DNA duplex and is well accepted in the form of a nucleoside triphosphate by many DNA-polymerases.[53] Also, the 8-oxo group of compound **4** (base pair motif I in Fig. 7) is well accommodated in a duplex structure, as is demonstrated by the stable base pairs formed with dC. The corresponding 8-oxoguanine (Fig. 8C, base pair motif II in Fig. 7) has a slightly negative effect on the base pair stability compared to that of compound **4**.[19,20] These observations are in line with findings by our laboratory and by others that 7-substituents of moderate size located within the major groove of B-DNA have no or only a little influence on the duplex stability compared to modifications occurring in the minor groove.[54] The better base discrimination of 2′-deoxyimmunosine compared to 8-oxo-2′-deoxyguanosine results from the absence of a 7-hydrogen. This 7-hydrogen can act as hydrogen bond donator while 2′-deoxyimmunosine cannot. The 8-oxo groups have no noticeable negative effect on the duplex stability, as is demonstrated for oligonucleotides containing the immunosine base or 8-oxoguanine. Moreover, the 8-oxo group can act as hydrogen bond acceptor, and binds water molecules or other ligands in the major groove of DNA.

Conclusion

The stereoselective and regioselective nucleobase anion glycosylation of 5-amino-thiazolo[4,5-*d*]pyrimidine-2,7(3*H*,6*H*)-dione has many difficulties because of its bad solubility and the presence of two "lactam functions" which are both prone to glycosylation. Amino group protection with a lipophilic amidine residue resulted in the base derivatives **10** and **18**, which could be glycosylated with 1-chloro-2-deoxy-3,5-di-*O*-(*p*-toluoyl)-α-D-*erythro*-pentofuranose (**6**) in a regioselective and stereoselective way. This selectivity results from the more acidic (and nucleophilic) character of the five-membered ring lactam and the bulky protecting groups of the nucleobase derivative **18**. Compared to dG, the nucleoside **4** is more labile under alkaline conditions but more stable in an acidic medium. Compound **4** has the same capability to form a strong base pair with dC and no base pair with dA;

it cannot form "dG" quartets. The molecular shape models of the 2′-deoxyimmunosine base and related 7-modified derivatives are shown in Fig. 8. Compared to guanine and 7-deazaguanine, the sulfur atom of the immunosine nucleobase is more space demanding. However, the spatial requirements are similar to that of 8-oxoguanine.

Oligonucleotides containing **4** in place of dG within GpC motives have the potential to act as immunostimulatory agents.

Experimental

General

All chemicals were purchased from Acros, or Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). Solvents were of laboratory grade. TLC: Aluminium sheets, silica gel 60 F254 (0.2 mm, VWR International, Darmstadt, Germany). Flash column chromatography (FC): silica gel 60 (VWR International, Darmstadt, Germany) at 0.4 bar. UV Spectra: U-3200 UV-vis spectrometer (Hitachi, Japan). Reverse-phase HPLC was carried out on a 250 × 10 mm RP-18 LiChrosorb column (Merck) with a Merck-Hitachi HPLC pump (model L-7100) connected with a variable wavelength monitor (model L-7400). NMR spectra: Avance-DPX-250, Avance-DPX-300 spectrometer (Bruker, Rheinstetten, Germany); δ values in ppm relative to Me_4Si as internal standard (^1H and ^{13}C) or external 85% H_3PO_4 (^{31}P), J values in Hz. Melting points were determined by a Linström apparatus and are not corrected. Element analyses were performed by Mikroanalytisches Laboratorium Beller, Göttingen, Germany. MALDI-TOF mass spectra were recorded with an Applied Biosystems Voyager DE PRO spectrometer with 3-hydroxypicolinic acid (3-HPA) as a matrix.

Melting curves were measured with a Cary-100 Bio UV/Vis spectrophotometer (Varian, Australia) equipped with a Cary thermo-electrical controller. The calculation of thermodynamic data was performed with the program MeltWin (version 3.0) using the curve fitting of the melting profiles according to a two-state model.

5-Aminothiazolo[4,5-*d*]pyrimidine-2,7(3*H*,6*H*)-dione (5). Compound **5** was prepared as described by Baker and Chatfield.[24,25] δ_H (250 MHz; DMSO-d$_6$; Me_4Si) 12.09 (1H, s, NH), 10.99 (1H, s, NH) and 6.77 (2H, s, NH_2).

5-{[(Dimethylamino)methylidene]amino}thiazolo[4,5-*d*]pyrimidine-2,7(3*H*,6*H*)-dione (10). To a suspension of 5-aminothiazolo[4,5-*d*]pyrimidine-2,7(3*H*,6*H*)-dione (**5**) (1.0 g, 5.43 mmol) in MeOH (30 cm^3), *N*,*N*-dimethylformamide dimethyl acetal (6.0 cm^3, 44.9 mmol) was added and stirred at r.t. for 4 h, resulting in a clear solution. The reaction mixture was adsorbed onto silica gel 60 (2.0 g), and applied on the top of a silica gel column (10 × 5.5 cm). Elution with CH_2Cl_2–MeOH–triethylamine (20 : 1 : 0.2) yielded compound **10** (0.91 g, 70%) as a yellowish solid (Found: C, 40.06; H, 3.35; N, 29.11. $C_8H_9N_5O_2S$ requires C, 40.16; H, 3.79; N, 29.27%); TLC (silica gel, CH_2Cl_2–MeOH 20 : 1) R_f 0.31; UV/Vis: λ_{max}(MeOH)/nm 317 (ε/dm^3 mol^{-1} cm^{-1} 19 100), 289 (sh, 14 600), 246 (14 800) and 212 (17 200); δ_H (250 MHz; DMSO-d$_6$; Me_4Si) 12.19 (1H, s, NH), 11.75 (1H, s, NH), 8.50 (1H, s, N=CH), 3.15 (3H, s, NCH_3) and 3.04 (3H, s, NCH_3).

5-{[(Di-*n*-butylamino)methylidene]amino}thiazolo[4,5-*d*]pyrimidine-2,7(3*H*,6*H*)-dione (18). Compound **5** (1.00 g, 5.43 mmol) was treated with *N*,*N*-dimethyllformamide dibutyl acetal[30] (4.0 cm^3) in the same way as described for **10** at 40 °C. The reaction mixture was evaporated to dryness. The residue was purified by FC (silica gel, column, 15 × 5.5 cm, CH_2Cl_2–CH_3OH 100 : 1) to yield compound **18** (1.32 g, 75%) as colorless solid (Found: C, 52.11; H, 6.54; N, 21.69. $C_{14}H_{21}N_5O_2S$ requires C, 51.99; H, 6.54; N, 21.65%); TLC (silica gel, CH_2Cl_2–CH_3OH 40 : 1): R_f 0.16; UV/Vis: λ_{max}(MeOH)/nm: 319 (ε/dm^3 mol^{-1} cm^{-1} 23 600), 290 (16 700), 249 (16 000); δ_H (250 MHz; DMSO-d$_6$; Me_4Si) 12.16 (1H, s, H–N(3), 11.71 (1H, s, H–N(6)), 8.49 (1H, s, N=CH–C(2)), 3.49–3.35 (4H, m, 2 × NCH_2), 1.54 (4H, m, 2 × CH_2), 1.27 (4H, m, 2 × CH_2) and 0.90 (6H, t, J 7.0, 2 × CH_3).

Glycosylation of 5-aminothiazolo[4,5-*d*]pyrimidine-2,7(3*H*,6*H*)-dione with 1-chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-*erythro*-pentofuranose (6). To a solution of **5** (300 mg, 1.63 mmol) in anhydrous DMF (10 cm^3), NaH (60% suspended in oil, 75 mg, 1.87 mmol) was added under stirring. Stirring was continued at r.t. for 15 min; then 1-chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-*erythro*-pentofuranose (**6**)[55] (951 mg, 2.45 mmol) was added, and stirring was continued for 30 min. The mixture was filtered; the filtrate was evaporated to dryness and the residue was subjected to FC (silica gel, column 10 × 5.5 cm, CH_2Cl_2–MeOH 100 : 1) to yield an anomeric mixture of **7/8** (254 mg, 29%) as yellowish foam of 5-amino-3-[2-deoxy-3,5-di-*O*-(*p*-toluoyl)-D-*erythro*-pentofuranosyl]thiazolo[4,5-*d*]pyrimidine-2,7(3*H*,6*H*)-diones (**7/8**): TLC (silica gel, CH_2Cl_2–MeOH 20 : 1, one spot): R_f = 0.20; HPLC: two peaks, the ratio is 2 : 1.

Glycosylation of 10 with 1-chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-*erythro*-pentofuranose (6). To the solution of compound **10** (500 mg, 2.09 mmol) in dry CH_3CN (200 cm^3), NaH (60% suspended in oil, 92 mg, 2.30 mmol) was added under stirring at r.t. Stirring was continued at r.t. for 15 min, then 1-chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-*erythro*-pentofuranose (894 mg, 2.30 mmol) was added in portions within 20 min. The stirring was continued for another 30 min. Insoluble material was filtered off and the filtrate was evaporated to dryness. The residue was applied to a FC (silica gel, column 10 × 5.5 cm, CH_2Cl_2–CH_3OH 100 : 1) to yield an anomeric mixture of **11/12** as yellowish solid (680 mg, 55%).

3-[2-Deoxy-3,5-di-*O*-(*p*-toluoyl)-D-*erythro*-pentofuranosyl]-5-{[(dimethylamino)methylidene]amino}thiazolo[4,5-*d*]pyrimidine-2,7(3*H*,6*H*)-dione (11/12). TLC (silica gel, CH_2Cl_2–MeOH 40 : 1, one spot): R_f 0.22; HPLC: two peaks, the ratio is 2 : 1. δ_H (250 MHz; DMSO-d$_6$; Me_4Si) taken from the mixture: Isomer I: 8.69 (NH) and 5.79 (q, J 5.7 Hz, H–C(3′)); Isomer II: 8.64 (NH) and 5.51 (q, J 7.7, H–C(3′)).

3-(2-Deoxy-D-*erythro*-pentofuranosyl)-5-{[(dimethylamino)methylidene]amino}thiazolo[4,5-*d*]pyrimidine-2,7(3*H*,6*H*)-dione (13/14). The anomeric mixture of **11/12** (1.2 g, 2.03 mmol) was dissolved in 0.1 M $NaOCH_3$/MeOH (40 cm^3) and was stirred at r.t. for 2 h. The reaction mixture was adsorbed onto silica gel 60 (1.6 g), and applied on the top of a silica gel column (10 × 5.5 cm). Elution with CH_2Cl_2–MeOH 10 : 1 afforded a mixture of **13/14** as a colorless solid (592 mg, 82%). TLC (silica gel, CH_2Cl_2–CH_3OH

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10 : 1): R_f 0.26; δ_H (250 MHz; DMSO-d$_6$; Me$_4$Si) taken from the mixture: Isomer I: 11.93 (s, NH), 8.65 (s, H–N=CH) and 6.36 (t, *J* 6.9, H–C(1′)); Isomer II: 11.92 (s, NH), 8.92 (s, H–N=CH) and 6.21 (t, *J* 7.6, H–C(1′)).

Dimethoxytritylation of the anomeric mixture 13/14. The mixture of nucleosides **13/14** (500 mg, 1.41 mmol) was co-evaporated with pyridine (3 × 10 cm^3) and then dissolved in pyridine (20 cm^3). The solution was treated with 4,4′-dimethoxytriphenylmethyl chloride (713 mg, 2.11 mmol) at room temperature for 1 h, MeOH (3 cm^3) was added, and stirring was continued for 10 minutes. The solution was concentrated to half of the volume, and CH_2Cl_2 (70 cm^3) was added. The organic layer was washed with aqueous $NaHCO_3$ (5%, 50 cm^3), dried over Na_2SO_4, filtered and evaporated, and the residue was applied to FC (silica gel, column 20 × 5 cm, CH_2Cl_2–acetone 4 : 1).

3-[2-Deoxy-5-*O*-(4,4′-dimethoxytriphenylmethyl)-β-D-*erythro*-pentofuranosyl]-5-{[(dimethylamino)methylidene]amino}thiazolo-[4,5-*d*]pyrimidine-2,7(3*H*,6*H*)-dione (15). From the slower migrating zone, compound **15** was isolated (497 mg, 54%) as a colorless foam (Found: C, 62.20; H, 5.30; N, 10.46. $C_{34}H_{35}N_5O_7S$ requires C, 62.09; H, 5.36; N, 10.65%); TLC (silica gel, CH_2Cl_2–acetone 4 : 1): R_f 0.22; UV/Vis: λ_{max} (MeOH)/nm 319 (ε/dm^3 mol^{-1} cm^{-1} 18 800), 282 (15 000) and 235 (28 800); δ_H (250 MHz; DMSO-d$_6$; Me$_4$Si) 11.90 (1H, s, H–N), 8.64 (1H, s, N=CH), 7.37–6.78 (15H, m, arom. H), 6.46 (1H, m, H–C(1′)), 5.27 (1H, d, *J* 4.7, OH–C(3′), 4.32 (1H, m, H–C(3′)), 3.86 (1H, m, H–C(4′)), 3.72 (8H, m, H–C(5′), 2 × OCH_3), 3.16 (3H, s, N–CH_3), 3.05 (3H, s, N–CH_3), 2.78 (1H, m, H–C(2′)-β) and 2.15 (m, 1H, H–C(2′)-α).

3-(2-Deoxy-5-*O*-(4,4′-dimethoxytriphenylmethyl)-α-D-*erythro*-pentofuranosyl)-5-{[(dimethylamino)methylidene]amino}thiazolo-[4,5-*d*]pyrimidine-2,7(3*H*,6*H*)-dione (16). The faster migrating zone yielded compound **16** (253 mg, 26%) as a colorless foam (Found: C, 62.23; H, 5.29; N, 10.47. $C_{34}H_{35}N_5O_7S$ requires C, 62.09; H, 5.36; N, 10.65%); TLC (silica gel, CH_2Cl_2–acetone 4 : 1): R_f 0.38; UV/Vis: λ_{max}(MeOH)/nm 319 (ε/dm^3 mol^{-1} cm^{-1} 18 100), 283 (13 700) and 235 (26 900); δ_H (250 MHz; DMSO-d$_6$; Me$_4$Si) 11.84 (1H, s, H–N), 8.78 (1H, s, N=CH), 7.41–6.86 (15H, m, arom. H), 6.33 (1H, t, *J* 7.5, H–C(1′)), 5.48 (1H, d, *J* 5.0, OH–C(3′), 4.32 (1H, m, H–C(3′)), 4.15 (1H, m, H–C(4′), 3.72 (6H, s, 2 × OCH_3), 3.22 (2H, m, H–C(5′), 3.03 (3H, s, N–CH_3), 2.90 (3H, s, N–CH_3), 2.72 (1H, m, H–C(2′)-β) and 2.56 (1H, m, H–C(2′)-α).

3-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-5-{[(dimethylamino)-methylidene]amino}thiazolo[4,5-*d*]pyrimidine-2,7(3*H*,6*H*)-dione (13). Compound **15** (320 mg, 0.49 mmol) was dissolved in 1% trichloroacetic acid in CH_2Cl_2 (40 cm^3). The solution was stirred at r.t. for 20 min, and then neutralized with triethylamine. The reaction mixture was adsorbed onto silica gel 60 (1.0 g), and applied to the top of a silica gel column (10 × 5.5 cm). Elution with CH_2Cl_2–CH_3OH (10 : 1) gave a white solid. Crystallization from EtOH afforded compound **13** (127 mg, 73%) as colorless needles (Found: C, 43.90; H, 4.90; N, 19.66. $C_{13}H_{17}N_5O_5S$ C, 43.94; H, 4.82, N, 19.71%); m.p. 196 °C (from EtOH); TLC (silica gel, CH_2Cl_2–CH_3OH 10 : 1): R_f 0.26; UV/Vis: λ_{max} (MeOH)/nm 318 (ε/dm^3 mol^{-1} cm^{-1} 19 300), 290 (sh, 13 900), 246 (13 300) and 215 (17 100); δ_H (250 MHz; DMSO-d$_6$; Me$_4$Si) 11.93 (1H, s, NH), 8.65 (1H, s, N=CH), 6.36 (1H, t, *J* 6.9, H–C(1′)), 5.23 (1H, d, *J* 4.6, OH–C(5′)), 4.68 (1H, t, *J* 5.7, OH–C(3′)), 4.31 (1H, m, H–C(3′)), 3.69 (1H, m, H–C(4′)), 3.58, 3.62–3.39 (2H, m, H–C(5′)), 3.20 (3H, s, H–NCH_3), 3.06 (3H, s, H–NCH_3), 2.88 (1H, m, H–C(2′)) and 2.06 (1H, m, H–C(2′)).

3-(2-Deoxy-α-D-*erythro*-pentofuranosyl)-5-{[(dimethylamino)-methylidene]amino}thiazolo[4,5-*d*]pyrimidine-2,7(3*H*,6*H*)-dione (14). Compound **16** (810 mg, 1.23 mmol) was treated as described for compound **15**. Purification by FC (silica gel, column 10 × 5 cm, CH_2Cl_2–CH_3OH 10 : 1) gave a colorless solid. Crystallization from EtOH afforded **14** (319 mg, 73%) as a colorless solid (Found: C, 43.79; H, 4.85; N, 19.60. $C_{13}H_{17}N_5O_5S$ requires C, 43.94; H, 4.82; N, 19.71%); TLC (silica gel, CH_2Cl_2–CH_3OH 10 : 1): R_f 0.26; UV/Vis: λ_{max} (MeOH)/nm 319 (ε/dm^3 mol^{-1} cm^{-1} 20 000), 291 (sh, 14 600), 247 (14 100) and 216 nm (16 700); δ_H (250 MHz; DMSO-d$_6$; Me$_4$Si): 11.92 (1H, s, NH), 8.92 (1H, s, N=CH), 6.21 (1H, t, *J* 7.5, H–C(1′)), 5.47 (1H, d, *J* 4.0, OH–C(5′)), 4.69 (1H, t, *J* 5.44, OH–C(3′)), 4.15 (2H, m, H–C(5′)), 3.61 (1H, m, H–C(3′)), 3.40 (1H, m, H–C(4′)), 3.17 (3H, s, NCH_3), 3.06 (3H, s, NCH_3), 2.88–2.83 (1H, m, H–C(2′)-β) and 2.50 (H, m, H–C(2′)-α).

5-Amino-3-(2-deoxy-β-D-*erythro*-pentofuranosyl)thiazolo[4,5-*d*]pyrimidine-2,7-(3*H*,6*H*)-dione (4). A solution of compound **13** (300 mg, 0.84 mmol) in saturated NH_3–CH_3OH (CH_3OH saturated with NH_3 at 0 °C, 50 cm^3) was stirred at room temperature for 72 h in a sealed bottle. The reaction mixture was adsorbed onto silica gel 60 (1.0 g), and applied to the top of a silica gel column (10 × 5 cm). Elution with CH_2Cl_2–CH_3OH 6 : 1 afforded **4** (229 mg, 91%) as colorless solid (Found: C, 39.94; H, 4.00; N, 18.70. requires $C_{10}H_{12}N_4O_5S$ C, 40.00; H, 4.03; N, 18.66%); Crystallization from EtOH afforded colorless crystals; m.p. 178°C (decomp.); TLC (silica gel, CH_2Cl_2–MeOH 6 : 1): R_f 0.23; UV/Vis: λ_{max} (MeOH)/nm 302 (ε/dm^3 mol^{-1} cm^{-1} 8800), 246 (8500) and 216 (25 900); δ_H (250 MHz; DMSO-d$_6$; Me$_4$Si) 11.22 (s, 1H, NH), 6.92 (s, 2H, NH_2), 6.24 (1H, t, *J* 7.1, H–C(1′)), 5.17 (1H, d, *J* 3.6, OH–C(5′)), 4.66 (1H, m, OH–C(3′)), 4.31 (1H, d, *J* 2.3, H–C(3′)), 3.70 (1H, m, H–C(4′)), 3.53 (2H, m, H–C(5′)), 2.90 (1H, m, H–C(2′)) and 1.98 (m, 1H, H–C(2′)).

5-Amino-3-(2-deoxy-α-D-*erythro*-pentofuranosyl)thiazolo[4,5-*d*]pyrimidine-2,7-(3*H*,6*H*)-dione (9). Compound **14** (300 mg, 0.85 mmol) was treated as described for **4**. FC (silica gel, column 10 × 5 cm, CH_2Cl_2–CH_3OH 6 : 1) afforded compound **9** (225 mg, 88%) as a white solid (Found: C, 39.92; H, 3.93; N, 18.49. requires $C_{10}H_{12}N_4O_5S$ C, 40.00, H, 4.03, N, 18.66%); TLC (silica gel, CH_2Cl_2–MeOH 6 : 1): R_f 0.23; UV/Vis λ_{max}(MeOH)/nm 302 (ε/dm^3 mol^{-1} cm^{-1} 9600), 247 (9100) and 217 (28 100); δ_H (250 MHz; DMSO-d$_6$; Me$_4$Si) 11.24 (1H, s, H–N), 6.89 (2H, s, NH_2), 6.11 (1H, t, *J* 7.4, H–C(1′)), 5.17 (1H, d, *J* 4.8 Hz, OH–C(5′)), 4.68 (1H, t, *J* 5.1 Hz, OH–C(3′)), 4.07 (2H, m, H–C(5′)), 3.58 (1H, m, H–C(3′)), 3.36 (1H, m, H–C(4′)), 2.74 (1H, m, H–C(2′)) and 2.46–2.35 (m, 1H, H–C(2′)).

3-[2-Deoxy-5-*O*-(4,4′-dimethoxytriphenylmethyl)-β-D-*erythro*-pentofuranosyl]-5-{[(dimethylamino)methylidene]amino}thiazolo-[4,5-*d*]pyrimidine-2,7(3*H*,6*H*)-dione 3′-(2-cyanoethyl)diisopropylphosphoramidite (17). To the solution of compound **15** (350 mg, 0.53 mmol) in dry CH_2Cl_2 (10 cm^3), *i*Pr_2EtN (0.17 cm^3, 0.80 mmol) and (2-cyanoethyl)diisopropylphosphoramido chloridite (0.26 cm^3, 1.17 mmol) were added. The solution was stirred

at r.t. for 10 min. The solution was diluted with CH_2Cl_2 (40 cm^3) and washed with 5% aq. $NaHCO_3$ solution. The mixture was extracted with CH_2Cl_2 (2 × 20 cm^3), and the combined organic phase dried (Na_2SO_4), filtered, and evaporated. The residue was purified by FC (column 15 × 5 cm, CH_2Cl_2–acetone 9 : 1) to afford a colorless foam. This was dissolved in CH_2Cl_2 (3 cm^3) and added gradually to stirring cyclohexane (80 cm^3) cooled to −30 °C. The precipitate was isolated by filtration and the powder was dried under vacuum, yielding **17** as a colorless foam (360 mg, 80%). TLC (silica gel, CH_2Cl_2–acetone 4 : 1): R_f 0.32; δ_P (250 MHz; $CDCl_3$; H_3PO_4): 150.0, 149.8 ppm.

Glycosylation of 18 with 2-deoxy-3,5-di-*O*-(*p*-toluoyl)-α-D-*erythro*-pentofuranose chloride (6). To a solution of compound **18** (580 mg, 1.79 mmol) in MeCN (200 cm^3), KOH (626 mg, 8.94 mmol) and TDA-1 tris(3,6-dioxaheptyl)amine (0.2 cm^3, 0.60 mmol) was added and the mixture was stirred at r.t for 20 min. Then 1-chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-*erythro*-pentofuranose (765 mg, 1.97 mmol) was added in portions over 10 min, stirring was continued for another 20 min, and the reaction mixture was filtered and evaporated to dryness. The residue was purified by FC (silica gel, column 15 × 5.5 cm, CH_2Cl_2–MeOH 200 : 1).

3-[2-Deoxy-3,5-di-*O*-(*p*-toluoyl)-β-D-*erythro*-pentofuranosyl)]-5-{[(di-*n*-butylamino)methylidene]amino}thiazolo[4,5-*d*]pyrimidine-2,7(3*H*,6*H*)-dione (19). From the main zone, compound **19** was obtained as a colorless foam (629 mg, 55%) (Found: C, 62.56; H, 6.28; N, 9.98. $C_{35}H_{41}N_5O_7S$ requires C, 62.20, H, 6.12, N, 10.36%); TLC (silica gel, CH_2Cl_2–MeOH 40 : 1): R_f 0.34; UV/Vis: λ_{max} (MeOH)/nm 323 (ε/dm^3 mol^{-1} cm^{-1} 22 200), 284 (14 600) and 242 (41 700); δ_H (250 MHz; DMSO-d_6; Me_4Si) 11.97 (1H, s, HN), 8.68 (1H, s, CH=N), 7.95–7.75 (4H, m, arom. H), 7.39–7.21 (4H, m, arom. H), 6.50 (1H, q, *J* 4.6, H–C(1′)), 5.78 (1H, m, H–C(3′)), 4.58–4.35 (3H, m, H–C(5′), H–C(4′)), 3.46–3.34 (4H, m, NCH_2), 2.38 (3H, s, CH_3), 2.34 (3H, s, CH_3), 1.54–1.06 (8H, m, 4 × CH_2,), 0.88 (3H, t, *J* 7.3, CH_3) and 0.76 (3H, t, *J* 7.3, CH_3).

3-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-5-{[(di-*n*-butylamino)-methylidene] amino}thiazolo[4,5-*d*]pyrimidine-2,7(3*H*,6*H*)-dione (20). Compound **19** (380 mg, 0.56 mmol) was dissolved in 0.1 M $NaOCH_3$ (30 cm^3) and stirred at r.t. for 4 h. The solid residue, obtained after evaporation, was purified by FC (silica gel, column 10 × 5 cm, CH_2Cl_2–CH_3OH 20 : 1) to yield a colorless solid, which was crystallized from ethyl acetate to give **20** (200 mg, 81%) as a colorless solid (Found: C, 52.00; H, 6.59; N, 15.75. $C_{19}H_{29}N_5O_5S$ requires C, 51.92, H, 6.65, N, 15.93%); TLC (silica gel, CH_2Cl_2–MeOH 20 : 1): R_f 0.2; UV/Vis λ_{max} (MeOH)/nm 321 (ε/dm^3 mol^{-1} cm^{-1} 23 400), 292 (15 400), 250 (13 900) and 216 (17 000); δ_H (250 MHz; DMSO-d_6; Me_4Si) 11.89 (1H, s, H–N), 8.62 (1H, s, CH=N), 6.34 (1H, t, *J* 6.9, H–C(1′)), 5.21 (1H, d, *J* 4.6, OH–C(5′)), 4.67 (1H, t, *J* 5.7, OH–C(3′)), 4.33 (1H, m, H–C(3′)), 3.69 (1H, m, H–C(4′)), 3.62–3.41 (6H, m, H–C(5′), 2 × N–CH_2), 2.91 (1H, m, H–C(2′)), 2.06 (1H, m, H–C(2′)), 1.58 (4H, m, 2 × CH_2), 1.28 (4H, m, 2 × CH_2) and 0.91 (6H, t, *J* 7.2, 2 × CH_3).

5-Amino-3-(2-deoxy-β-D-*erythro*-pentofuranosyl)thiazolo[4,5-*d*]pyrimidine-2,7-(3*H*,6*H*)-dione (4). Compound **19** (256 mg, 0.58 mmol) was dissolved in concentrated ammonia solution (30 cm^3) and stirred at room temperature for 72 h. The reaction mixture was adsorbed onto silica gel 60 (1.0 g), and applied to the top of a silica gel column (8 × 5 cm). Elution with CH_2Cl_2–CH_3OH (5 : 1) afforded a colorless solid. Crystallization from EtOH afforded colorless crystals of **4** (132 mg, 76%). Analytical data were the same as that for compound **4** obtained from compound **13**.

3-(2-Deoxy-5-*O*-(4,4′-dimethoxytriphenylmethyl)-β-D-*erythro*-pentofuranosyl)-5-{[(di-*n*-butylamino)methylidene]amino}thiazolo[4,5-*d*]pyrimidine-2,7(3*H*,6*H*)-dione (21). Compound **20** (220 mg, 0.50 mmol) was co-evaporated with pyridine (3 × 10 cm^3) and then dissolved in pyridine (15 cm^3). This solution was treated with 4,4′-dimethoxytriphenylmethyl chloride (270 mg, 0.79 mmol) at room temperature for 1 hour, MeOH (3 cm^3) was added, and stirring continued for 10 minutes. The solution was concentrated to half of the volume, and CH_2Cl_2 (50 cm^3) was added. The organic layer was washed with aqueous $NaHCO_3$ (5%, 50 cm^3), and the organic phase was dried over Na_2SO_4, filtered and evaporated. The residue was purified by FC (column 20 × 5 cm, CH_2Cl_2–acetone 20 : 1), yielding **21** (220 mg, 60%) as a white foam (Found: C, 64.28; H, 6.32; N, 9.40. $C_{40}H_{47}N_5O_7S$ requires C 64.76, H 6.39, N 9.44%); TLC (silica gel, CH_2Cl_2–acetone 4 : 1): R_f 0.38; UV/Vis: λ_{max}(MeOH)/nm 322 (ε/dm^3 mol^{-1} cm^{-1} 21 300), 283 (15 700), 235 (28 500); δ_H (250 MHz; DMSO-d_6; Me_4Si) 11.82 (1H, s, H–N), 8.60 (1H, s, CH=N), 7.42–6.69 (13H, m, arom H), 6.44 (1H, q, *J* 4.1, H–C(1′)), 5.22 (1H, m, OH–C(3′)), 4.30 (1H, m, H–C(3′)), 3.86 (1H, m, H–C(4′)), 3.71 (6H, s, 2 × OCH_3), 3.50–3.18 (6H, m, H–C(5′), 2 × N–CH_2), 2.81 (1H, m, H–C(2′)), 2.16 (1H, m, H–C(2′)), 1.58–1.50 (4H, m, 2CH_2), 1.36–1.17 (4H, m, 2 × CH_2) and 0.96–0.8 (6H, m, 2 × CH_3).

3-(2-Deoxy-5-*O*-(4,4′-dimethoxytriphenylmethyl)-β-D-*erythro*-pentofuranosyl-5-{[(di-n-butylamino)methylidene]amino}thiazolo[4,5-*d*]pyrimidine-2,7(3*H*,6*H*)-dione 3′-(2-cyanoethyl)diisopropylphosphoramidite (22). To the solution of compound **21** (160 mg, 0.22 mmol) in dry CH_2Cl_2 (10 cm^3), (iPr)$_2$EtN (0.060 cm^3, 0.33 mmol) and (2-cyanoethyl)diisopropylphosphoramido chloridite (0.074 cm^3, 0.33 mmol) were added. The solution was stirred at r.t. for 15 min. The solution was diluted with CH_2Cl_2 (40 cm^3) and washed with 5% aq. $NaHCO_3$ solution. The mixture was extracted with CH_2Cl_2 (2 × 20 cm^3). The combined organic phase was dried (Na_2SO_4), filtered, and evaporated. The residue was purified by FC (column 15 × 5 cm, CH_2Cl_2–acetone 15 : 1) to afford a white foam. The foamy residue was dissolved in CH_2Cl_2 (3 cm^3) and added gradually to stirring cyclohexane (80 cm^3) cooled to −30 °C. The precipitate was isolated by filtration and the resulting powder was dried under vacuum, yielding compound **22** as a colorless foam (161 mg, 80%). TLC (silica gel, CH_2Cl_2–acetone 4 : 1): R_f 0.5; δ_P (250 MHz; $CDCl_3$; H_3PO_4) 150.0, 149.8.

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