pH-Dependent mismatch discrimination of oligonucleotide duplexes containing 2'-deoxytubercidin and 2- or 7-substituted derivatives: protonated base pairs formed between 7-deazapurines and cytosine

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ABSTRACT

Oligonucleotides incorporating 2'-deoxytubercidin (1a), its 2-amino derivative 2a and related 2-, or 7-substituted analogs (1d, 2b–d, 3 and 4) are synthesized. For this purpose, a series of novel phosphoramidites are prepared and employed in solid-phase synthesis. Hybridization experiments performed with 12mer duplexes indicate that 7-halogenated nucleosides enhance the duplex stability both in antiparallel and parallel DNA, whereas 2-fluorinated 7-deaza-2'-deoxyadenosine residues destabilize the duplex structure. The 7-deazaadenine nucleosides 1a, 1d and their 2-amino derivatives 2a–d form stable base pairs with dT but also with dC and dG. The mispairing with dC is pH-dependent. Ambiguous base pairing is observed at pH 7 or under acid conditions, whereas base discrimination occurs in alkaline medium (pH 8.0). This results from protonated base pairs formed between 1a or 2a and dC under neutral or acid condition, which are destroyed in alkaline medium. It is underlined by the increased basicity of the pyrrolo[2,3-d]pyrimidine nucleosides over that of the parent purine compounds (pK_a values: 1a = 5.30; 2a = 5.71; dA = 3.50).

INTRODUCTION

Mutations in the DNA molecule are the basis of evolution. It is widely accepted that tautomerism of the canonical nucleobases and the formation of wobble base pairs play an important role in this phenomenon (1,2). To keep the number of errors low, enzymatic proofreading during nucleoside triphosphates incorporation takes place with the help of polymerases (3). DNA mutation is caused by mismatches of the normal bases because of a failure of proofreading during DNA replication (4). DNA is also damaged continuously by oxidation, by depurination, by light or other processes occurring within the cellular environment. The daily number of errors in a human is estimated to be several thousands. This damage is removed by repair enzymes (5).

Several diagnostic tools have been developed to detect such single nucleotide polymorphisms (SNPs) by hybridization in solution or on polymer surfaces (biochips). Modified nucleosides are used in these protocols as fluorescent dyes to be anchored to them without disturbing the DNA structure (6,7). 7-Deazapurine nucleoside triphosphates are commonly used for these purposes (6–8). Thus, the knowledge about their recognition properties and base discrimination is of mutual interest. Mismatch discrimination is evaluated from the difference in melting temperatures (T_m) between matched and mismatched base pairs within an oligonucleotide duplex. For a given mismatch, the properties of the modified nucleosides incorporated in the DNA chain and the environmental conditions are of utmost importance for the stability of base pairs. Although studies on the mispairing of modified nucleosides have been performed, little attention has been paid to the influence of the pH values of the reaction medium on the recognition of canonical and modified nucleosides.

Among the modified nucleosides, 7-deazapurine (pyrrolo[2,3-d]pyrimidine) nucleosides and 7-substituted derivatives (purine numbering is used throughout the discussion) have attracted attention because they closely resemble the structure of purine nucleosides and are therefore ideal shape mimics of the canonical DNA constituents. They are well accepted by DNA polymerases and made a significant contribution to DNA and RNA sequencing and diagnostics (6–9). Reporter groups that are necessary to generate high-sensitivity probes are usually introduced at the 7-position of a 7-deazapurine giving them steric freedom in duplex DNA. Substituents of
moderate size incorporated into the DNA chain have shown to increase duplex stability with the potential of a better mismatch discrimination (10–14).

2'-Deoxytubercidin (1a) and its 2-amino derivative 2a can substitute 2'-deoxyadenosine (dA) without significantly changing the base pair stability with dT (15,16) (see also Table 2). Studies on the pK\textsubscript{a} values of 7-deazapurine nucleosides show that compared to the parent dA (pK\textsubscript{a} = 3.50) (17) compounds 1a (pK\textsubscript{a} = 5.30) (18) and 2a (pK\textsubscript{a} = 5.71; Supplementary Data) are much more easily protonated. Moreover, it is shown that the pK\textsubscript{a} values of nucleobases present in stacked oligonucleotides can be significantly higher due to the attractive force of the phosphodiester backbone for the protons (17). Thus, the pK\textsubscript{a} values of nucleobases are shifted by one or two pK\textsubscript{a} units towards neutral conditions. This indicates that 7-deazaadenine nucleosides such as 1a or 2a as constituents of oligonucleosides might be protonated already under neutral conditions. In order to investigate this matter in more detail, 2'-deoxytubercidin (1a) as well as 2', 7-substituted derivatives (1d, 2a–d, 3) or 4 were incorporated into oligonucleotides and their hybridization properties were studied (Scheme 1). For this, the phosphoramidites (5a–d and 6) were synthesized and the base pair stability as well as the pH-dependent mismatch discrimination of oligonucleotides were investigated.

**MATERIALS AND METHODS**

**General**

All chemicals were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Solvents were of laboratory grade. Snake-venom phosphodiesterase (EC 3.1.15.1, *Crotalus adamanteus*) and alkaline phosphatase (EC 3.1.3.1, *Escherichia coli*) were gifts from Roche Diagnostics GmbH (Taufkirchen, Germany). All chemicals were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Solvents were of laboratory grade. All chemicals were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Solvents were of laboratory grade. Snake-venom phosphodiesterase (EC 3.1.15.1, *Crotalus adamanteus*) and alkaline phosphatase (EC 3.1.3.1, *Escherichia coli*) were gifts from Roche Diagnostics GmbH (Taufkirchen, Germany). The phosphoramidites related to compounds 1a, 1d and 4 were synthesized as described previously: 4-benzoylamino-7-[2-deoxy-5-O-(4,4'-dimethoxytriphenylmethyl)-\beta-D-erythro-pentofuranosyl]-7H-pyrrrolo[2,3-d]pyrimidine \(3'(2\text{-cyanoethyl})\)-N,N-diisopropylphosphoramidite (19), 7-[2-deoxy-5-O-(4,4'-dimethoxytriphenylmethyl)-\beta-D-erythro-pentofuranosyl]-4-[(dimethoxymethyl)imino]-5-iodo-7H-pyrrrolo[2,3-d]pyrimidine \(3'(2\text{-cyanoethyl})\)-N,N-diisopropylphosphoramidite (20) and 7-[2-deoxy-5-O-(4,4'-dimethoxytriphenylmethyl)-\β-D-erythro-pentofuranosyl]-2-formylamino-7H-pyrrrolo[2,3-d]pyrimidine \(3'(2\text{-cyanoethyl})\)-N,N-diisopropylphosphoramidite (21). The standard phosphoramidites are commercial materials bought from Proligo (Hamburg, Germany). Thin-layer chromatography (TLC) was performed on TLC aluminium sheets covered with silica gel 60 F\textsubscript{254} (0.2 mm, VWR International, Darmstadt, 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). NMR spectra were measured on an Avance-250 or AMX-500 spectrometers (Bruker, Rheinetten, Germany). Chemical shifts (\(\delta\)) are given in p.p.m. relative to internal Me\textsubscript{3}Si or external H\textsubscript{3}PO\textsubscript{4} (\(31P\)). The \(J\)-values are given in Hz. Elemental analyses were performed by the Mikroanalytisches Laboratorium Beller, Göttingen, Germany.

**Oligodeoxyribonucleotides**

The oligonucleotide synthesis was performed on an ABI 392-08 synthesizer (Applied Biosystems, Weiterstadt, Germany) on a 1.0 \(\mu\)mol scale using the phosphoramidites 5a–d, 6 as well as those of the canonical 2'-deoxyribonucleosides (Proligo, Hamburg, Germany) following the synthesis protocol for 3'-cyanoethyl phosphoramidite chemistry (22). The phosphoramidites related to compounds 1a (19), 1d (20) and 4 (21) were also employed. The average coupling yield of the modified phosphoramidites was always \(>98\%\). After cleavage from the solid-support, the oligonucleotides were deprotected in 25% aq. NH\textsubscript{3} for 16–18 h at 60°C. The synthesis of oligonucleotides incorporating the 2-fluoro nucleoside 3 used BPA-protected (tert-butylphenoxyacetyl) canonical phosphoramidites and employing ultra mild deprotection conditions (25% aq. NH\textsubscript{3}, room temperature, 2 h). If the deprotection was performed at elevated temperature (25% aq. NH\textsubscript{3}, 60°C, 20–24 h), the 2-fluoro substituent was displaced by an amino group. This conversion can be used to synthesize oligonucleotides containing the diamino nucleoside 2a using the ‘fluoro’ phosphoramidite 6 instead of 5a. The oligonucleotides were purified by reversed-phase HPLC. The detailed procedure for oligonucleotide purification is shown in Supplementary Data.

The compositions of oligonucleotides was determined by reversed-phase HPLC (RP-18) after tandem enzymatic hydrolysis with snake-venom phosphodiesterase (EC 3.1.15.1, *C.adamanteus*) followed by alkaline phosphatase (EC 3.1.3.1, *E.coli* from Roche Diagnostics GmbH, Germany) (11)
UV thermal denaturation curves were acquired on a Cary 1/3 UV/VIS spectrophotometer (Varian, Australia) equipped with a Cary thermoelectrical controller. All thermal measurements were conducted in 0.1 M NaCl, 10 mM MgCl₂ and 10 mM sodium cacodylate buffer, with 5 μM single-strand concentration. The extinction coefficients at 260 nm of monomers were calculated from the sum of the extinction coefficients of the monomeric 2′-deoxyribonucleosides corrected by the hypochromicity. The hypochromicity (h = [(ε_{monomer} - ε_{polymer}) × (ε_{monomer})⁻¹] × 100%) was determined from the absorbance before and after enzymatic digestion with snake-venom phosphodiesterase (EC 3.1.15.1, C. adamanteus) [for details see (11)]. The hypochromicity was ~20% for all oligonucleotides (ε_{260} of monomers: Cl₂C₄Gd 6350, daA 15400, dG 11700, dC 7600, m²C₄d 6300, 2a 8100, 2b 8200, 2c 7700, 2d 7800, 3 9800, 4 4100). Absorbance versus temperature spectra were collected at 260 nm over a range of 10–85°C with 0.1°C increments and a heating rate of 1.0°C/min. Samples were annealed by heating rapidly to 85°C for 10–15 min, followed by cooling slowly to 10°C. The thermodynamic data were calculated with the program Meltwin 3.0 (23).

5-Chloro-7-(2-deoxy-β-D-erythro-pentofuranosyl)-4-(dimethylamino)methylidene]amino-2-formylamino-7H-pyrrolo[2,3-d]pyrimidine (7b). Compound 7b was prepared from 5-chloro-7-(2-deoxy-β-D-erythro-pentofuranosyl)-7H-pyrrolo[2,3-d]pyrimidin-2,4-diamine (2b) (25) (500 mg, 1.67 mmol) and N,N-dimethylformamide dimethylether (3.0 ml, 22.4 mmol) as described for 7a. FC (silica gel, column 4 × 10 cm, elution with CH₂Cl₂/MeOH, 95:5) resulted in a colorless foam (518 mg, 81%). TLC (silica gel, CH₂Cl₂/MeOH, 9:1): Rₓ 0.33. UV (MeOH): δ = 2.10–2.17, 2.38–2.50 [2m, 2 H, H-C(2′)’]; 3.14, 3.19 (2s, 6 H, Me₂N); 3.50–3.52 [2m, 2 H, H-C(5)’]; 3.77–3.80 [m, 1 H, H-C(4)’]; 4.31–4.33 [m, 1 H, H-C(3)’]; 4.94 [t”, 1 H, J = 5.4 Hz, OH-C(5)’]; 5.27 [d, 1 H, H-C(1)’]; 5.31, 5.32 (2m, 2 H, H-C(5)); 5.40–5.42 [2m, 1 H, H-C(3)’]; 5.49 [t’, 1 H, J = 5.4 Hz, OH-C(5)’]; 6.47 [dd, 1 H, J = 5.9, 8.3 Hz, H-C(1)’]; 7.51 [s, 1 H, H-C(6)]; 8.84 (s, 1 H, N = CH); 9.48 (d, 1 H, J = 10.1 Hz, NH); 10.47 [d, J = 10.1 Hz, 1 H, COH]. Anal. calc. for C₁₅H₁₉ClN₆O₄ (382.80): C, 47.06; H, 5.00; N, 21.95; found: C, 47.15; H, 5.06; N, 21.81.

5-Bromo-7-(2-deoxy-β-D-erythro-pentofuranosyl)-4-(dimethylamino)methylidene]amino-2-formylamino-7H-pyrrolo[2,3-d]pyrimidine (7c). Compound 7c was prepared from 5-bromo-7-(2-deoxy-β-D-erythro-pentofuranosyl)-7H-pyrrolo[2,3-d]pyrimidin-2,4-diamine (2c) (480 mg, 1.39 mmol) and N,N-dimethylformamide dimethylether (3.0 ml, 22.4 mmol) as described for 7a. FC (silica gel, column 4 × 10 cm, elution with CH₂Cl₂/MeOH, 95:5) resulted in a colorless foam (450 mg, 76%). TLC (silica gel, CH₂Cl₂/MeOH, 9:1): Rₓ 0.33. UV (MeOH): 238, 274, 328 nm (ε = 20 800, 15 600, 9400). 1H NMR (DMSO-d₆): δ = 2.13–2.17, 2.39–2.45 [2m, 2 H, H-C(2′)’]; 3.17, 3.19 (2s, 6 H, Me₂N); 3.35–3.54 [2m, 2 H, H-C(5)’]; 3.78–3.79 [m, 1 H, H-C(4)’]; 4.30–4.32 [2m, 1 H, H-C(3)’]; 4.93 [t’, 1 H, J = 5.4 Hz, OH-C(5)’]; 5.27 [d, 1 H, H-C(1)’]; 5.32, 5.37 [2m, 2 H, H-C(5)]; 6.47 [dd, 1 H, J = 5.9, 8.3 Hz, H-C(1)’]; 7.51 [s, 1 H, H-C(6)]; 8.84 (s, 1 H, N = CH); 9.48 (d, 1 H, J = 10.1 Hz, NH); 10.47 [d, J = 10.1 Hz, 1 H, COH]. Anal. calc. for C₁₅H₁₉BrN₆O₄ (427.25): C, 42.17; H, 4.48; N, 19.67; found: C, 42.33; H, 4.40; N, 19.43.
2-formylamino-7H-pyrrolo[2,3-d]pyrimidine (8a). Compound 7a (300 mg, 0.86 mmol) was co-evaporated with anhydrous pyridine (three times) and then dissolved in pyridine (2.0 ml). To this solution 4,4′-dimethoxytriphenylmethyl chloride (DMT-Cl) (348 mg, 1.03 mmol) was added and the mixture was stirred at room temperature for 3 h. The reaction was quenched by the addition of MeOH and the mixture was evaporated to dryness. It was dissolved in CH₂Cl₂ (3.0 ml) and subjected to FC (column 4 × 9 cm, elution with CH₂Cl₂/MeOH, 98:2) to give a colorless foam (460 mg, 82%). TLC (silica gel, CH₂Cl₂/MeOH, 98:2): Rₖ 0.23. UV (MeOH): λₘₐₓ = 236, 323 (ε = 35 600, 17 500). ¹H NMR (DMSO-d₆): δ = 2.24–2.28, 2.50–2.57 [2m, 2 H, H-C(2)]; 3.10–3.17 [m, 8 H, Me₂N, H-C(5)]; 3.71 (s, 6 H, 2MeO); 3.89–3.91 [m, 1 H, H-C(4)]; 4.34–4.36 [m, 1 H, H-C(3)]; 5.36 [d, 1 H, J = 4.0 Hz, OH-C(3)]; 6.45–6.52 [m, 2 H, H-C(1); H-C(5)]; 6.79–6.84 (m, 4 H, arom. H); 7.16–7.37 [m, 10 H, 9 arom. H, H-C(6)]; 8.62 (s, 1 H, N = CH); 9.47 (d, 1 H, J = 10.2 Hz, NH); 10.40 (d, J = 10.2 Hz, 1 H, COH). Anal. calc. for C₃₆H₃₈N₆O₆ (650.72): C, 66.45; H, 6.00; N, 12.75.

5-Chloro-7-[2-deoxy-5-O-(4,4′-dimethoxytriphenylmethyl)β-6-erythro-pentofuranosyl]-4-[(dimethylamino)methylidene]-aminom-2-formylamino-7H-pyrrolo[2,3-d]pyrimidine (8b). Compound 8b was prepared from 7b (500 mg, 1.31 mmol) and DMT-Cl (594 mg, 1.75 mmol) as described for 8a. FC (column 4 × 9 cm, elution with CH₂Cl₂/MeOH, 98:2) produced a colorless foam (781 mg, 87%). TLC (silica gel, CH₂Cl₂/MeOH, 95:5): Rₖ 0.26. UV (MeOH): λₘₐₓ = 237, 248, 327 nm (ε = 34 800, 32 200, 18 000). ¹H NMR (DMSO-d₆): δ = 2.21–2.24, 2.50–2.58 [2m, 2 H, H-C(2)]; 3.05–3.19 [m, 8 H, Me₂N, H-C(5)]; 3.72 (s, 6 H, 2MeO); 3.89–3.91 [m, 1 H, H-C(4)]; 4.33–4.35 [m, 1 H, H-C(3)]; 5.32 [d, 1 H, J = 4.1 Hz, OH-C(3)]; 6.48 [d, 1 H, J = 6.6 Hz, H-C(1)]; 6.81–6.86 (m, 4 H, arom. H); 7.21–7.31 [m, 10 H, 9 arom. H, H-C(6)]; 8.84 (s, 1 H, N = CH); 9.48 (d, 1 H, J = 10.0 Hz, NH); 10.50 (d, J = 10.0 Hz, 1 H, COH). Anal. calc. for C₃₆H₃₈BrN₆O₆ (675.72): C, 66.45; H, 5.89; N, 12.91; found: C, 66.36; H, 6.00; N, 12.75.

7-[2-Deoxy-5-O-(4,4′-dimethoxytriphenylmethyl)β-6-erythro-pentofuranosyl]-4-[(dimethylamino)methylidene]aminom-2-formylamino-7H-pyrrolo[2,3-d]pyrimidine 3′-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite (5a). Compound 8a (200 mg, 0.31 mmol) dissolved in anhydrous CH₂Cl₂ (3.0 ml) under Ar was reacted with 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (100 µl, 0.42 mmol) in the presence of (Pr₃)₂N=O (100 µl) at room temperature. After 30 min, the reaction mixture was diluted with CH₂Cl₂ and the solution was washed with a 5% aqueous NaHCO₃ solution, followed by brine. The organic solution was dried over anhydrous Na₂SO₄, filtered and concentrated. The residue was submitted to FC (column 3 × 9 cm, CH₂Cl₂/acetone, 95:5) yielding a colorless foam (206 mg, 78%). TLC (silica gel, CH₂Cl₂/acetone, 9:1): Rₖ 0.2, 0.26. ³¹P NMR (CDCl₃): 149.6, 149.8.

5-Chloro-7-[2-deoxy-5-O-(4,4′-dimethoxytriphenylmethyl)β-6-erythro-pentofuranosyl]-4-[(dimethylamino)methylidene]-aminom-2-formylamino-7H-pyrrolo[2,3-d]pyrimidine (5b). Compound 8b (400 mg, 0.58 mmol) was treated with (Pr₃)₂N=O (157 µl) and 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (157 µl, 0.70 mmol) as described for 5a. FC (column 3 × 9 cm, CH₂Cl₂/acetone, 95:5) resulted in a colorless foam (363 mg, 71%). TLC (CH₂Cl₂/acetone, 9:1): Rₖ 0.23, 0.30. ³¹P NMR (CDCl₃): 149.7, 149.9.

7-[2-Deoxy-5-O-(4,4′-dimethoxytriphenylmethyl)β-6-erythro-pentofuranosyl]-4-[(dimethylamino)methylidene]aminom-2-formylamino-7H-pyrrolo[2,3-d]pyrimidine 3′-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite (5c). Compound 8c (400 mg, 0.55 mmol) was treated with (Pr₃)₂N=O (157 µl) and 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (157 µl, 0.70 mmol) as described for 5a. FC (column 3 × 9 cm, CH₂Cl₂/acetone, 95:5) resulted in a colorless foam (384 mg, 75%). TLC (CH₂Cl₂/acetone, 9:1): Rₖ 0.23, 0.30. ³¹P NMR (CDCl₃): 149.8, 150.0.

7-[2-Deoxy-5-O-(4,4′-dimethoxytriphenylmethyl)β-6-erythro-pentofuranosyl]-4-[(dimethylamino)methylidene]aminom-5-iodo-7H-pyrrolo[2,3-d]pyrimidine 3′-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite (5d). Compound 8d (250 mg, 0.32 mmol) was treated with (Pr₃)₂N=O (80 µl) and 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (100 µl, 0.42 mmol) as described for 5a. FC (column 3 × 9 cm, CH₂Cl₂/acetone, 95:5) resulted in a colorless foam (249 mg, 80%). TLC (CH₂Cl₂/acetone, 9:1): Rₖ 0.23, 0.30. ³¹P NMR (CDCl₃): 149.9, 150.1.

7′-Pyrrolo[2,3-d]pyrimidin-2,4-diamine (10). A suspension of 4-chloro-7′-pyrrolo[2,3-d]pyrimidin-2-amine (24)
column (4 × 9 cm), and the product was eluted stepwise with CH2Cl2/MeOH (98:2, 300 ml) and CH2Cl2/MeOH (9:1, 600 ml). The product-containing fractions were combined and the solvent was evaporated to give a yellowish solid (405 mg, 85%). TLC (silica gel, CH2Cl2/MeOH, 9:1): Rf 0.27. UV (MeOH): λmax = 221, 269 (ε = 15 400, 10 800). 1H NMR (DMSO-d6): δ = 2.11–2.18, 2.39–2.50 [2m, 2 H, H-C(2')]; 3.48–3.59 [m, 1 H, H-C(5')]; 3.79–3.81 [m, 1 H, H-C(4')]; 4.31–4.33 [m, 1 H, H-C(3')]; 4.94 ['t', 1 H, J = 5.4 Hz, OH-C(5')]; 5.28 [d, 1 H, J = 4.0 Hz, OH-C(3')]; 6.33 ['t', 1 H, J = 7.0 Hz, H-C(5')]; 6.60 [d, 1 H, J = 3.5 Hz, H-C(5')]; 7.30 [d, 1 H, J = 3.5 Hz, H-C(6)]; 7.55 (br. s, 2 H, NH2). 19F NMR (DMSO-d6): δ = −54.2. Anal. calc. for C14H18FN5O3 (323.32): C, 53.6; H, 6.6; F, 5.3. Yield: 73%.}

7-(2-Deoxy-β-D-erythro-pentofuranosyl)-4-(dimethylenomethylidenediamino)-2-fluoro-7-pyrrrolo[2,3-d]pyrimidine (14). A solution of compound 3 (268 mg, 1.0 mmol) in MeOH (10 ml) was stirred with N,N-dimethylformamide dimethylacetal (2.0 ml, 14.9 mmol) for 18 h at room temperature. After evaporation, the residue was applied to FC (silica gel, column 4 × 10 cm, elution with CH2Cl2/MeOH, 95:5) yielding a yellowish foam (272 mg, 84%). TLC (silica gel, CH2Cl2/MeOH, 9:1): Rf 0.44. UV (MeOH): λmax = 226, 312, 320 nm (ε = 14 400, 11 000, 20 100). 1H NMR (DMSO-d6): δ = 2.15–2.22, 2.43–2.50 [2m, 2 H, H-C(2')]; 3.13, 3.20 (2s, 6 H, Me2N); 3.49–3.58 [m, 2 H, H-C(5')]; 3.79–3.81 [m, 1 H, H-C(4')]; 4.33–4.34 [m, 1 H, H-C(3')]; 4.96 ['t', 1 H, J = 5.2 Hz, OH-C(5')]; 5.31 [d, 1 H, J = 4.0 Hz, OH-C(3')]; 6.41 ['t', 1 H, J = 7.0 Hz, H-C(1')]; 6.55 [d, 1 H, J = 3.5 Hz, H-C(5')]; 7.47 [d, 1 H, J = 3.5 Hz, H-C(6)]; 8.76 (s, 1 H, NH). 19F NMR (DMSO-d6): δ = −53.6. Anal. calc. for C14H18FN5O3 (323.32): C, 52.0; H, 5.57; N, 21.63.}

7-(2-Deoxy-β-D-erythro-pentofuranosyl)-2-fluoro-7H-pyrrrolo[2,3-d]pyrimidin-4-amine (I). Into a suspension of powdered KOH (494 mg, 85%, 7.4 mmol) and TDA-1 (0.2 ml, 0.63 mmol) in MeCN (30 ml), compound II (456 mg, 3.00 mmol) was added. After the mixture was stirred for 5 min, sugar halide 12 (26) (1.38 g, 3.55 mmol) was added during 5 min and the stirring was continued for 10 min. Insoluble material was filtered off, the precipitate was washed with MeCN and the filtrate was evaporated to dryness. The residue was applied to FC (silica gel column 4 × 12 cm, elution with CH2Cl2/MeOH, 100:0 → 99:1). The product-colorless foam was collected and evaporated to give a colorless foam (1.12 g, 74%). TLC (silica gel, CH2Cl2/MeOH, 98:2): Rf 0.23. 1H NMR (CDCl3): δ = 2.42, 2.44 (2s, 6 H, 2Me); 2.72–2.77 [m, 2 H, H-C(2')]; 4.57–4.71 [m, 3H, H-C(4')]; 5.50 (br. s, 2 H, NH2); 5.69–5.71 [m, 1 H, H-C(3')]; 6.36 [d, 1 H, J = 3.7 Hz, H-C(5')]; 6.70 ['t', 1 H, J = 7.0 Hz, H-C(1')]; 7.09 [d, 1 H, J = 3.7 Hz, H-C(6)]; 7.23–7.30, 7.93–7.99 (2m, 8 H, 2C=C=H). 19F NMR (DMSO-d6): δ = −97.4. Anal. calc. for C27H25FN4O5 (504.51): C, 70.1; H, 5.7; N, 7.0 Hz, H-C(1').

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9: 4.0 g, 23.73 mmol) in dioxane (60 ml) and 25% sq. NH3 (160 ml) was introduced into an autoclave and stirred at 100°C for 24 h. The clear solution was evaporated to remove ammonia (1 half the volume). The solution was applied to a Servolit AD-4 column (4 × 20 cm, resin 0.1–0.2 mm; Serva, Germany), the column was washed with H2O (200 ml) and the product was eluted with H2O/PrOH (5:1, 500 ml). The product-containing fractions were combined and the solvent was evaporated to give compound 10 as a yellowish foam (3.22 g, 91%). TLC (CH2Cl2/MeOH, 5:1): Rf 0.25. 1H NMR (DMSO-d6): δ = 6.51 [d, 1 H, J = 3.4 Hz, H-C(5)]; 6.70 (br. s, 2 H, NH2); 6.81 [d, 1 H, J = 3.4 Hz, H-C(6)]; 7.85 (br. s, 2 H, NH2); 11.43 (s, 1 H, NH). 13C-NMR (DMSO-d6): δ = 154.3, 153.8, 149.6, 119.4, 100.7, 95.0. Anal. calc. for C6H8N2 (149.15): C, 48.32; H, 4.73; N, 46.95; found: C, 48.30; H, 4.80; N, 46.80.
2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (134 µl, 0.60 mmol) as described for 5a. 2-Cyanoethyl-N,N-diisopropylchlorophosphoramidite (134 µl, 0.60 mmol) as described for 5a. FC (column 3 x 9 cm, CH₂Cl₂/acetone, 95:5) resulted in a colorless foam (289 mg, 70%). TLC (CH₂Cl₂/acetone, 95:5): Rf 0.32, 0.41. ³¹P NMR (CDCl₃): 149.7, 149.9.

RESULTS AND DISCUSSION

Monomers

The phosphoramidites of 2'-deoxytubercidin (1a) and its 7-ido-derivative (1d) as well as that of compound 4 have already been described (19–21). Compound 2a was prepared according to (24) and the synthesis of its 7-halogenated derivatives 2b–d refers to (25). As the N,N-dialkyllaminomethylidene protecting groups had already been successfully employed in the case of related 2,6-diaminopurine nucleosides (13,16,27), now a similar strategy was chosen for 2a–d. Nucleosides 2a–d were treated with N,N-dimethylformamide dimethylacetal in methanol yielding the bis-amidine [7a–d] (Scheme 2). After work-up, the N,N-dimethylaminomethylidene residue was partially hydrolyzed during silica gel FC resulting in a mixture of compounds [7a–d] and 7a–d. This was established on the basis of TLC and NMR data. As a typical example, the analytical data of compound [7c] is given in Supplemental Data. A complete and selective conversion of the amidine residue at position-2 to a formyl group was accomplished by the addition of traces of water to the methanolic solution of [7c] while stirring at 30–40°C for 48 h. This resulted in compounds 7a–d. As problems regarding the stability of amidine protection were reported for 2-amino-7-deaza-2'-deoxy-7-propynyladenosine (28), the half-lives of deprotection were measured for compounds 7a–d UV-spectrophotometrically (25% aq. NH₃ at 40°C). The apparent values for the complete deprotection are 53 min for 7a, 35 min for 7b, 36 min for 7c and 50 min for 7d. Subsequently, the 5'-hydroxyl groups were protected with the 4,4'-dimethoxytrityl (DMT) residues to give nucleosides 8a–d. Phosphitylation of the latter performed in anhydrous CH₂Cl₂ in the presence of Pr₂EtN and 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite, furnished the phosphoramidites 5a–d.

Next, the syntheses of the 2-fluoro-7-deazaadenine nucleoside 3 and its phosphoramidite were performed (Scheme 3). As the diazotization/fluorination reaction required strong acid conditions (HF/pyridine) (29), it resulted in the decomposition of the 2,6-diamino nucleoside 2a. Thus, the nucleobase 10 was used instead of the nucleoside. As a precursor the 2-amino-6-chloro-7-deazapurine (9) (24) was employed, which was converted to the diamino compound 10 in aqueous ammonia (autoclave, 100°C). The diazotization/fluorination reaction was performed under the same conditions as done for 2a by dropwise addition of BuNO₂ affording the 2-fluoro base 11. The low yield of 11 (30%) is caused by the partial fluorination at the 6-position giving 2,6-difluoro-7-deazapurine, in which the 6-fluoro group was displaced by nucleophiles such as MeOH (~2-fluoro-6-methoxy-7-deazapurine) (Supplementary Data). Nucleobase anion glycosylation of 11 with 2-deoxy-3,5-di-O-(p-toluoyl)-α-D-erythro-pentofuranosyl chloride (12) yielded the toluoyl-protected β-D-nucleoside 13 (74% yield; Scheme 3). Compound 13 was converted to the 2-fluoro nucleoside 3 in methanolic ammonia at room temperature. The 2-amino group of 3 was protected by the N,N-dimethylaminomethylidene residue to give 14, which shows a half-life value of 19 min (25% aqeous ammonia, room temperature). Subsequently, compound 14 was converted into the 5'-O-DMT-derivative 15 under standard conditions. Phosphitylation of 15 was performed in anhydrous CH₂Cl₂ in the presence of Pr₂EtN and 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite, furnishing the phosphoramidite 6. The reactions performed with the 2-fluoro nucleosides were carried out at room temperature in order to avoid the displacement of the 2-fluoro substituent. As 2'-deoxy-2-fluorotubercidin is a convertible nucleoside, it can be used to generate DNA containing modified nucleosides with various substituents at the 2-position by using elevated temperature and/or different deprotection conditions leading to fluorine displacement.

All compounds were characterized by ¹H-, ¹³C-, ³¹P- or ¹⁹F-NMR spectra as well as by elemental analysis. ¹³C-NMR shift assignment was made according to gated-decoupled spectra and/or different deprotection conditions leading to fluorine displacement.

Scheme 2. (i) N,N-Dimethylformamide dimethylacetal, methanol, 40–50°C, 24 h. (ii) water, 30–40°C, 48 h. (iii) 4,4'-Dimethoxytritylphenylmethyl chloride, anhydrous pyridine. (iv) 2-Cyanoethyl-N,N-diisopropylchlorophosphoramidite, N,N-diisopropylethylamine, dichloromethane.
Oligonucleotides

Base-pairing properties of oligonucleotides containing the modified nucleosides 1a, 1d, 2a–d, 3 and 4. For all hybridization experiments studying the influence of the base-modified nucleosides on the duplex stability, the duplex 5′-d(TAGGTCAGCTGA) (16) 3′-d(ATCCAGTTATGA) (17) was used as reference. The modified duplexes contain single or multiple incorporations of the base-modified compounds at various positions. The base pairing of the oligonucleotides with the four canonical nucleosides opposite to the modification sites was investigated. For compounds 1a, 1d, 2a, 2b, 3 and 4 the mismatch discrimination was studied at various pH values.

Duplex stability of oligonucleotides with anti-parallel strand (aps) orientation. The base pair stability of 2′-deoxytubercidin (1a) or its 7-halogenated derivatives in oligonucleotide duplexes has already been studied (11,15,20). Very little is known about the influence of the 7-halogen substituents of the 2-amino-2′-deoxytubercidin derivatives 2b–d on the base pairing properties (28). Also the effect of small substituents introduced at the 2-position of pyrrolo[2,3-d]pyrimidine on the base pair stability is unknown and is now studied with the 2-fluorinated 2′-deoxytubercidin 3.

Moreover, the effect of the amino group in the 2- or 6-position of a 7-deazapurine is investigated by comparing the duplex stability and mismatch discrimination of 2-amino-7-deazapurine-2′-deoxyribonucleoside (4) with 1a. Hybridization experiments were performed with oligonucleotides containing 2a–d as well as 3 and 4. For comparison, oligonucleotides incorporating 1a and its 7-iodo derivative 1d at the same position as 2a–d were investigated. Table 2 summarizes

Table 1. 

Table 1. 13C-NMR chemical shifts (δ) of 7-deazapurine 2′-deoxyribonucleosides

| Compound | C(2) | C(4) | C(6) | C(8) | C(10) | C(12) | C(14) | C(16) | C(18) | C(20) | C(22) |
|----------|------|------|------|------|--------|--------|--------|--------|--------|--------|--------|
| 7a       | 160.8| 152.3| 107.9| 101.0| 121.9  | 152.1  | 82.3   | e      | 71.1   | 87.1   | 62.1   |
| 7b       | 160.7| 152.7| 104.0| 105.1| 118.9  | 151.4  | 82.0   | e      | 70.4   | 87.0   | 61.7   |
| 7c       | 160.2| 152.8| 105.1| 101.1| 118.9  | 151.8  | 82.4   | e      | 71.0   | 85.5   | 64.4   |
| 7d       | 160.9| 152.8| 104.2| 105.2| 118.9  | 151.1  | 82.1   | e      | 70.8   | 85.3   | 64.1   |
| 8a       | 160.7| 152.7| 105.3| 89.6  | 121.8  | 152.3  | 82.4   | e      | 70.7   | 85.4   | 64.8   |
| 8b       | 160.9| 152.7| 105.3| 89.6  | 121.8  | 152.3  | 82.4   | e      | 70.7   | 85.4   | 64.3   |
| 8c       | 160.9| 152.7| 105.3| 89.6  | 121.8  | 152.3  | 82.4   | e      | 70.7   | 85.4   | 64.4   |
| 8d       | 160.7| 152.5| 107.2| 54.8  | 126.7  | 151.9  | 82.4   | e      | 70.8   | 85.6   | 64.3   |
| 8f       | 160.9| 152.7| 105.3| 89.6  | 121.8  | 152.3  | 82.4   | e      | 70.7   | 85.4   | 64.4   |
| 8g       | 160.9| 152.7| 105.3| 89.6  | 121.8  | 152.3  | 82.4   | e      | 70.7   | 85.4   | 64.3   |
| 1a       | 161.3| 157.3| 103.7| 99.4  | 124.1  | 149.5  | 83.2   | 39.6   | 70.9   | 81.7   | 62.0   |
| 3′       | 160.0| 160.2| 101.5| 101.3| 122.4  | 151.6  | 83.7   | e      | 71.8   | 88.1   | 62.8   |
| (JC,F)   | 196.6| 20.2  | 3.6   |       |        |        |        |        |        |        |        |
| 11f      | 159.3| 159.2| 100.2| 99.6  | 121.0  | 151.5  |        |        |        |        |        |
| (JC,F)   | 197.5| 20.4  | 3.3   | 2.3   |        |        |        |        |        |        |        |
| 13f,g    | 159.9| 158.9| 101.7| 100.3| 121.6  | 152.4  | 84.2   | 38.5   | 75.5   | 82.6   | 64.8   |
| (JC,F)   | 206.1| 19.0  | 3.8   | 2.3   |        |        |        |        |        |        |        |
| 14f      | 158.6| 162.5| 109.1| 100.4| 123.5  | 152.3  | 82.9   | e      | 71.0   | 87.3   | 62.0   |
| (JC,F)   | 203.6| 14.6  | 3.1   | 3.2   |        |        |        |        |        |        |        |
| 15f      | 159.4| 163.4| 110.0| 101.9| 124.2  | 153.2  | 83.6   | e      | 71.6   | 86.3   | 65.0   |
| (JC,F)   | 200.2| 14.4  | 4.7   |        |        |        |        |        |        |        |        |

*a*Measured in DMSO-d6.
*b*First heading row = systematic numbering.
*c*Second heading row = purine numbering.
*d*Tentative.
*e*Superimposed by DMSO.
*f*Measured in CDCl3.
*g*The second row shows the JC,F coupling constants.

Scheme 3. (i) Aqueous ammonia, dioxane, 100°C. (ii) HF/pyridine, BuNO2, –60 → –50°C. (iii) 2-Deoxy-3,5-di-(p-tolyl)-α-D-erythro-pentofuranosyl chloride (12), KOH, TDA-1, MeCN. (iv) Methanolic ammonia. (v) N,N-Dimethylformamide dimethylacetal, methanol. (vi) 4,4′-Dimethoxytritylmethyl chloride, anhydrous pyridine. (vii) 2-Cyanoethyl-N,N-diisopropylchlorophosphoramidite, N,N-diisopropylethylamine, dichloromethane.
Table 2. $T_m$ values and thermodynamic data of oligonucleotide duplexes containing 1a, 1d, 2a–d, 3 and 4 opposite to dT

| Duplex | $T_m$ (°C) | $\Delta T_m$ (°C) | $\Delta G_m$ (kcal/mol) |
|--------|------------|-----------------|----------------------|
| 5′-dTGGACCAATGACT | 16 | 47 | -10.9 |
| 3′-dTACCGAGTCAATGACT | 17 | 47 | -10.4 |
| 5′-dTGGACCAATGACT | 18 | 46 | -10.5 |
| 3′-dTACCGAGTCAATGACT | 19 | 46 | -10.6 |
| 5′-dTGGACCAATGACT | 20 | 48 | -10.0 |
| 3′-dTACCGAGTCAATGACT | 21 | 50 | -11.3 |
| 5′-dTGGACCAATGACT | 22 | 51 | +1.3 |
| 3′-dTACCGAGTCAATGACT | 23 | 47 | 0 |
| 5′-dTGGACCAATGACT | 24 | 47 | -10.8 |
| 3′-dTACCGAGTCAATGACT | 25 | 50 | -11.4 |
| 5′-dTGGACCAATGACT | 26 | 53 | +3.0 |
| 3′-dTACCGAGTCAATGACT | 27 | 53 | +3.0 |
| 5′-dTGGACCAATGACT | 28 | 53 | +3.0 |
| 3′-dTACCGAGTCAATGACT | 29 | 55 | +2.7 |
| 5′-dTGGACCAATGACT | 30 | 53 | +3.0 |
| 3′-dTACCGAGTCAATGACT | 31 | 54 | +3.5 |
| 5′-dTGGACCAATGACT | 32 | 56 | +3.0 |
| 3′-dTACCGAGTCAATGACT | 33 | 58 | +2.8 |
| 5′-dTGGACCAATGACT | 34 | 53 | +3.0 |
| 3′-dTACCGAGTCAATGACT | 35 | 54 | +2.3 |
| 5′-dTGGACCAATGACT | 36 | 44 | -3 |
| 3′-dTACCGAGTCAATGACT | 37 | 47 | 0 |

*a Measured at 260 nm in 0.1 M NaCl, 10 mM MgCl2 and 10 mM sodium cacodylate buffer, pH 7.0, with 5 mM single-strand concentration.

**$T_m$ increase per modification.

Figure 1. Base pair motifs in aqDNA.

![Figure 1](image_url)

The $T_m$ values of oligonucleotides containing 1a, 1d, 2–4 with one to six modifications.

In a first series of experiments, the influence of 7-substituents on the oligonucleotide duplexes stability was studied replacing dA-residues in the duplex 16–17 (47°C) by compounds 1a, 1d and 2a–d. According to Table 2 the replacement of one or two dA-residues by 2′-deoxytubercidin (1a: $T_m = 46°C$ for duplex 18–17 and 16–19) has very little influence on the duplex stability. Similar results were observed for the non-functionalized 2-amino-2′-deoxytubercidin (2a: $T_m = 47°C$ for duplex 22–17 and 16–23). This shows that the 2-amino group present in the non-halogenated 2a contributes very little to the stability of 2a–dT base pairs (motif iia, Figure 1). However, the 7-halogen substituents in 2a stabilize the duplex significantly (see $T_m$ values of duplexes incorporating 2b–d in Table 2). The stabilizing effect of 7-halogenated 7-deazapurin-2,6-diamine nucleosides (2b–d: $\Delta T_m = 2.0–3.0°C$ per modification) is stronger than that of the 7-substituted 2′-deoxytubercidin (1d: $\Delta T_m = 1.0°C$). These results show that the additional 2-amino group of compound 2a being not functionalized at 7-position does not strengthen the base pair (47°C) in comparison to 2′-deoxytubercidin (46°C), whereas in the case of 7-halogenated nucleosides the 2-amino group of 2b–d makes a contribution to the base pair stability (2d: 50°C or 53°C for duplexes 33–17 and 16–34 versus 1d: 48°C or 50°C for duplexes 20–17 or 16–21). This might result from the better proton donor properties of the 2-amino group of the 7-halogenated nucleosides 2b–d. A comparison of the $pK_a$ values of 2a (5.71) with 7-halogen derivatives 2b: 4.86; 2c: 4.85; 2d: 4.75 shows that 7-halogen substituents reduce the basicity of the 7-deazapurin-2,6-diamine. At the same time, the 2-amino group can become a better proton donor, thereby strengthening the 2b–d–dT base pair (motif iib–d, Figure 1).

In order to prove the effect of multiple incorporations of the halogenated nucleosides, we determined $T_m$ values in dependence of an increasing number of modified bases. The oligonucleotides containing 2b or 2c in a consecutive manner or in distant position were synthesized. The total number of incorporations was increased in duplexes from 1 to 6. It was found that the $T_m$ values increased steadily by an increasing number of modified residues (Table 2). The linear relationship of $T_m$ values with the modification numbers of 2b and 2c is shown in Figure 2A (2b) and B (2c).

In the second series of hybridization experiments, the stabilizing effect of the 2-amino versus the 6-amino group was evaluated. For this, oligonucleotides containing the 2-amino-7-deazapurine nucleoside 4 were synthesized (Table 2). It
was observed that the duplex 16-36 (47°C) incorporating 4 shows the same stability as that containing 1a having an amino function in the 6-position (46°C for duplex 18-17). This indicates that the amino group in the 2- (4) or 6-position (1a) of 7-deazapurines plays a similar role on the base pair stability when pairing with dT. Obviously, a bidentate base pair is formed between 4 and dT (motif iv, Figure 1).

The effect of 2-halogen substituent on the duplex stability was also investigated. For this purpose the 7-deaza-2-fluoro-2′-deoxyadenosine (3) was incorporated at exactly the same positions as the other modified derivatives. According to Table 3, compound 3 decreases the duplex stability by 3°C per modification (duplex 16-35). Owing to its negative effect, multiple incorporations of 3 were not undertaken. The destabilization of the nucleoside 3 results from the presence of the 2-fluoro group. Its electron-withdrawing property decreases the proton acceptor ability of nitrogen-1 causing weaker hydrogen bonding within the base pair (motif iii, Figure 1), which is underlined by the pK_a-value (<1.5) of 3 (Supplementary Data). In addition, the 2-fluoro substituent induces steric strain with the 2-oxo group of dT.

Stability of duplexes with parallel chain orientation. It is already reported that 7-halogenated 2′-deoxytubercidin derivatives, such as 1d, show a stabilizing effect on parallel-stranded (ps) DNA (30). Here, the base pairing of the 2-amino derivatives 2a–d in ps DNA was investigated. In order to induce parallel chain orientation two ‘iGd–dC’ and two ‘MeiCd–dG’ base pairs were introduced instead of the dG–dC pairs. The duplexes 37-17 and 16-38 with modifications in different positions served as standards. According to Table 3 the substitution of dA residues by the non-halogenated nucleoside 2a decreases the stability of the ps duplexes by 1°C per modification (duplex 37-23) (Table 3), whereas the incorporation of 7-halogenated nucleosides 2b–d into the ps DNA led to an increase of the ps duplex stability by 1–2.0°C per modification depending on the sequences (see T_m values of duplexes incorporating 2b–d in Table 3). These results show that the 7-halogen substituents introduced in 2-amino-7-deazaadenine nucleosides are well accommodated in the grooves of ps-DNA and stabilize ps duplexes structure. Similar to dA–dT base pair motif v, the motifs via-d are suggested for the 2a–d–dT pairs in ps DNA (Figure 3).

Mismatch discrimination. It is generally accepted that transition mutations in vivo proceed via the formation of base mispairs during DNA replication. Several mechanisms have been postulated to explain base mispair formation including rare tautomeric forms (1,31), ionized bases (32) and wobble base pairs (33,34). Different motifs have been proposed for hydrogen bonding between cytosine and adenine, involving major (1,35) or minor tautomeric forms of the bases (36). Most of the models lack direct experimental evidence. Hunter et al. (37) have reported the crystal structure of a dA–dC base pair, which shows that the protonated adenine can form a bidentate base pair with cytosine. However, due to the low basicity of dA (pK_a = 3.50) the protonated dA–dC base pair is not observed in neutral solution (38). As the basicity of 7-deazaadenine is significantly increased, such a mispair seems more easily formed between 2′-deoxy-7-deazaadenosine (pK_a = 5.30) and dC. In order to investigate this property in more detail, the mismatch discrimination of 2′-deoxytubercidin and its derivatives 1d or 2–4 was studied at various pH values.

Base recognition of compounds 1a, 1d, 2a–d, 3 and 4 towards the four canonical nucleosides under neutral conditions. In a first series of experiments, the base pair discrimination of the nucleosides 1a, 1d, 2a–d, 3 and 4 was investigated under neutral conditions. For this, the T_m values of 12mer duplexes

Figure 3. Base pair motifs in ps DNA.
containing these modified nucleosides located opposite to the four canonical constituents were measured. From the data shown in Table 4 it is apparent that 7-deaza-2'-deoxyadenosine (1a) generates a strong base pair not only with dT but also with dC (Tm = 45°C) an dG (45°C). Similar results were found for its 2-amino derivative 2a. A base pair formed by 1a or 2a with dG is not unexpected, as stable dG–dA base pairs have been already detected. Various motifs for dA–dG or ‘C/A’ dG pairs have been reported previously (39–42). The observation of the highly stable base pair of 2a and dC was already reported by Saito and co-workers (16) but without giving an explanation for this phenomenon. Our hybridization experiments show that the base pair of 1a and dC is strong, while the corresponding dA–dC interaction is very weak (Tm = 43°C versus Tm = 36°C; Table 4). Thus, these properties are caused by the replacement of the purine by the pyrrolo[2,3-d]pyrimidine system and not by the additional 2-amino group. The study on substituted 7-deazaadenine nucleosides provides further proof.

The same hybridization experiments as discussed above were performed with oligonucleotide duplexes containing the halogenated compounds 1d, 2b–d and 3. According to Table 4, one incorporation of 1d opposite to dC results in a ΔTm = −6°C, whereas a decrease of 3°C is observed for one 1a–dC mismatch. Similar results were found between compounds 2b–d and 2a (−5°C versus −1°C). This shows that the introduction of 7-halogen substituents improves the base pair discrimination slightly. Nevertheless, a strong base pair is still observed between 7-halogenated nucleosides 1d, 2b–d and dC. 7-Halogenated nucleosides also form stable mismatches with dG. In the case of the 2-fluorinated nucleoside 3, a strong base pair with dG was observed as well (Tm = 45°C), while significant base discrimination towards dC was now occurring (ΔTm = −13°C) (Table 4). A different situation is observed for the 2-amino-7-deazapurine nucleoside 4. It shows a base discrimination towards dG (ΔTm = −10°C) due to the absence of 6-amino group but generates a relatively stable mispair with dC and dA. Inspection of the pk2 values of the various nucleosides reveals that they are decreasing in the following order: 5.71 (2a) > 5.30 (1a) > 5.08 (4) > 3.50 (dA) > less than 1.5 (3) (Supplementary Data). A similar relationship is found for the Tm values of duplexes containing dA*–dC base pairs (dA*; modified nucleosides): 2a (46°C) > 1a (43°C) > 4 (41°C) > dA (36°C) > 3 (31°C). The ΔTm values (|Tm(dA*–dC) − Tm(dA–dT)|) increased in the reverse order: |−1°C| (2a) < |−3°C| (1a) < |−6°C| (4) < |−12°C| (dA) < |−13°C| (3). This implies a relationship between mispairing and nucleobase protonation. Consequently, the Tm values of duplexes containing modified dA-residues pairing with dC or dT were measured at various pH values.

**Base discrimination of compounds 1a, 1d, 2a, 3 and 4 against dC under acidic or alkaline conditions.** Duplexes containing the base pairs of 1a,d–dC, 2a,b–dC, 3–dC and 4–dC were investigated in buffer solutions at pH values 6.5, 7.0, 8.0 and 9.0. For comparison, the Tm of the duplexes incorporating the corresponding dA*–dT base pair were measured under the same conditions. The data are summarized in Table 5. It is apparent that the stability of the 1a–dC pair was enhanced under acid conditions (47°C at pH 6.5), while strongly decreased under basic conditions (39°C at pH 8.0 and 35°C at pH 9.0) (Table 5). Figure 4 clearly shows the
relationship between the stability of 1a-dC base pair and pH value of the buffer solution. Similar results were found for 2a (Table 5). When the pH value is 6.5, the duplex with a 2a-dC base pair (49°C) is even more stable than that incorporating a 2a-dT base pair (48°C).

Similar results as found for non-halogenated nucleosides 1a or 2a are observed for 7-halogenated compounds 1d and 2b. The stability of duplexes incorporating 1d-dC or 2b-dC pairs decreased with increasing pH values: $T_{m}^{\text{pH 6.5}} > T_{m}^{\text{pH 7.0}} > T_{m}^{\text{pH 8.0}} > T_{m}^{\text{pH 9.0}}$. This demonstrates that the 7-deazapurine nucleosides 1a, 1d, 2a, and 2b show an enhanced base discrimination towards dC under alkaline conditions. This phenomenon can be clearly seen from Figure 5A–D. It is apparent that compounds 1a, 1d, 2a, and 2b show much stronger discrimination towards dC under alkaline condition than that in neutral or acidic medium as indicated from the order of $-\Delta T_{m} = -\Delta T_{m}^{\text{pH 6.5}} > -\Delta T_{m}^{\text{pH 7.0}} > -\Delta T_{m}^{\text{pH 8.0}} > -\Delta T_{m}^{\text{pH 9.0}}$. The various $T_{m}$ values of the corresponding duplexes with 1a, 1d, 2a, 2b, and dA opposite to dC at various pH values were visualized in the inserted figures, which reflects the destabilizing effect of alkaline buffer solution on ‘c7Ad*’–dC base pair and the stabilizing effect of mild acid conditions. However, when the pH values are in the range of 6.5–9.0, such a behaviour is not observed for the dA–dC or 3–dC pairs as indicated from the similar $\Delta T_{m}$ values ($\sim 13^\circ C$) obtained in acidic, neutral or alkaline medium (Table 5 and Figure 5E, F).

According to the p$K_{a}$ values of the monomeric nucleosides 1a (5.30) and 2a (5.71), one might argue that the p$K_{a}$ values are too low to cause protonation under neutral or weak alkaline conditions. However, from p$K_{a}$ studies on oligonucleotides it is known that the p$K_{a}$ values of nucleobases within stacked oligonucleotides can be raised significantly by the attractive force of the phosphodiester backbone for the protons, and by the stabilization caused by hydrogen bonding (17). In our case, a p$K_{a}$ increase of 1–2 p$K_{a}$ values is likely to occur. Thus, the protonated base pairs are easily formed between the 7-deazapurine nucleosides 1a or 2a and dC under neutral conditions when they are as constituents of oligonucleotides. According to the 7-deazapurine structure a protonation in the five-member ring can be excluded. Possible protonation sites are only nitrogen-1 or nitrogen-3 (Scheme 4). Rosemeyer and Seela (43) reported that 15-N-NMR studies on 7-deazadenine nucleosides show that the protonation site of 1a is N-1 (1ap; Scheme 4, right). Therefore, the 1a-dC or 1d-dC base pairs should form according to bidentate motifs viia,b with nucleosides 1a or 1d in the N-1 protonated state (Figure 6). Similarly, bidentate base pair motifs viia–d are suggested for 2a–d-dC mispairs (Figure 6). Our findings are consistent with earlier observations reported by Hunter et al. (37) on a protonated dA–dC base pair (motif ix) existing in the crystal structure. Also, Saito and co-workers have observed that a wobble base pair of 4-amino-6-methoxy-9-(2'-deoxy-β-D-erythro-pentofuranosyl)-7H-pyrimido(4,5-b)indole and dC (44).

The 2-amino-7-deazapurine nucleoside 4 also generates stable base pair with dC under acidic condition (pH 6.5), whereas the stability of 4-dC base pair is kept in the same range in neutral or basic buffer solution (4.0, 7.0, 8.0 and 9.0) (Table 5). This might be due to the formation of different base pair motifs under acid or neutral and basic conditions. The protonated base pair (motif x) is likely to be generated in acid condition, whereas a wobble base pair xi is present in neutral and basic conditions (Figure 6). In the case of 2-fluoro-7-deazadene nucleoside 3 and dA, rather strong base discrimination was observed against dC keeping the $T_{m}$ values constant at different pH values (6.5, 7.0, 8.0, 9.0) (Table 5 and Figure 5E, F). This results from the properties of dA or compound 3 which are not protonated under these conditions...
conditions, underlined by the low-pK values of dA (3.5) and 3 (<1.5).

Regarding the capability to form strong base pairs with both dT and dC, the pyrrolo[2,3-d]pyrimidines described above show similar properties to N6-methoxyaminopurine and N6-methoxy-2,6-diaminopurine 2'-deoxyribonucleosides reported by Brown and co-workers (45,46). In that case, the formation of two tautomeric structures (amino and imino form) induced by the N6-methoxy group was discussed. The present study does not rule out the formation of c7dA–dC base pairs where 7-deazaadenine nucleosides are in a rare tautomer form. It does not seem necessary, however, to invoke this hypothesis, since the 6-amino group of nucleosides 1a, d and both 2- and 6-amino groups of 2a–d were found in their 1H-NMR spectrum as singlets and the number of hydrogens is two according to the integrals of each peak. Furthermore, the structure of compound 2b was confirmed by the single-crystal X-ray analysis (47).

CONCLUSION

(i) Synthesis. The novel 2-fluoro-7-deazaadenine nucleoside 3 and various 7-deazapurine nucleoside phosphoramidites (5a–d and 6) were synthesized and applied to the solid-phase synthesis of oligonucleotides containing 7-deazapurin-2,6-diamine nucleosides 2a–d or 2-fluoro-2'-deoxytubercidin (3). From 2-fluorinated phosphoramidite 6, oligonucleotides incorporating 3 can be selectively prepared when deprotection was carried out in mild conditions (25% aq. NH3, room temperature), whereas the deprotection performed at elevated temperature (25% aq. NH3, 60°C) leads to the displacement of 2-fluoro group by amino residue giving oligonucleotides containing 2a.

(ii) Base pair stability. 7-Halogen substituents introduced in the 7-deazapurine nucleosides (1d or 2b–d) stabilize the base pair with dT in both apos and psDNA whereas a 2-fluoro group decreases the base pair stability. The 7-halogenated 7-deazapurin-2,6-diamine nucleosides show stronger stabilizing effects on duplex stability than that of 7-halogenated 2'-deoxytubercidin. 7-Deazapurine nucleosides with amino groups in the 6-position (1a), in the 2-position (4) or in both positions (2a) show similar base pair stability when pairing with dT.

(iii) Mismatch discrimination. 7-Deaza-2'-deoxyadenosine (1a) as well as its 2-amino derivative 2a form strong base pairs not only with dT but also with dC. The 7-halogenated nucleosides 1d or 2b–d show slightly enhanced base pair discrimination compared with the non-functionalized nucleosides 1a or 2a. From pH-dependent Tm measurements it is apparent that the base discrimination of 1a, 2a as well as of their 7-halogenated derivatives 1d, 2b towards dC is strongly improved in weakly alkaline medium. As the Tm increase can be correlated to the pK values of the nucleosides or to the pH values of buffer solution, protonated base pairs are suggested for the interaction of 1a, 2a, and of the 7-halogenated compounds 1d or 2b–d with dC. A strong base discrimination against dC was observed for 7-deaza-2-fluoro-2'-deoxyadenosine (3) already in neutral medium, which is similar to that of dA (3.5; c3F7Ad <1.5). As the strength of the base pairs of compounds 1a, 1d or 2a–d with dT or dC depends on the pH value, this phenomenon can be used to generate pH-dependent molecular switches.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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