DNA with stable fluorinated dA and dG substitutes: syntheses, base pairing and ^{19}F-NMR spectra of 7-fluoro-7-deaza-2′-deoxyadenosine and 7-fluoro-7-deaza-2′-deoxyguanosine

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Fluorinated DNA containing stable fluorine substituents in the "purine" base were synthesized for the first time. For this, the phosphoramidites of 7-fluoro-7-deaza-2′-deoxyadenosine and 7-fluoro-7-deaza-2′-deoxyguanosine were prepared and oligonucleotides were synthesized. The 7-fluoro substitution leads to increased duplex stability and more selective base pairing compared to the non-functionalized 7-deazapurine oligonucleotides. ^{19}F NMR spectra of fluorinated nucleosides, single stranded oligonucleotides and DNA duplex show only a single signal for one fluorine modification. The NMR sensitive ^{19}F spin or the positron emitting ^{18}F isotope make these compounds applicable for DNA detection or imaging *in vitro* and *in vivo.*

Introduction

Fluorinated nucleosides develop extraordinary pharmacological activities as chemotherapeutic agents for the treatment of cancer or as antiviral drugs.[1] Fluorinated oligonucleotides are stabilized against enzymatic degradation and polyfluorinated oligonucleotides are employed in fluorous affinity purification.[2] The fluorine isotopes are used for molecular imaging or as molecular tags. The positron emitting isotope [^{18}F] ($\tau = 109.8$ min) finds clinical application in *in vivo* imaging by positron emission tomography (PET) using [^{18}F] labelled molecules. By this means, the hypermetabolic activity of proliferating cells can be followed in tissues. On the other hand, the NMR sensitive ^{19}F isotope is a valuable tool to study DNA or RNA secondary structure[3] by NMR spectroscopy or to investigate the binding of nucleic acids to proteins or other cellular components. Sugar fluorinated nucleosides,[4] in particular the 2′-deoxy-2′-fluoroarabino nucleosides have been used as nucleoside mimics in DNA and RNA chemistry, and molecular biology.[5–8] However, a fluorine atom in the 2′-position of the sugar moiety of nucleosides and oligonucleotides induces conformational changes depending on the antipodal position of incorporation (2′-ribo or 2′-arabino).[8–12] This effect can be circumvented when the fluorine atom is introduced into the nucleobase.

From a steric point of view, the fluorine atom introduced into a nucleobase is expected to lead only to minor perturbation of the molecule shape. This results from the small van der Waals radius of fluorine (1.47 Å) compared to other halogens and being similar to that of hydrogen (1.20 Å). As 5-fluorinated pyrimidine nucleosides are readily accessible and reasonably stable, 5-fluorinated uracil/thymine and cytosine nucleosides have been incorporated in DNA or RNA either chemically by solid-phase synthesis or enzymatically employing polymerases.[13] Single crystal X-ray analyses showed that a fluorine atom present in the 5-position of a pyrimidine moiety is well accommodated in the duplex structures.[14,15] It was also reported that a 5-fluorouridine residue at a single site of RNA stabilizes the duplex due to more favorable stacking interactions.[16]

Compared to the easier access to fluorinated pyrimidine nucleosides, the introduction of fluorine atoms into purine nucleosides encounters difficulties, as fluorine substituents in the 2- or 8-position are labile and subject to nucleophilic displacement reactions (purine numbering is used throughout the general section). A fluorine in the 2-position of adenosine or 2′-deoxyadenosine[17] is easily displaced by nucleophiles such as amines.[18] 2-Fluoro-7-deaza-2′-deoxyadenosine is more stable and oligonucleotides with this modification were prepared recently.[19] Nevertheless, the replacement of a hydrogen atom by fluorine in the 2-position has an unfavorable effect on the dA–dT base pair stability.[19] By changing the fluorination side of a purine base from the 2- to the 8-position, the situation becomes even more complex already on the nucleoside level.[20] The preparation encounters synthetic difficulties and defluorination reactions are occurring in basic and acidic media.[21] Thus, there has been no report on the incorporation of a purine nucleobase fluorinated in the 8-position of the adenine or the guanine moiety, neither in DNA, RNA or in short oligonucleotides.

Our laboratory and others have selected the 7-position of 7-deazapurines as the target site for fluorination reactions. Recently, the syntheses of 7-deaza-7-fluoro-2′-deoxyadenosine (**1b**) and 7-deaza-7-fluoro-2′-deoxyguanosine (**2b**) have been reported[22] and the X-ray analysis of **1b** was performed.[23] Now, we are studying the behavior of oligonucleotides incorporating the fluorinated 7-deazapurine nucleosides **1b** and **2b**. The non-functionalized nucleosides **1a** and **2a**, as well as other halogenated derivatives including **1c** and **2c**, were already introduced into the DNA molecule (Scheme 1).[19,24–26] This manuscript describes for the

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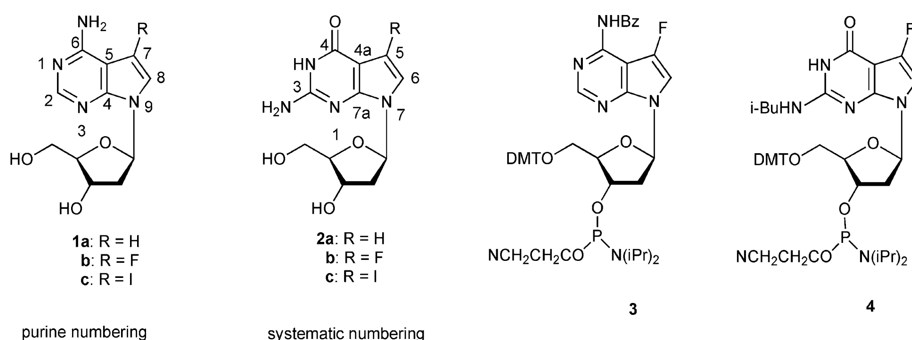


Scheme 1 Structure of nucleosides and the corresponding phosphoramidites.

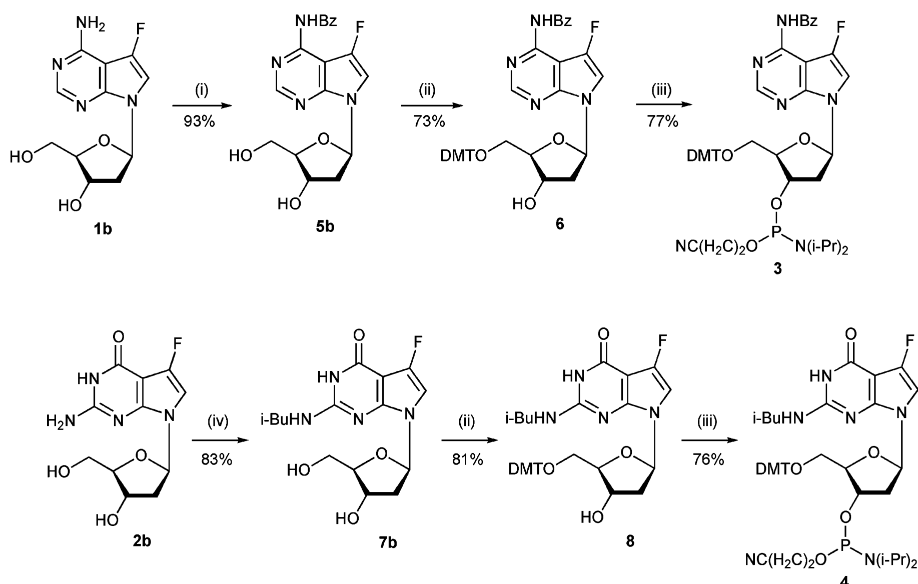
first time the solid-phase synthesis of stable fluorinated oligonucleotides with fluorine atoms in the "purine" moiety employing the phoshoramidites **3** and **4**. It reports on the thermal and chemical stability of base pairs formed by the 7-fluorinated nucleosides **1b** or **2b**. It demonstrates that the ^{19}F NMR signal can be used to detect fluorinated "purine" bases in oligonucleotide single strands and duplexes by ^{19}F-NMR spectroscopy. Thus the modification has the potential to be employed in DNA detection or imaging.

Results and discussion

1. Synthesis and properties of the monomers

The synthesis of 7-fluoro-7-deaza-2′-deoxyadenosine (**1b**) and 7-fluoro-7-deaza-2′-deoxyguanosine (**2b**) has been already reported by our laboratory.[22] The phosphoramidites **3** and **4** were prepared from **1b** and **2b** in a three-step route (Scheme 2). As amino protecting groups, the benzoyl residue (Bz) was chosen for compound **1a**[27] and the isobutyryl residue (*i*-Bu) for **2a**.[28,29] These protecting groups were also employed here for the 7-fluorinated nucleosides using the protocol of transient protection[30] (**1b**, **2b** → **5b**, **7b**, Scheme 2). The amino-protected intermediates **5b** and **7b** were converted into the 5′-*O*-DMT derivatives **6** and **8** under standard conditions with DMT-Cl in pyridine. Phosphitylation with 2-cyanoethyl-*N*,*N*-diisopropylphosphoramidochloridite in anhydrous CH_2Cl_2 (*i*-Pr_2EtN) furnished the phosphoramidites **3** and **4** (Scheme 2).

The structures of all new compounds were established by ^{1}H-, ^{13}C-, ^{19}F- and ^{31}P-NMR spectroscopy, as well as by mass spectrometry and elemental analyses (Table 1 and experimental part). Assignment of the ^{13}C-NMR resonance was made on the basis of gated-decoupled ^{1}H-^{13}C-NMR spectra and by using J_{CF} coupling constants as described in earlier publications.[22,31] Table 1 summarizes the ^{13}C NMR chemical shifts and Table 2 the ^{19}F chemical shifts and the J_{CF} coupling constants. The observed C-7 chemical shifts of the 7-fluorinated 7-deazapurine nucleosides **1b** and **2b**[22]($\delta \approx 140$ ppm) are found downfield compared to the non-functionalized nucleosides **1a** and **2a** ($\delta \approx 100$ ppm).[24,25] The other 7-halogen substitutions lead either to a small downfield shift ($\Delta = 1$–2 ppm upon 7-chlorination)[24,25] or an up-field shift ($\Delta \approx -15$ ppm upon bromination and $\Delta \approx -50$ ppm upon iodination).[24,25,32,33] The 7-fluoro atom causes $^{1}J_{C7,F}$, $^{2}J_{C8,F}$, and $^{2}J_{C5,F}$ couplings, as was observed for the sugar modified nucleosides



Scheme 2 *Reagents and conditions*: (i) TMSCl, BzCl, pyridine; (ii) 4,4′-dimethoxytriphenylmethyl chloride, anhydrous pyridine; (iii) 2-cyanoethyl-*N*,*N*-diisopropylphosphoramidochloridite, *N*,*N*-diisopropylethylamine, dichloromethane; (iv) TMSCl, isobutyric anhydride, pyridine.

Table 1 ^{13}C NMR chemical shifts (δ) of nucleosides[a]

	C(2)[b]	C(4)	C(4a)	C(5)	C(6)	C(7a)	C(1′)	C(2′)	C(3′)	C(4′)	C(5′)	CO	OMe
	C(2)[c]	C(6)	C(5)	C(7)	C(8)	C(4)							
1a	151.6	157.5	102.9	99.6	121.6	149.7	83.3	[d]	71.2	87.3	62.2	—	
1b[22]	152.7	155.8	92.3	142.6	103.9	145.8	82.4	[d]	70.9	87.2	61.9	—	
5a[24]	150.0	151.0	109.4	103.1	124.3	152.0	82.9	[d]	70.9	87.3	61.9	165.7	
5b	150.1	151.2	101.9	141.6	108.9	147.3	82.5	[d]	70.9	87.4	61.8	166.5	
6	150.1	151.2	102.0	141.6	108.8	147.3	82.5	[d]	70.6	85.5	64.2	166.6	55.0
2a[25]	152.5	158.5	100.0	102.1	116.7	150.5	82.2	[d]	70.8	86.9	61.9	—	
2b[22]	153.0[e]	156.9[e]	89.9	145.6	99.5	147.6[e]	82.6	[d]	70.8	87.4	61.8	—	
7a[25]	152.5	158.5	100.0	102.1	116.7	150.5	82.2	[d]	70.8	86.9	61.9		
7b	147.4	154.9	93.6	145.4	102.2	143.5	82.3	34.8	70.9	87.3	61.8	180.2	
8	147.4	154.9	93.8	145.3	102.2	143.5	82.3	34.8	70.5	85.4	64.2	180.2	54.9

[a] Measured in d_6-DMSO at 298 K. [b] Systematic numbering. [c] Purine numbering. [d] Superimposed by DMSO. [e] Tentative.

Table 2 ^{19}F NMR chemical shifts (δ)[a,b] and J_{FC}[c] coupling constants of nucleosides

Compound	δ (F-7)[b]	$^1J_{C7,F7}$	$^2J_{C8,F7}$	$^2J_{C5,F7}$	$^3J_{C4,F7}$	$^3J_{C6,F7}$
1b	−167.95	248.1	28.4	15.8	[d]	[d]
9	−168.30	245.3	30.2	15.7	[d]	[d]
10	−168.00	244.8	26.8	15.7	[d]	[d]
5	−163.79	248.1	28.4	15.8	[d]	[d]
6	−163.60	247.8	27.9	15.3	[d]	[d]
2b	−167.89	247.8	27.1	12.4	[d]	[d]
7b	−165.93	247.8	26.8	13.8	3.2	2.6
8	−165.94	247.9	26.5	13.8	3.1	2.8

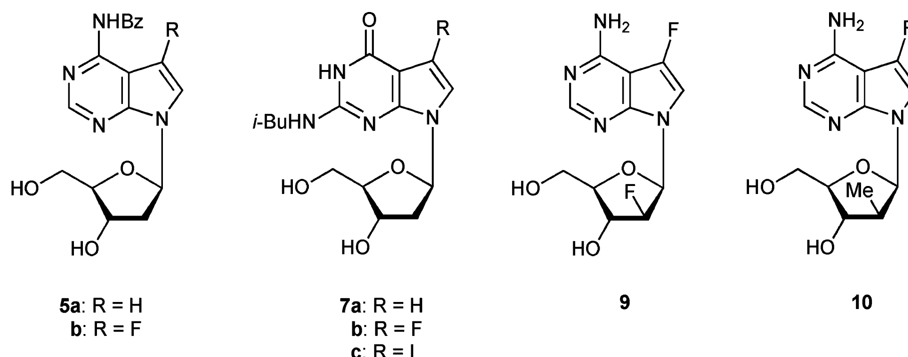
[a] Measurements were performed in d_6-DMSO at 25 °C. [b] Data are taken from ^{19}F NMR spectra with $CFCl_3$ as reference. [c] Data are taken from ^{13}C NMR spectra. [d] Smaller than 1 Hz.

9[22] and **10**.[34] The protected 7-fluoro-7-deaza-2′-deoxyguanosine derivatives show long range couplings ($^3J_{C4,F}$ and $^3J_{C6,F}$) (Table 2).

Next, the stability of compounds **5b** and **7b** was studied. The half life values of both compounds were determined UV-spectrophotometrically in 25% aqueous NH_3 at 40 °C in a sealed vessel. In the "dG" series, the half life is 94 min for **7b**, which is similar to that of **7a** (109 min).[28] The corresponding values for the "dA" derivatives are significantly longer with 530 min for **5b**, compared to 320 min for **5a**[28] (Scheme 3). Both **5b** and **5a** were fully deprotected at 60 °C in 25% aqueous NH_3. However, in the case of 7-fluoro-7-deaza-2′-deoxyguanosine, the deprotection of **7b** was accompanied by partial decomposition (see HPLC profile of Fig. 1a compared to 1b). This decomposition was not observed in the case of the non-halogenated derivative **7a** or the corresponding 7-iodo derivative **7c** (Fig. 1c). Consequently, mild deprotection conditions were chosen for **7b** and deprotection was complete after 24 hours' treatment in 25% ammonia, as monitored by HPLC, without significant decomposition (Fig. 1d). Thus, for the synthesis of oligonucleotides containing **2b**, canonical phosphoramidites with *tert*-butylphenoxyacetyl (tac) protecting groups were employed, which can be deprotected in aqueous ammonia at room temperature.

2. Synthesis and duplex stability of oligonucleotides containing the fluorinated nucleosides 1b and 2b

Oligonucleotide synthesis was carried out on a solid phase with an ABI 392-08 synthesizer at a 1 μmol scale employing the phosphoramidites **3** and **4**, as well as standard building blocks. The coupling yields were always higher than 95%. For oligonucleotides without modification, or those containing **1b**, the synthesis of oligonucleotides was performed by employing the DMT-on mode. After cleavage from the solid support, the oligonucleotides were deprotected in 25% aq. NH_3 for 16 h at 60 °C. The synthesis of oligonucleotides incorporating **2b** used *tert*-butylphenoxyacetyl (tac) protected canonical phosphoramidites employing the DMT-off mode and mild deprotection conditions (25% aq. NH_3, room temperature, 24 h). The oligonucleotides were purified by reversed-phase HPLC. The detailed procedure for oligonucleotide purification is reported in the experimental part. The molecular masses of all synthesized oligonucleotides were determined by



Scheme 3 Structure of nucleosides.

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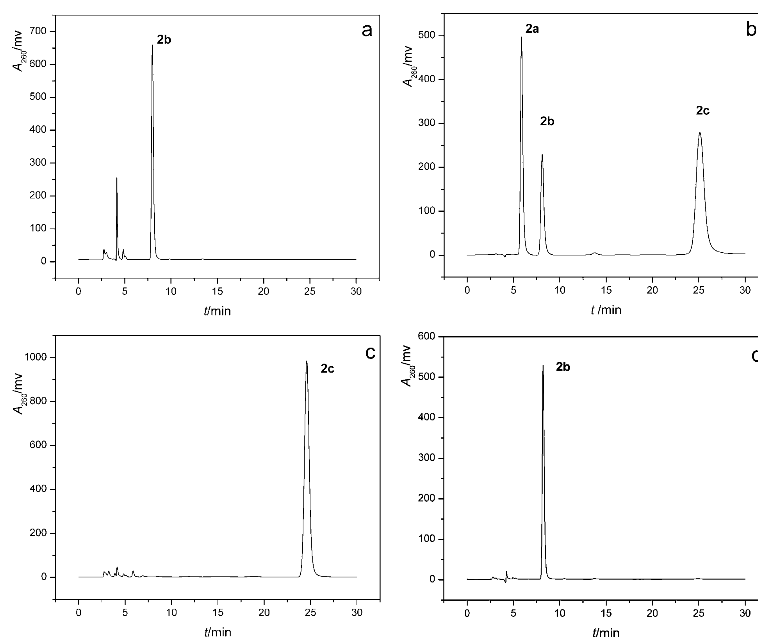

Fig. 1 Reversed-phase HPLC (RP-18) profiles of deprotection of **7b** with 25% aqueous ammonia at 60 °C for 16 h (a); an artificial mixture of compounds **2a–c** (b); deprotection of **7c** with 25% aqueous ammonia at 60 °C for 16 h (c) and deprotection of **7b** with 25% aqueous ammonia at room temperature (d). The mixtures were analyzed by reversed-phase HPLC at 260 nm on a RP-18 column (250 × 4 mm). Gradient: 4% MeCN, 96% buffer [buffer: 0.1M $(Et_3NH)OAc$ (pH 7.0)–MeCN, 95 : 5]. Flow rate = 0.7 cm^3 min^{-1}.

MALDI-TOF mass spectra (Table 6, experimental part). They were in agreement with the calculated values.

The base pair stabilities of halogenated 7-deazapurine nucleosides with Cl, Br, I in the 7-position of 7-deaza-2′-deoxyadenosines and 7-deaza-2′-deoxyguanosines have already been reported.[19,24–26,33] However, due to synthetic problems, nothing is known about fluorinated derivatives. Therefore, the effect of 7-fluorine substitution in 7-deazapurines has now been studied for the first time by comparing the duplex stabilities and mismatch discrimination of the 7-fluorinated nucleosides (**1b**, **2b**) with the parent compounds (**1a**, **2a**) and the 7-iodo derivatives (**1c**, **2c**). The hybridization experiments were performed using the duplexes 5′-d(TAGGTCAATACT) (**11**) and 3′-d(ATCCAGTTATGA) (**12**) as references. The modified duplexes contain single or multiple incorporations of fluorine-modified molecules at various positions. The base pairing of the oligonucleotides with the four canonical nucleosides opposite to the modification sites was also investigated.

Table 3 summarizes the T_m values of duplex oligonucleotides containing **1a–c**, **2a–c** with one to twelve modifications. According to the Table, the replacement of one or two dA-residues by 2′-deoxytubercidin (**1a**) has a slight destabilizing effect on the non-self complementary duplex stability (T_m = 50 °C for duplex **13**·**12** and **11**·**14**). For comparison, compound **1b** introduced at the same positions stabilizes the duplexes (T_m = 51 °C, 53 °C for **15**·**12** and **11**·**16**) as well as compound **1c**. Also, the 7-deaza-2′-deoxyguanosine (**2a**) has a destabilizing effect on the duplex in place of dG (T_m = 50 or 49 °C with one or more modifications). Like in the "2′-deoxyadenosine" series, the 7-fluorination of the 2′-deoxyguanosine analogue (→**2b**) led to the stabilization of the duplexes (T_m = 51–53 °C with one or more modifications). This stabilizing effect is comparable to that of the 7-iodo derivative **2c** (Table 3), which also stabilizes the duplexes under the same conditions. The step-wise stability increase by the various halogen substituents is most clearly seen in the case of the self-complementary dodecamers **29–32**. Both the fluorinated and the iodinated residues have a positive effect on the base pair stability. The duplex stability increases with the increasing van der Waals radii of the 7-halogens (Table 3).

Fig. 2 shows the space-filling molecular models of adenine, 7-deazaadenine and the halogenated 7-deazapurines. It is apparent that the fluorine substituent is less space demanding than the other halogens. So, in enzymatic reactions within the limited space in a catalytic active center of an enzyme, the fluoro compounds are expected to be excellent substrates with favorable properties over the other halogenated derivatives. As the non-functionalized 7-deazapurine nucleosides (**1a** and **2a**) in the form of their triphosphates are already excellent substrates for the polymerase chain reaction[35] or sequencing protocols[36], it is expected that the 7-fluoro compounds **1b** and **2b** are also excellent mimics of the canonical DNA constituents.

Apart from Watson–Crick base pairing, we wanted to investigate the mismatch discrimination of the fluorinated nucleosides **1b** and **2b**. Therefore, the base pairing of compounds **1b**, **2b** against the four canonical nucleosides was studied. For this investigation, the T_m values of 12-mer duplexes containing the modified nucleosides located opposite the four canonical constituents were prepared and T_m values were measured (Table 4 and Table 5). From earlier investigations[19] and the data shown in Table 5, it is apparent that 7-deaza-2′-deoxyadenosine (**1a**) generates a strong base pair not only with dT (T_m = 50 °C) but also with dG (T_m = 47 °C) and dC (T_m = 44 °C), while a significant discrimination occurs against dA (T_m = 33 °C). Although the 7-fluoro derivative **1b** strengthens the base pairs with dT (T_m = 53 °C), dG (T_m = 51 °C) and dA

Table 3 T_m values and thermodynamic data of oligonucleotide duplexes containing **1a–c**, **2a–c**[a]

Duplexes		T_m/°C	ΔG[b]			T_m/°C	ΔG[b]
5′-d(TAGGTCAATACT) 3′-d(ATCCAGTTATGA)	**11** **12**	51	−13.0	5′-d(TAGGTCAATACT) 3′-d(ATCCA**2a**TTATGA)	**11** **21**	50	−12.0
5′-d(TAGGTC**1a**ATACT) 3′-d(ATCCAGTTATGA)	**13**[19] **12**	50	−12.7	5′-d(TAGGTCAATACT) 3′-d(ATCCA**2a**TTAT**2a**A)	**11** **22**[26]	50	−11.8
5′-d(TAGGTCAATACT) 3′-d(ATCC**1a**GTT**1a**TGA)	**11** **14**[19]	50	−11.3	5′-d(TA**2a2a**TCAATACT) 3′-d(ATCCAGTTATGA)	**23**[26] **12**	49	−12.0
5′-d(TAGGTC**1a**ATACT) 3′-d(ATCC**1a**GTT**1a**TGA)	**13** **14**	48	−11.0	5′-d(TA**2a2a**TCAATACT) 3′-d(ATCCA**2a**TTAT**2a**A)	**23** **22**	49	−11.5
5′-d(TAGGTC**1b**ATACT) 3′-d(ATCCAGTTATGA)	**15** **12**	51	−10.7	5′-d(TAGGTCAATACT) 3′-d(ATCCA**2b**TTATGA)	**11** **24**	51	−10.7
5′-d(TAGGTCAATACT) 3′-d(ATCC**1b**GTT**1b**TGA)	**11** **16**	53	−13.0	5′-d(TAGGTCAATACT) 3′-d(ATCCA**2b**TTAT**2b**A)	**11** **25**	52	−11.5
5′-d(TAGGTC**1b**ATACT) 3′-d(ATCC**1b**GTT**1b**TGA)	**15** **16**	52	−11.8	5′-d(TA**2b2b**TCAATACT) 3′-d(ATCCAGTTATGA)	**26** **12**	52	−11.6
5′-d(TAGGTC**1b1b**TACT) 3′-d(ATCCAGTTATGA)	**17** **12**	53	−12.4	5′-d(TA**2b2b**TCAATACT) 3′-d(ATCCA**2b**TTAT**2b**A)	**26** **25**	53	−12.2
5′-d(T**1b**GGTC**1b1b**T**1b**CT) 3′-d(ATCCAGTTATGA)	**18** **12**	52	−12.0				
5′-d(T**1b**GGTC**1b1b**T**1b**CT) 3′-d(ATCC**1b**GTT**1b**TGA)	**18** **16**	53	−11.7				
5′-d(TAGGTC**1c**ATACT) 3′-d(ATCCAGTTATGA)	**19**[19] **12**	51	−11.9	5′-d(TAGGTCAATACT) 3′-d(ATCCA**2c**TTAT**2c**A)	**11** **27**[28]	52	−11.9
5′-d(TAGGTCAATACT) 3′-d(ATCC**1c**GTT**1c**TGA)	**11** **20**[19]	54	−13.2	5′-d(TA**2c2c**TCAATACT) 3′-d(ATCCAGTTATGA)	**28**[28] **12**	51	−11.6
5′-d(TAGGTC**1c**ATACT) 3′-d(ATCC**1c**GTT**1c**TGA)	**19** **20**	54	−12.2	5′-d(TA**2c2c**TCAATACT) 3′-d(ATCCA**2c**TTAT**2c**A)	**28** **27**	53	−12.1
5′-d(**ATATATATATAT**)[c] 3′-d(**TATATATATATA**)	**29**[24] **29**	33	0.22	5′-d(**1bT1bT1bT1bT1bT1bT**)[c] 3′-d(**T1bT1bT1bT1bT1bT1b**)	**31** **31**	48	−1.9
5′-d(**1aT1aT1aT1aT1aT1aT**)[c] 3′-d(**T1aT1aT1aT1aT1aT1a**)	**30**[24] **30**	36	0.0	5′-d(**1cT1cT1cT1cT1cT1cT**) 3′-d(**T1cT1cT1cT1cT1cT1c**)	**32**[33] **32**	59	−11.4

[a] Measured at 260 nm in 1 M NaCl, 0.1 M $MgCl_2$, 60 mM Na-cacodylate, pH 7, at 5 μM + 5 μM of single strand concentration. [b] ΔG values are given in Kcal mol^{-1} and within 10% errors. [c] 15 μM of single strands.

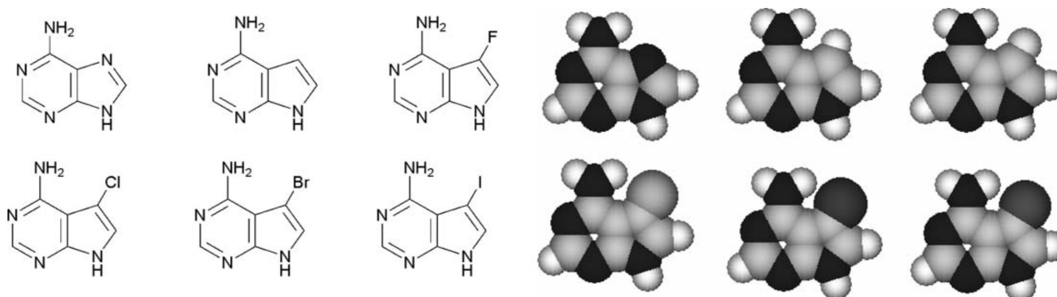

Fig. 2 Structure of nucleobases (left) and the corresponding space-filling models (right). Van der Waals radii (Å) are: H = 1.2; F = 1.47; Cl = 1.75; Br = 1.85; I = 1.98. Electronegativities are: H = 2.2; F = 3.98; Cl = 3.16; Br = 2.96; I = 2.66.

(T_m = 39 °C) when compared to the parent compound **1a**, the pairing discrimination against dC is significantly better in the case of the fluorinated nucleoside **1b** than **1a**. The mispairing of **1b** with dG is not unexpected as stable dA–dG base pairs (Fig. 3, motif I) have already been detected in many other cases including dA–dG or c^7A_d–dG pairs (Fig. 3, motif II).[37–40] It was already reported that the stability of this mispair is sequence dependent. Also, tandem base pairs are better stabilized than single substitutions. Table 4

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Table 4 T_m values (°C) of duplexes 5′-d(TAGGTXAATACT)-3′·3′-d(ATCCAYTTATGA) with mismatches opposite to dG and **2a**,**b**[a,b]

X·Y	Duplex	T_m/°C	ΔT_m/°C	ΔG/Kcal mol^{-1}
C·G	**11·12**	51		−13.0
G·G	**33·12**	30	−21	−6.4
A·G	**34·12**	35	−16	−7.3
T·G	**35·12**	36	−15	−7.8
C·**2a**	**11·21**	50		−12.0
G·**2a**	**33·21**	30	−20	−5.9
A·**2a**	**34·21**	36	−14	−7.5
T·**2a**	**35·21**	38	−12	−8.3
C·**2b**	**11·24**	51		−10.7
G·**2b**	**33·24**	33	−18	−7.1
A·**2b**	**34·24**	31	−20	−6.5
T·**2b**	**35·24**	38	−13	−7.8

[a] Measured at 260 nm in 1 M NaCl, 0.1 M $MgCl_2$, 60 mM Na-cacodylate buffer, pH 7.0, with 5 μM single-strand concentration. [b] The data in parentheses are ΔT_m ($T_m^{\text{base mismatch}} - T_m^{\text{base match}}$).

Table 5 T_m values (°C) of duplexes 5′-d(TAGGXCAATACT)·3′-d(ATCCYGTTYTGA) with mismatches opposite to A and **1a–c**[a,b]

X·Y	Duplex	T_m/°C	ΔT_m/°C	ΔG/Kcal mol^{-1}
T·A	**11·12**	51		−13.0
A·A	**36·12**	40	−11	−8.7
G·A	**37·12**	47	−4	−10.1
C·A	**38·12**	37	−14	−7.9
T·**1a**	**11·14**	50		−11.3
A·**1a**	**36·14**	33	−16	−6.9
G·**1a**	**37·14**	47	−3	−10.6
C·**1a**	**38·14**	44	−6	−9.7
T·**1b**	**11·16**	53		−13.0
A·**1b**	**36·16**	39	−14	−8.4
G·**1b**	**37·16**	51	−2	−11.0
C·**1b**	**38·16**	43	−10	−9.8
T·**1c**	**11·20**	54		−13.2
A·**1c**	**36·20**	41	−13	−8.8
G·**1c**	**37·20**	53	−1	−12.1
C·**1c**	**38·20**	43	−11	−10.0

[a] Measured at 260 nm in 1 M NaCl, 0.1 M $MgCl_2$, 60 mM Na-cacodylate buffer, pH 7.0, with 5 μM single-strand concentration. [b] The data in parentheses are ΔT_m ($T_m^{\text{base mismatch}} - T_m^{\text{base match}}$).

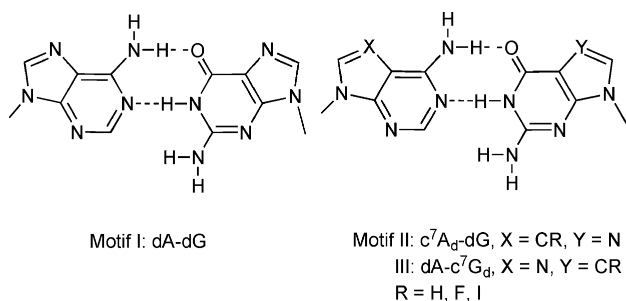

Fig. 3 Base pair motifs of dA–dG, c^7A_d–dG and dA–c^7G_d.

shows the mispairing of dA–**2a**,**b** (Fig. 3, motif III). Obviously a dA (or **1a–c**)–dG (or **2a**,**b**) pair between two dA–dT pairs (dA–dG, T_m = 35 °C, Table 4) is less stable than that between two dG–dC base pairs (dA–dG, T_m = 47 °C, Table 5).

The base pairing of protonated dA with dC[41] or of the protonated 7-deaza-2′-deoxyanosine with dC[19] was already reported. In the case of **1a**, it was found that a decreasing pH value[19] resulted in higher T_m values, which is in line with protonated base pair formation (Fig. 4, motifs IV and V). As the pH value of protonation of compound **1a** is higher (pK_a = 5.08) compared to dA (pK_a = 3.5), it is obvious that at neutral pH, compound **1a** forms stronger mispairs with dC than dA. Table 5 shows the base discrimination of oligonucleotides with **1a–c** against dC. The ΔT_m values are calculated on the basis of the corresponding dA (c^7A_d)–dT base-pair T_m values to eliminate the effect of the second modification. Compared to the 7-non functionalized compound **1a** (ΔT_m = −6 °C), the 7-fluorinated compound **1b** (ΔT_m = −10 °C) shows a better mismatch discrimination being similar to dA (ΔT_m = −14 °C). Therefore, at pH 7.0, a higher proportion of protonated species is formed in the case of compound **1a** (pK_a 5.1) compared to **1b** (pK_a 4.4).[18] Thus, the 7-fluorinated 7-deaza-2′-deoxyadenosine (**1b**) behaves more as a dA analogue than the non-fluorinated **1a**. The proposed base pair motifs are shown in Fig. 4.

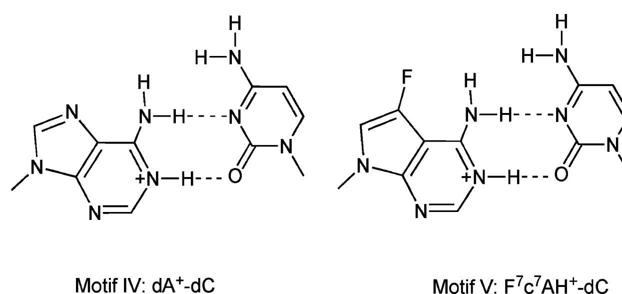

Fig. 4 Base pare motifs for dA and **1b** with dC.

^{19}F NMR spectroscopy is widely used for the structure elucidation of monomeric nucleosides as well as for oligonucleotides.[3,10,42] As the fluorine nucleus is surrounded on average by 9 electrons, rather than by a single electron in the case of hydrogen, the range of fluorine chemical shifts and the sensitivity to local environment changes are used as sensitive reporters. At present, fluorinated purine nucleosides cannot be used for this purpose due to stability problems. The ^{19}F spectra of the fluorinated nucleosides **2b** and the oligonucleotide **24** incorporating **2b** can be used, and spectra were measured in aqueous buffer solution. The data for the monomers measured in DMSO-d_6 are already summarized in Table 2. All 7-fluorinated 7-deazapurine nucleosides show only one ^{19}F NMR signal at approximately −165 ppm when measured in d_6-DMSO (see Table 2). This signal is not split when the proton-coupled mode is used. As we wanted to confirm that the measurement was correct, the corresponding spectrum of the bis-fluorinated compound **9** (Scheme 3, see above) was undertaken. The latter shows only one singlet for the 7-fluorine atom at −168.30 ppm (Table 2) and a multiplet at −199.68 ppm for the 2′-fluoro substituent with a $^2J_{F,H2'}$ coupling and two $^3J_{F,H}$ couplings.[22] Thus, it was confirmed that a possible coupling of the 7-fluorine atom with any proton is smaller than 1 Hz, which is the detection limit of our NMR machine.

In aqueous solutions (0.1 M NaCl, 10 mM $MgCl_2$, and 10 mM Na-cacodylate buffer), the chemical shift of 7-fluorine of **2b** is shifted downfield to −165.96 ppm compared to −166.67 in d_6-DMSO (Fig. 5). Next, the oligonucleotide **24** incorporating one **2b** residue was measured also showing only one signal at

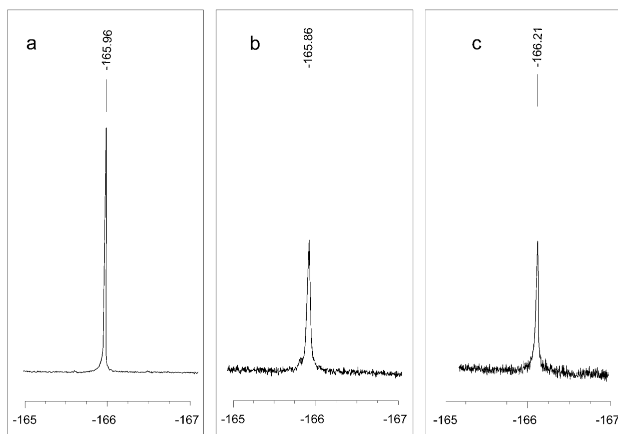

Fig. 5 ^{19}F NMR spectra ($CFCl_3$ as external standard and measured at 298 K) of compound **2b** (a); oligonucleotide **24** (b) and duplex **11·24** (c). Samples were dissolved in 0.4 mM in 1 M NaCl, 100 mM $MgCl_2$ and 60 mM Na-cacodylate buffer, pH 7.0.

−165.86 ppm with an almost identical chemical shift to that of the monomeric **2b** measured in the same solvent (Fig. 5). This indicates that in the single-stranded chain, the microenvironment and the solvation of the 7-fluoro substituent are rather similar to that of the monomeric nucleoside. The situation changes when compound **2b** is part of a base pair stack as in the duplex **11·24**. Now, the fluorine signal is shifted upfield to −166.21 ppm.

3. Conclusion

7-Fluoro substituents were introduced in 7-deaza-2′-deoxyadenosine (→**1b**) and 7-deaza-2′-deoxyguanosine (→**2b**) and corresponding phosphoramidites (**3**, **4**) were prepared as well as oligonucleotides containing this modification. The 7-fluoro substituent introduced in the 7-position of 7-deaza-2′-deoxydenosine (**1b**) stabilizes the base pair with dT and improves mismatch discrimination. A stabilization of the base pair of **2b** with dC is observed. While fluorine in the 2- or 8-position of purine nucleosides is labile, the 7-fluorinated 7-deazapurine nucleosides are stable enough to be detected by ^{19}F NMR spectroscopy in single stranded oligonucleotides or duplex DNA. Thus, the fluorinated 7-deazapurines are applicable to DNA detection and imaging *in vitro* as well as in living systems.

Experimental

General

All chemicals were purchased from Acros, Aldrich, Sigma, or Fluka (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). Solvents were of laboratory grade. Thin-layer chromatography (TLC) was performed on a TLC aluminium sheet covered with silica gel 60 F254 (0.2 mm, VWR International, Germany). Column flash chromatography (FC): silica gel 60 (VWR International, Darmstadt, Germany) at 0.4 bar; UV-spectra were recorded on a U-3200 spectrophotometer (Hitachi, Japan), λ_{max} in nm, ε in $dm^3\ mol^{-1}\ cm^{-1}$. NMR spectra: Avance DPX 250 or DPX 300 spectrometers (Bruker Germany); δ values in ppm relative to Me_4Si as internal standard, $CFCl_3$ as external standard for ^{19}F, or 85% H_3PO_4 for ^{31}P. The *J*-values are given in Hz. Reversed-phase HPLC was carried out on a 4 × 250 mm RP-18 (10 μm) LiChroscorb column (VWR International) with a Merck-Hitachi HPLC pump (Model 655 A-12) connected with a variable wavelength monitor (model 655-A), a controller (model L-500) and an integrator (model D-2000). Elemental analyses were performed by the Mikroanalytisches Laboratorium Beller, Göttingen, Germany. MALDI-TOF mass spectra were recorded on a Biflex-III spectrometer (Bruker, Leipzig, Germany) in the reflector mode and on an Applied Biosystems Voyager DE PRO spectrometer in the linear mode. Half-life values (τ) and the melting curves were measured with a Cary-100 Bio UV/Vis spectrophotometer (Varian, Australia) and all values were measured more than twice and they were repeatable.

Synthesis and characterization of oligonucleotides

The oligonucleotide synthesis was performed on an ABI 392–08 synthesizer, model 392–08 (Applied Biosystems, Weiterstadt, Germany) at a 1 μmol scale (trityl on mode) employing phosphoramidites and following the synthesis protocol for 3′-cyanoethyl phosphoramidites (User's Manual of the DNA Synthesizer, Applied Biosystems, Weiterstadt, Germany). The average coupling yield was always higher than 95%. After cleavage from the solid-support, the oligonucleotides were deprotected in 25% aq. NH_3 for 16 h at 60 °C or 24 h at r.t. for oligonucleotides containing **2b**. The DMT-containing oligonucleotides were purified by reversed-phase HPLC (RP-18) with the following solvent gradient system [A: 0.1 M $(Et_3NH)OAc$ (pH 7.0)–MeCN 95 : 5; B: MeCN; gradient *I*: 0–3 min 10–15% B in A, 3–15 min 15–50% B in A, 15–20 min 50–10% B in A, flow rate 1.0 $cm^3\ min^{-1}$]. Then, the mixture was evaporated to dryness, and the residue was treated with 2.5% $Cl_2CHCOOH–CH_2Cl_2$ for 3 min at 0 °C to remove the 4,4′-dimethoxytrityl residues. Oligonucleotides containing **2b** were synthesized in the DMT-off mode to avoid acidic conditions after deprotection of the amino group. The detritylated oligomers were purified by reversed-phase HPLC with the gradient *II*: 0–25 min 0–20% B in A, 25–30 min 20% B in A, 30–35 min 20–0% B in A, flow rate 1.0 $cm^3\ min^{-1}$. The oligomers were desalted on a short column (RP-18, silica gel) using H_2O for elution of the salt, while the oligomers were eluted with $MeOH–H_2O$ (3 : 2). The oligonucleotides were lyophilized on a Speed Vac evaporator to yield colourless solids which were stored frozen at −24 °C.

The molecular masses of the oligonucleotides were determined in positive mode by MALDI-TOF Biflex-III mass spectrometry (Bruker Saxonia, Leipzig, Germany) for compounds containing **1b** or in negative mode by an Applied Biosystems Voyager DE PRO spectrometer for compounds containing **2a** or **2b** with 3-hydroxypicolinic acid (3-HPA) as a matrix. The detected masses were identical with the calculated values (Table 6).

N^4-(Benzoyl)-7-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-fluoro-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine (5b)

To a stirred suspension of 4-amino-7-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-fluoro-7*H*-pyrrolo[2,3-*d*]pyrimidine (**1b**,[22] 220 mg, 0.82 mmol) in 4.1 cm^3 of dry pyridine was added trimethyl chlorosilane (TMSCl, 0.53 cm^3, 4.1 mmol) at r.t. After 30 min, benzoyl chloride (0.49 cm^3, 4.1 mmol) was added. Stirring

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Table 6 Molecular masses determined from MALDI-TOF mass spectra

	Mass (calcd)	Mass (found)
5′-d(TAGGTC**1b**ATACT)-3′ (**15**)	3659.7	3659.0
5′-d(AGT**1b**TTG**1b**CCTA)-3′ (**16**)	3676.6	3677.4
5′-d(TAGGTC**1b1b**TACT)-3′ (**17**)	3676.6	3677.3
5′-d(T**1b**GGTC**1b1b**T**1b**CT)-3′ (**18**)	3711.6	3711.3
5′-d(**1b**T**1b**T**1b**T**1b**T**1b**T**1b**T)-3′ (**31**)	3742.6	3743.0
5′-d(AGTATT**2a**ACCTA)-3′ (**21**)	3641.6	2642.0
5′-d(AGTATT**2b**ACCTA)-3′ (**24**)	3659.7	3660.5
5′-d(A**2b**TATT**2b**ACCTA)-3′ (**25**)	3676.6	3677.0
5′-d(TA**2b2b**TCAATACT)-3′ (**26**)	3676.6	3677.1

was continued for 2 h and the mixture was cooled in an ice bath. Water (0.82 cm^3) was added and after 5 min, aqueous ammonia (1.64 cm^3, 25%) was added and the mixture was kept stirring at r.t. for 30 min. The reaction mixture was then evaporated to dryness, dissolved in methanol, adsorbed on silica gel (1.5 g) and applied to flash chromatography (FC) on the top of a silica gel column (10 × 2 cm). Elution with CH_2Cl_2–CH_3OH (30 : 1–20 : 1) yielded **5b** as colourless solid (284 mg, 93%) (Found: C, 58.10; H, 4.70; N, 14.86%. $C_{18}H_{17}FN_4O_4$ requires C, 58.06; H, 4.60; N, 15.05%); TLC (silica gel, CH_2Cl_2–CH_3OH, 10 : 1): R_f 0.31; λ_{max}(MeOH)/nm 312 (ε/dm^3 mol^{-1} cm^{-1} 6100), 276 (6400) and 227 (25300); δ_H (250 MHz; [d_6]DMSO; Me_4Si) 2.20–2.30 (1 H, m, 2′-H_α), 2.44–2.54 (1 H, m, 2′-H_β), 3.54 (2 H, m, 5′-H), 3.84 (1 H, m, 4′-H), 4.36 (1 H, m, 3′-H), 4.98 (1 H, m, 5′-OH), 5.32 (1 H, s, 3′-OH), 6.73 (1 H, t, *J* 6.3, 1′-H), 7.48–7.68 (3 H, m, arom. H), 7.75 (1 H, d, *J* 1.9, 6-H), 8.66 (1 H, s, 2-H), 8.05 (2 H, m, arom. H) and 11.27 (1 H, br s, NH).

N^4-(Benzoyl)-7-[2-deoxy-5-*O*-(4,4′-dimethoxytriphenylmethyl)-β-D-*erythro*-pentofuranosyl]-5-fluoro-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine (6)

Compound **5b** (564 mg, 1.51 mmol) was dried by repeated co-evaporation with anhydrous pyridine (3 × 3 cm^3) and dissolved in anhydrous pyridine (3 cm^3). This solution was treated with 4,4′-dimethoxytriphenylmethyl chloride ((MeO)$_2$TrCl; 623 mg, 1.84 mmol) at r.t. while stirring (3 h). The mixture was coevaporated with toluene to remove pyridine and the oily residue was applied to FC (silica gel, column 2.5 × 10 cm). Elution with CH_2Cl_2–CH_3OH (100 : 1) furnished **6** as slight yellow foam (744 mg, 73%) (Found: C, 69.29; H, 5.36; N, 8.15%. $C_{39}H_{35}FN_4O_6$ requires C, 69.42; H, 5.23; N 8.30%); TLC (silica gel, CH_2Cl_2–CH_3OH, 20 : 1): R_f 0.19; λ_{max}(MeOH)/nm 312 (ε/dm^3 mol^{-1} cm^{-1} 6200), 275 (9900) and 229 (41 700); δ_H(250 MHz; [d_6]DMSO; Me_4Si) 2.33 (1 H, m, 2′-H_α), 2.60 (1 H, m, 2′-H_β), 3.13–3.21 (2 H, m, 5′-H), 3.73 (6 H s, 2 × OCH_3), 3.97 (1 H, m, 4′-H), 4.38 (1 H, m, 3′-H), 5.39 (1 H, d, *J* 4.4, 3′-OH), 6.75 (1 H, t, *J* 6.5, 1′-H), 6.84–6.87 (4 H, m, arom. H), 7.22–7.28 (7 H, m, arom. H), 7.38 (2 H, m, arom. H), 7.54–7.66 (4 H, m, arom. H and 6-H), 8.05 (2 H, m, arom. H), 8.67 (1 H, s, 2-H) and 11.30 (1 H, br s, NH).

N^4-(Benzoyl)-7-[2-deoxy-5-*O*-(4,4′-dimethoxytriphenylmethyl)-β-D-*erythro*-pentofuranosyl]-5-fluoro-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine 3′-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (3)

To a solution of compound **6** (337 mg, 0.5 mmol) in anhydrous CH_2Cl_2 (4.5 cm^3) were added *N*,*N*-diisopropylethylamine (0.26 cm^3, 1.48 mmol) and 2-cyanoethyldiisopropylphosphoramidochloride (167 µl, 0.75 mmol) while stirring at r.t. The stirring was continued for 30 min and the reaction mixture diluted with CH_2Cl_2 (15 cm^3). The solution was washed with an aqueous solution of 5% $NaHCO_3$ (2 × 20 cm^3). The organic layer was separated and dried over Na_2SO_4, filtrated and evaporated to an oil. The residue was applied to FC (silica gel, column 2.5 × 8 cm), elution with CH_2Cl_2–acetone–Et_3N 10 : 1 : 0.2) gave a colorless foam (338 mg, 77%); TLC (silica gel, CH_2Cl_2–acetone 10 : 1): R_f 0.5; δ_P(101 MHz; $CDCl_3$; H_3PO_4) 149.9 and 150.0.

7-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-3,7-dihydro-2-(isobutyrylamino)-5-fluoro-4*H*-pyrrolo[2,3-*d*]pyrimidin-4-one (7b)

Compound **2b**[22] (180 mg, 0.63 mmol) was dried by repeated co-evaporation with pyridine (3 × 5 cm^3). The residue was suspended in pyridine (3.7 cm^3) and Me_3SiCl (0.53 cm^3, 4.2 mmol) was added at r.t. while stirring. After 30 min, the solution was treated with isobutyric anhydride (0.53 cm^3, 3.2 mmol) and the mixture was kept stirring at r.t. for 3 h. The mixture was cooled in an ice bath, H_2O (0.6 cm^3) was added, followed by the addition of 25% aqueous ammonia (1.2 cm^3) after 5 min. The solution was stirred for another 15 min and evaporated. The residue was applied to FC (silica gel, column 2.5 × 8 cm, eluted with CH_2Cl_2–CH_3OH 30 : 1→15 : 1) to yield **7b** as a colourless solid (186 mg, 83%) (Found: C, 50.74; H, 5.30%. $C_{15}H_{19}FN_4O_5$ requires C, 50.84; H 5.40%); TLC (silica gel, CH_2Cl_2–CH_3OH, 10 : 1): R_f 0.19; λ_{max}(MeOH)/nm 294 (ε/dm^3 mol^{-1} cm^{-1} 12 600); δ_H(250.13 MHz; [d_6]DMSO; Me_4Si) 1.08 (6 H, m, 2 × CH_3), 2.13 (1 H, m, 2′-H), 2.35 (1 H, m, 2′-H), 2.73 (1 H, m, Me_2CH), 3.50 (2 H, m, 5′-H), 3.77 (1 H, m, 4′-H), 4.30 (1 H, m, 3′-H), 4.94 (1 H, br s, 5′-OH), 5.26 (1 H, br s, 3′-OH), 6.43 (1 H, t, *J* 6.6, 1′-H), 7.19 (1 H, s, 6-H), 11.59 (1 H, br s, NH) and 12.00 (1 H, br s, NH); *m*/*z* (ESI-TOF) 377.1248 (M + Na^+. $C_{15}H_{19}FN_4O_5Na$ requires 377.1237).

7-[2-Deoxy-5-*O*-(4,4′-dimethoxytriphenylmethyl)-β-D-*erythro*-pentofuranosyl]-3,7-dihydro-2-(isobutyrylamino)-5-fluoro-4*H*-pyrrolo[2,3-*d*]pyrimidin-4-one (8)

Compound **7b** (150 mg, 0.42 mmol) was dried by repeated co-evaporation with anhydrous pyridine and dissolved in anhydrous pyridine (2.3 cm^3). The solution was treated with 4,4′-dimethoxytriphenylmethyl chloride (300 mg, 0.9 mmol) at r.t. while stirring (3 h). The reaction mixture was evaporated and the residue was applied to FC (silica gel, column 2.5 × 10 cm, eluted with CH_2Cl_2–H_3OH 50 : 1) to yield **8** as a slightly yellowish foam (225 mg, 81%) (Found: C, 65.86; H, 5.60; N, 8.54%. $C_{36}H_{37}FN_4O_7$ requires C, 65.84; H, 5.68; N, 8.53%); TLC (silica gel, CH_2Cl_2–CH_3OH, 20 : 1): R_f 0.12; λ_{max}(MeOH)/nm 276 (ε/dm^3 mol^{-1} cm^{-1} 15 200), 283 (15 200) and 297 (14 200); δ_H (250 MHz; [d_6]DMSO; Me_4Si) 1.12 (6 H, m, 2 × CH_3), 2.22 (1 H, m, 2′-H_α), 2.46 (1 H, m, 2′-H_β), 2.75 (1 H, m, Me_2CH), 3.13 (2 H, m, 5′-H), 3.73 (6 H, s, 2 × OCH_3), 3.90 (1 H, m, 4′-H), 4.30 (1 H, m, 3′-H), 5.34 (1 H, d, *J* 2.4, 3′-OH), 6.43 (1 H, t, *J* 6.3 Hz, 1′-H), 6.83 (4 H, m, arom. H), 7.03 (1 H, s, 6-H), 7.21–7.38 (9 H, m, arom. H) and 11.59 (2 H, br s, 2 × NH); *m*/*z* (ESI-TOF) 679.2560 (M + Na^+. $C_{36}H_{37}FN_4O_7Na$ requires 679.2544).

7-[2-Deoxy-5-*O*-(4,4′-dimethoxytriphenylmethyl)-β-D-*erythro*-pentofuranosyl]-3,7-dihydro-2-(isobutyrylamino)-5-fluoro-4*H*-pyrrolo[2,3-*d*]pyrimidin-4-one 3′-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (4)

To a solution of compound **8** (200 mg, 0.30 mmol) in anhydrous CH_2Cl_2 (3 cm^3) were added *N*,*N*-diisopropylethylamine (0.18 cm^3, 1.09 mmol) and 2-cyanoethyldiisopropylphosphoramidochloride (192 μl, 0.85 mmol) while stirring at r.t. The stirring was continued for 30 min and the reaction mixture diluted with CH_2Cl_2 (15 cm^3). The solution was washed with an aqueous solution of 5% $NaHCO_3$ (2×20 cm^3). The organic layer was separated, dried over Na_2SO_4, and evaporated. The residue was applied to FC (silica gel, column, 2.5×8 cm, CH_2Cl_2–acetone–Et_3N 10 : 1 : 0.2) to give a colourless foam (200 mg, 77%); TLC (silica gel, CH_2Cl_2–acetone 10 : 1): R_f 0.5; δ_P(101 MHz; $CDCl_3$; H_3PO_4) 148.6. and 149.1.

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