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Pyrazolo[3,4-*d*]pyrimidine nucleic acids: adjustment of dA-dT to dG-dC base pair stability

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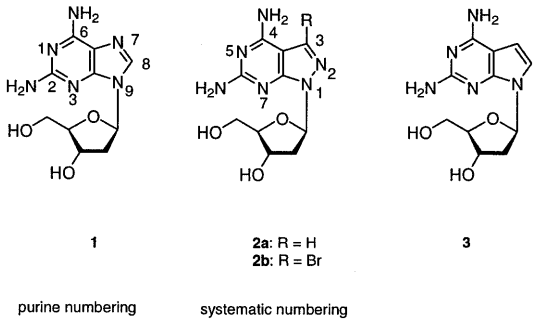
ABSTRACT

Oligonucleotides incorporating 8-aza-7-deazapurin-2,6-diamine (pyrazolo[3,4-*d*]pyrimidin-4,6-diamine) nucleoside 2a or its 7-bromo derivative 2b show enhanced duplex stability compared to those containing dA. While incorporation of 2a opposite dT increases the T_m value only slightly, the 7-bromo compound 2b forms a very stable base pair which is as strong as the dG-dC pair. Compound 2b shows a similar base discrimination in duplex DNA as dA. The base-modified nucleosides 2a,b have a significantly more stable *N*-glycosylic bond than the rather labile purin-2,6-diamine 2′-deoxyribonucleoside 1. Base protection with acyl groups, with which we had difficulties in the case of purine nucleoside 1, was effective with pyrazolo[3,4-*d*]-pyrimidine nucleosides 2a,b. Oligonucleotides containing 2a,b were obtained by solid phase synthesis employing phosphoramidite chemistry. Compound 2b harmonizes the stability of DNA duplexes. Their stability is no longer dependent on the base pair composition while they still maintain their sequence specificity. Thus, they have the potential to reduce the number of mispairs when hybridized in solution or immobilized on arrays.

INTRODUCTION

The stability of natural DNA depends on the base pair composition. The guanine-cytosine pair is more stable than the adenine-thymine pair, which is attributed to the third hydrogen bond. Oligonucleotide duplexes forming other tridentate base pairs are expected to show a similar stability to dG-dC. However, it has been reported that the additional amino group of 2-amino-2′-deoxyadenosine (**1**) stabilizes the dA-dT pair only very little, resulting in a T_m increase of only 1–2°C per modified residue (1–7). Moreover, stabilization does not correspond to an increasing number of purin-2,6-diamine 2′-deoxyribonucleoside (**1**) residues (8). A stronger stabilization as reported for DNA is found for duplex RNA and for DNA–RNA hybrids (7,9). High base pair stability is observed when 2-aminoadenine is introduced into peptide nucleic acids (PNA) (10) or hexitol nucleic acids (11).

The unusual behavior of oligonucleotide duplexes containing tridentate **1**-dT base pairs has prompted us to search for more stable tridentate base pairs with dA-dT base recognition. Finally, the 'dA-dT' base pair should exhibit the same stability as a dG-dC pair. This problem was approached by replacing the purine moiety of compound **1** by other heterocyclic bases. 7-Deazapurines (pyrrolo[2,3-*d*]pyrimidines) (purine numbering is used throughout Results and Discussion) and 8-aza-7-deazapurines (pyrazolo[3,4-*d*]pyrimidines) have already been used in the case of adenine and guanine nucleosides to alter duplex stability (12–15). These heterocycles mimic the shapes of the purines and can substitute for purine bases in Watson–Crick base pairs. In the case of analogs of 2′-deoxyadenosine it has been observed that the 7-deaza-2′-deoxyadenosine-dT base pair is less stable than a dA-dT pair while the 8-aza-7-deaza-2′-deoxyadenine-dT pair shows the same stability. Furthermore, it was found that the 7-halogen substituents increase base pair stability significantly (13,14,16). However, it was not possible to increase the stability of the bidentate dA-dT pair to the level of a tridentate dG-dC pair when a modified base was substituted for a regular one. This manuscript reports on oligonucleotides containing the pyrazolo[3,4-*d*]pyrimidine nucleosides **2a**,**b** (Scheme 1). They represent structural analogs of **1** and are incorporated into oligonucleotides by solid phase synthesis employing phosphoramidite chemistry.



Scheme 1.

MATERIALS AND METHODS

General

Chemicals were purchased from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). Solvents were of laboratory grade. Elemental analyses were performed by Mikroanalytisches

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Laboratorium Beller (Göttingen, Germany). NMR spectra were measured on Avance DPX 250 or AMX 500 spectrometers (Bruker, Karlsruhe, Germany) operating at proton resonance frequencies of 250.13 and 500.14 MHz (125.13 MHz for the ^{13}C nuclei). Chemical shifts are in p.p.m. relative to TMS as internal standard. *J* values are given in Hz. UV spectra were recorded on a U 3200 spectrometer (Hitachi, Japan). Thin layer chromatography (TLC) was performed on 0.2 mm thick silica gel 60 F_{254} aluminum sheets (Merck, Germany) and column flash chromatography (FC) on silica gel 60 (Merck, Germany) at 0.4 bar (4×10^4 Pa).

Oligonucleotides

Synthesis and purification. The oligonucleotide syntheses were performed in an ABI 392-08 DNA synthesizer (Applied Biosystems, Weiterstadt, Germany) at the 1 µmol scale using the phosphoramidites **7b** and **11a**,**b** and those of **1** and the canonical 2′-deoxyribonucleosides (Applied Biosystems, Weiterstadt, Germany) following the synthesis protocol for 3′-cyanoethyl phosphoramidites (user manual for the 392 DNA synthesizer; Applied Biosystems, Weiterstadt, Germany). The phosphoramidite of nucleoside **1** was a commercial product from Glen Research (USA). After cleavage from the solid support the oligonucleotides were deprotected with 25% aqueous NH_3 (10–12 h at 60°C). Composition analysis of the oligomers was performed by tandem hydrolysis with snake venom phosphodiesterase followed by alkaline phosphatase as described (17).

Quantification of the constituents was made on the basis of peak areas, which were divided by the extinction coefficients of the nucleoside (ε_{260} values: dA, 15 400; dC, 7300; dG, 11 400; dT, 8800). Snake venom phophodiesterase (EC 3.1.15.1, *Crotallus durissus*) and alkaline phosphatase (EC 3.1.3.1, *Escherichia coli*) were supplied by Roche Diagnostics GmbH (Penzberg, Germany). The MALDI-TOF mass spectra were provided by Dr T.Wenzel (Bruker, Germany). MALDI-TOF data for the modified oligonucleotides are shown in Table 1. Oligonucleotide purification was performed by reverse phase HPLC (RP-18) with a Merck-Hitachi HPLC: 250 × 4 mm RP-18 column with gradients of 0.1 M (Et_3NH)OAc (pH 7.0)/MeCN 95:5 (A) and MeCN (B) (gradient I, 50 min 0–50% B in A, flow rate 1.0 ml/min; gradient II, 20 min 0–25% B in A, flow rate 0.7 ml/min plus 30 min 25–40% B in A, flow rate 1.0 ml/min. The concentrated oligonucleotide solutions were treated with 2.5% $CHCl_2COOH/CH_2Cl_2$ for 5 min at r.t. to remove the 4,4′-dimethoxytrityl residues. The detritylated oligomers were purified by reverse phase HPLC with the gradient III, 20 min 0–25% B in A, flow rate 1.0 ml/min). 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 oligomers were lyophilized on a Speed-Vac evaporator to yield colorless solids which were frozen at –24°C.

Table 1. Molecular masses (M^+) of oligonucleotides determined by MALDI-TOF mass spectroscopy

Oligomer	M^+ (calculated)	M^+ (found)
5′-d(TAGGTCAATACT) (**12**)	3644.4	3645
5′-d(AGTATTGACCTA) (**13**)	3644.4	3645
5′-d(TAGGTC**2a2a**TACT) (**16**)	3674.4	3677
5′-d(AGT**2a**TTG**2a**CCTA) (**17**)	3674.4	3675
5′-d(TAGGTC**2b2b**TACT) (**21**)	3832.5	3830
5′-d(AGT**2b**TTG**2b**CCTA) (**18**)	3832.5	3832

Determination of T_m values and thermodynamic data. Absorbance versus temperature profiles were measured on a Cary 1/1E UV/VIS spectrophotometer (Varian, Australia) equipped with a Cary thermoelectrical controller. The temperature was measured in the reference cell with a Pt-100 resistor. The thermodynamic data $\Delta H°$ and $\Delta S°$ were obtained by curve shape analysis using the program Meltwin 3.0 (18); the $\Delta G°$ values were calculated for 310°C. The CD spectra were recorded in 1.0 cm cuvettes with a Jasco-600 spectropolarimeter (Jasco, Japan) connected to a thermostatically controlled water bath (RCS-6; Lauda, Germany).

3-Bromo-1-(2-deoxy-β-D-erythro-pentofuranosyl)-1*H*-pyrazolo[3,4-*d*]-pyrimidin-4,6-diamine (2b)

A solution of 6-amino-3-bromo-1-[2-deoxy-β-D-erythro-pentofuranosyl]-4-isopropoxy-1*H*-pyrazolo[3,4-*d*]pyrimidine (**4b**) (19) (1.0 g, 2.6 mmol) in aqueous 25% NH_3 (80 ml) was heated at 70°C for 100 h in an autoclave. The solvent was reduced to a small volume while the residue precipitated. Crystallization of a small sample was performed in H_2O. The product was obtained as colorless needles (646 mg, 72%). Melting point 155°C. TLC (CH_2Cl_2/MeOH 9:1), R_f 0.2; UV (MeOH), λ_{max} 261, 278 nm (ε 8700, 9000). ^1H NMR [(D_6)DMSO], δ 2.16, 2.68 [2m, H_2-C(2′)], 3.38, 3.48 [2m, H_2-C(5′)], 3.77 [m, H-C(4′)], 4.36 [m, H-C(3′)], 4.72 [t, *J* = 4.9, HO-C(5′)], 5.19 [d, *J* = 4.1, HO-C(3′)], 6.32 ['t', *J* = 6.5, H-C(1′)], 6.39 (s, NH_2), 6.77 (br, NH_2). Calculated for $C_{10}H_{13}BrN_6O_3$ (345.2), C 34.80, H 3.80, N 24.35; found, C 34.97, H 3.97, N 24.21.

1-(2-Deoxy-β-D-erythro-pentofuranosyl)-N^4,N^6-(diphenoxyacetyl)-1*H*-pyrazolo[3,4-*d*]-pyrimidin-4,6-diamine (5a)

Compound **2a** (20) (1.5 g, 5.6 mmol) was dried by co-evaporation with pyridine. The residue was dissolved in pyridine (25 ml) and trimethylsilyl chloride (3.3 ml, 26 mmol) was added at r.t. with stirring. Stirring was continued for 15 min and a solution of 2,4,5-trichlorophenyl phenoxyacetate (5.4 g, 16.4 mmol) (21) in pyridine (15 ml) was added in one portion. The reaction mixture was stirred for 16 h at 40°C. It was cooled (ice bath) and H_2O (4.2 ml) was added. After 5 min the solution was diluted with aqueous 25% NH_3 (6 ml) and the mixture was concentrated to dryness. The residue was absorbed on silica gel and subjected to FC (CH_2Cl_2/MeOH 98:2, CH_2Cl_2/MeOH 9:1) yielding a colorless foam (900 mg, 30%). TLC (CH_2Cl_2/MeOH 9:1), R_f 0.4; UV (MeOH), λ_{max} 266 nm (ε 9100). ^1H NMR [(D_6)DMSO], δ 2.26, 2.82 [2m, H_2-C(2′)], 3.34, 3.48 [2m, H_2-C(5′)], 3.82 [m, H-C(4′)], 4.44 [m, H-C(3′)], 4.73 [t, *J* = 5.5, HO-C(5′)], 5.14 (s, OCH_2), 5.17 [d, *J* = 4.3, HO-C(3′)], 6.60 ['t', *J* = 6.3, H-C(1′)], 6.92–7.32 (m, arom. H), 8.41 [s, H-C(3)], 10.77 (s, NH), 11.30 (s, NH). Calculated for $C_{26}H_{26}N_6O_7$ (534.5), C 58.42, H 4.90, N 15.72; found, C 58.68, H 4.78, N 15.20.

1-[2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-N^4,N^6-(diphenoxyacetyl)-1*H*-pyrazolo[3,4-*d*]-pyrimidin-4,6-diamine (6a)

Compound **5a** (0.8 g, 1.5 mmol) was co-evaporated twice with pyridine. The residue was dissolved in pyridine (2 ml) and 4,4′-dimethoxytrityl chloride (0.6 g, 1.77 mmol) was added under stirring at r.t. The stirring was continued for 4 h, then the solution was diluted with MeOH (5 ml) and CH_2Cl_2 (25 ml). After washing with 5% aqueous sodium bicarbonate (3 × 20 ml) the organic layers were combined, dried (Na_2SO_4) and concentrated to dryness. The residue was purified by FC (CH_2Cl_2/acetone 95:5, CH_2Cl_2/acetone 9:1) yielding a colorless foam (310 mg, 25%). TLC (CH_2Cl_2/acetone 9:1), R_f 0.3; UV (MeOH), λ_{max} 266 nm (ε 8900). ^{1}H NMR [(D_6)DMSO], 2.32, 2.81 [2m, H_2-C(2′)], 2.99 [2m, H_2-C(5′)], 3.67 (2s, OCH_3), 3.95 [m, H-C(4′)], 4.53 [m, H-C(3′)], 5.15 (s, OCH_2), 5.33 [d, *J* = 4.7, HO-C(3′)], 6.62 [‘t’, *J* = 6.3, H-C(1′)], 6.69–7.31 (m, arom. H), 8.36 [s, H-C(3)], 10.83 (*s*, NH), 11.56 (*s*, NH). Calculated for $C_{47}H_{44}N_6O_9$ (836.9), C 67.45, H 5.30, N 10.04; found, C 66.95, H 5.41, N 10.15.

1-[2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-N^4,N^6-(diphenoxyacetyl)-1*H*-pyrazolo[3,4-*d*]-pyrimidin-4,6-diamine 3′-[(2-cyanoethyl) *N*,*N*-diisopropylphosphoramidite] (7a)

To a solution of compound **6a** (0.15 g, 0.18 mmol) in THF (0.5 ml) (i-Pr)$_2$EtN (0.12 ml, 0.71 mmol) and 2-cyanoethyl diisopropylphosphoramido chloridite (64 mg, 0.27 mmol) were added and stirred for 30 min at r.t. (argon atmosphere). The mixture was diluted with CH_2Cl_2 (20 ml) and 5% aqueous $NaHCO_3$ and extracted with CH_2Cl_2 (3 × 15 ml). The combined organic layers were dried (Na_2SO_4) and evaporated to give an oil. This was dissolved in CH_2Cl_2 and submitted to FC (eluant CH_2Cl_2/EtOAc 85:15) yielding a colorless foam (80 mg, 43%). TLC (CH_2Cl_2/EtOAc 85:15), R_f 0.8. ^{31}P NMR ($CDCl_3$), 149.2, 149.4.

1-(2-Deoxy-β-D-erythro-pentofuranosyl)-N^4,N^6-dibenzoyl-1*H*-pyrazolo[3,4-*d*]-pyrimidin-4,6-diamine (5b)

Compound **2a** (20) (1.0 g, 3.8 mmol) was co-evaporated twice with toluene and dissolved in pyridine (40 ml). Trimethylsilyl chloride (4.0 g, 36.8 mmol) was added with stirring at r.t. The solution was cooled to 0°C and PhCOCl (6.6 ml, 57 mmol) was added drop-wise over a period of 30 min. After stirring overnight at r.t. the solution was diluted with EtOAc (200 ml), washed with saturated aqueous sodium bicarbonate (200 ml) and ice-cold H_2O (200 ml). The aqueous layer was extracted with EtOAc (2 × 400 ml). The combined organic layers were evaporated to dryness and the residue dissolved in THF/MeOH/H_2O (5:4:1, 250 ml). The dark orange solution was cooled to 0°C and 2 N NaOH (25 ml) was added while stirring was continued for 40 min. The residue was purified by FC (eluant CH_2Cl_2/MeOH 98:2, CH_2Cl_2/MeOH 95:5) yielding an amorphous solid (1.1 g, 61%). TLC (CH_2Cl_2/MeOH 95:5), R_f 0.3; UV (MeOH), λ_{max} 245, 274 nm (ε 16 400, 15 200). ^{1}H NMR [(D_6)DMSO], 2.13, 2.67 [2m, H_2-C(2′)], 3.38, 3.52 [2m, H_2-C(5′)], 3.84 [m, H-C(4′)], 4.46 [m, H-C(3′)], 4.72 [t, *J* = 5.7, HO-C(5′)], 5.29 [d, *J* = 4.4, HO-C(3′)], 6.66 [‘t’, *J* = 6.5, H-C(1′)], 7.51–8.11 (m, arom. H), 8.40 (s, H-C3), 10.95 (s, NH), 11.49 (s, NH). Calculated for $C_{24}H_{22}N_6O_5$ (474.5), C 60.75, H 4.67, N 17.71; found, C 61.03, H 4.70, N 17.58.

1-[2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-N^4,N^6-dibenzoyl-1*H*-pyrazolo[3,4-*d*]-pyrimidin-4,6-diamine (6b)

As described for **6a** with **5b** (500 mg, 1.05 mmol) and 4,4′-dimethoxytriphenylmethyl chloride (460 mg, 1.35 mmol) in pyridine (3 ml). The residue was purified by FC (CH_2Cl_2/acetone 95:5, CH_2Cl_2/acetone 9:1) yielding a colorless foam (400 mg, 49%). TLC (CH_2Cl_2/acetone 9:1), R_f 0.4; UV (MeOH), λ_{max} 244, 275 nm (ε 16 400, 15 200).^{1}H NMR [(D_6)DMSO], 2.35, 2.67 [2m, H_2-C(2′)], 3.07, 3.10 [2m, H_2-C(5′)], 3.68, 3.69 (2s, OCH_3), 3.97 [m, H-C(4′)], 4.56 [m, H-C(3′)], 5.35 [d, *J* = 4.8, HO-C(3′)], 6.72–8.11 (m, arom. H), 8.36 [s, H-C(3)], 11.01 (s, NH), 11.54 (s, NH).

1-[2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-N^4,N^6-dibenzoyl-1*H*-pyrazolo[3,4-*d*]-pyrimidin-4,6-diamine 3′-[(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite] (7b)

As described for **7a** with **6b** (110 mg, 0.14 mmol), (i-Pr)$_2$EtN (75 µl, 0.43 mmol) and 2-cyanoethyl diisopropylphosphoramido chloridite (38 µl, 0.17 mmol) in THF (3 ml) at 30°C. The oily residue was submitted to FC (CH_2Cl_2/EtOAc 85:15) yielding a colorless foam (92 mg, 67%). TLC (CH_2Cl_2/EtOAc 85:15), R_f 0.8. ^{31}P NMR ($CDCl_3$), 149.82, 149.63.

1-(2-Deoxy-β-D-erythro-pentofuranosyl)-N^4,N^6-bis-[(di-*n*-butylamino)methylidene]-1*H*-pyrazolo[3,4-*d*]-pyrimidin-4,6-diamine (8a)

A solution of **2a** (300 mg, 1.13 mmol) in MeOH (5 ml) and *N*,*N*-di-*n*-butylformamide dimethyl acetal (22) (790 µl, 3.39 mmol) was added and stirred for 2 h at 40°C. The reaction mixture was evaporated to dryness and the residue adsorbed on silica gel. FC (CH_2Cl_2/MeOH 98:2→ CH_2Cl_2/MeOH 95:5) afforded two main products. From the faster migrating zone a colorless foam of **8a** (330 mg, 53%) was isolated. TLC (CH_2Cl_2/MeOH 95:5), R_f 0.4; UV (MeOH), λ_{max} 235, 274, 322 nm (ε 22 800, 13 800, 25 700). ^{1}H NMR ($CDCl_3$), 0.87–0.95 (m, CH_2CH_3), 1.26–1.38 (m, CH_2CH_3), 1.57–1.60 (m, CH_2CH_2), 2.42–2.92 [m, H_2-C(2′)], 3.26–3.72 [2m, H_2-C(5′), NCH_2], 3.88 [m, H-C(4′)], 4.39 [m, H-C(3′)], 4.78 [t, *J* = 5.8, HO-C(5′)], 5.21 [d, *J* = 4.8, HO-C(3′)], 6.55 [‘t’, *J* = 6.9, H-C(1′)], 7.93 [s, H-C(3)], 8.66 (s, N=CH), 8.88 (s, N=CH). Calculated for $C_{28}H_{49}N_8O_3$ (544.7), C 61.74, H 8.88, N 20.57; found, C 61.71, H 8.91, N 20.57.

1-(2-Deoxy-β-D-erythro-pentofuranosyl)-N^6-[(di-*n*-butylamino)methylidene]-1*H*-pyrazolo[3,4-*d*]-pyrimidin-4,6-diamine (9a)

The slower migrating zone furnished compound **9a** (80 mg, 17%) as a colorless foam. TLC (CH_2Cl_2/MeOH 95:5), R_f 0.3; UV (MeOH), λ_{max} 235, 275, 320 nm (ε 23 000, 14 900, 22 100). ^{1}H NMR [(D_6)DMSO], 0.88–0.95 (m, CH_2CH_3), 1.27–1.36 (m, CH_2CH_3), 1.53–1.62 (m, CH_2CH_2), 2.21 [m, H-C(2′)], 2.91 [m, H-C(2′)], 3.38–3.59 [2m, H_2-C(5′), NCH_2], 3.79 [m, H-C(4′)], 4.40 [m, H-C(3′)], 4.78 [t, *J* = 5.8, HO-C(5′)], 5.19 [d, *J* = 4.4, HO-C(3′)], 6.36 (*s*, NH_2), 6.40 [‘t’, *J* = 6.5, H-C(1′)], 7.77 [s, H-C(3)], 8.69 (s, N=CH). Calculated for $C_{19}H_{31}N_7O_3$ (405.5), C 56.28, H 7.71, N 24.18; found, C 55.98, H 7.52, N 24.05.

3-Bromo-1-(2-deoxy-β-D-erythro-pentofuranosyl)-*N*^{4},*N*^{6}-bis-[(di-*n*-butylamino)methylidene]-1*H*-pyrazolo[3,4-*d*]-pyrimidin-4,6-diamine (8b)

As described for **8a**, with **2b** (350 mg, 1 mmol) and *N*,*N*-di-*n*-butylformamide dimethyl acetal (720 µl, 3.1 mmol) in MeOH (7 ml) for 2 h at 40°C. After FC (CH_2Cl_2/MeOH 98:2, CH_2Cl_2/MeOH 95:5) two main products were isolated. The faster migrating zone gave **8b** as a foam (310 mg, 50%). TLC(CH_2Cl_2/MeOH 95:5), R_f 0.3; UV (MeOH), λ_{max} 235, 275, 320 nm (ε 22 500, 14 500, 22 100). ^{1}H NMR [(D_6)DMSO], 0.94–0.99 (m, CH_2CH_3), 1.32–1.44 (m, CH_2CH_3), 1.59–1.73 (m, CH_2CH_2), 2.21 [m, H-C(2′)], 2.91 [m, H-C(2′)], 3.32–3.78 [2m, H_2-C(5′), NCH_2], 3.89 [m, H-C(4′)], 4.40 [m, H-C(3′)], 4.78 [t, *J* = 5.8, HO-C(5′)], 5.19 [d, *J* = 4.4, HO-C(3′)], 6.81 ['t', *J* = 6.5, H-C(1′)], 8.69 (s, N=CH), 8.94 (s, N=CH). Calculated for $C_{28}H_{47}BrN_8O_3$ (623.6), C 53.93, H 7.60, N 17.97; found, C 54.01, H 7.52, N 18.05.

3-Bromo-1-(2-deoxy-β-D-erythro-pentofuranosyl)-*N*^{6}-[(di-*n*-butylamino)methylidene]-1*H*-pyrazolo[3,4-*d*]-pyrimidin-4,6-diamine (9b)

The slower migrating zone furnished **9b** as a colorless foam (75 mg, 16%). TLC (CH_2Cl_2/MeOH 95:5), R_f 0.3; UV (MeOH), λ_{max} 236, 276, 320 nm (ε 21 600, 14 000, 21 900). ^{1}H NMR [(D_6)DMSO], 0.90–0.96 (m, CH_2CH_3), 1.30–1.39 (m, CH_2CH_3), 1.59–1.73 (m, CH_2CH_2), 2.23 [m, H-C(2′)], 2.89 [m, H-C(2′)], 3.35–3.78 [2m, H_2-C(5′), NCH_2], 3.84 [m, H-C(4′)], 4.43 [m, H-C(3′)], 4.80 [t, *J* = 5.7, HO-C(5′)], 5.23 [d, *J* = 4.6, HO-C(3′)], 6.40 (s, NH_2), 6.81 ['t', *J* = 6.4, H-C(1′)], 8.65 (s, N=CH). Calculated for $C_{19}H_{30}BrN_7O_3$ (484.4), C 47.11, H 6.24, N 20.24; found, C 47.23, H 6.52, N 20.35.

1-[2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-*N*^{4}-[(di-*n*-butylamino)methylidene]-*N*^{6}-formyl-1*H*-pyrazolo[3,4-*d*]-pyrimidin-4,6-diamine (10a)

As described for **6a** with **8a** (80 mg, 0.15 mmol), 4,4′-dimethoxytriphenylmethyl chloride (60 mg, 0.18 mmol) in pyridine (0.5 ml). The residue was purified by FC (CH_2Cl_2/acetone 95:5, CH_2Cl_2/acetone 9:1) yielding a colorless foam (85 mg, 77%). TLC (CH_2Cl_2/acetone 9:1), R_f 0.3; UV (MeOH), λ_{max} 235, 276, 320 nm (ε 22 800, 20 000, 24 800). ^{1}H NMR ($CDCl_3$), 0.98–1.05 (m, CH_2CH_3), 1.14–1.48 (m, CH_2CH_3), 1.65–1.78 (m, CH_2CH_2), 2.42–2.92 [m, H_2-C(2′)], 3.35–3.70 [2m, H_2-C(5′), NCH_2], 3.71 (s, OCH_3), 4.06 [m, H-C(4′)], 4.39 [m, H-C(3′)], 5.21 [d, *J* = 4.8, HO-C(3′)], 6.55 ['t', *J* = 6.6, H-C(1′)], 6.76–7.96 (m, arom. H), 7.94 (d, *J* = 10.5, NH), 7.96 [s, H-C(3)], 8.81 (s, N=CH), 9.57 (d, *J* = 10.5, CHO). Calculated for $C_{41}H_{49}N_7O_6$ (735.9), C 66.92, H 6.71, N 13.32; found, C 66.85, H 6.56, N 13.40.

3-Bromo-1-[2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-*N*^{4}-[(di-*n*-butylamino)methylidene]-*N*^{6}-formyl-1*H*-pyrazolo[3,4-*d*]-pyrimidin-4,6-diamine (10b)

As described for **6a** with **8b** (310 mg, 0.5 mmol), 4,4′-dimethoxytriphenylmethyl chloride (202 mg, 0.6 mmol) in pyridine (2 ml). The residue was purified twice by FC (CH_2Cl_2/acetone 95:5, CH_2Cl_2/acetone 9:1) yielding a colorless foam (250 mg, 61%). TLC (CH_2Cl_2/acetone 9:1), R_f 0.3; UV (MeOH), λ_{max} 235, 276, 320 nm (ε 22 900, 21 100, 25 300). ^{1}H NMR [(D_6)DMSO], 0.90–0.96 (m, CH_2CH_3), 1.28–1.37 (*m*, CH_2CH_3), 1.61–1.68 (m, CH_2CH_2), 2.21 [m, H-C(2′)], 2.91 [m, H-C(2′)], 3.03 [2m, H_2-C(5′)], 3.35–3.52 (m, NCH_2), 3.69 (s, OCH_3), 3.90 [m, H-C(4′)], 4.48 [m, H-C(3′)], 5.32 [d, *J* = 4.7, HO-C(3′)], 6.46 ['t', *J* = 6.2, H-C(1′)], 6.73–7.31 (m, arom. H), 8.99 (s, N=CH), 9.56 (d, *J* = 9.89, NH), 10.77 (d, *J* = 9.88, CHO). Calculated for $C_{41}H_{48}BrN_7O_6$ (814.8), C 60.44, H 5.94, N 12.03; found, C 60.36, H 5.73, N 11.85.

1-[2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-*N*^{4}-[(di-*n*-butylamino)methylidene]-*N*^{6}-formyl-1*H*-pyrazolo[3,4-*d*]-pyrimidin-4,6-diamine 3′-[(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite] (11a)

As described for **7a** with **10a** (110 mg, 0.15 mmol), (i-Pr)$_2$EtN (78 µl, 45 mmol) and 2-cyanoethyl diisopropylphosphoramido chloridite (47 µl, 0.2 mmol) in THF (2 ml). The oily residue was submitted to FC (CH_2Cl_2/EtOAc 85:15) yielding a colorless foam (75 mg, 53%). TLC (CH_2Cl_2/EtOAc 85:15), R_f 0.8. ^{31}P NMR ($CDCl_3$), 149.58, 149.52.

3-Bromo-1-[2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-*N*^{4}-[(di-*n*-butylamino)methylidene]-*N*^{6}-formyl-1*H*-pyrazolo[3,4-*d*]-pyrimidin-4,6-diamine 3′-[(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite] (11b)

As described for **7a** with **10b** (90 mg, 0.11 mmol), (i-Pr)$_2$EtN (63 µl, 0.36 mmol) and 2-cyanoethyl diisopropylphosphoramido chloridite (31 µl, 0.14 mmol) in THF (1 ml). The oily residue was submitted to FC (CH_2Cl_2/EtOAc 85:15) yielding a colorless foam (80 mg, 72%). TLC (CH_2Cl_2/EtOAc 85:15), R_f 0.8. ^{31}P NMR ($CDCl_3$), 149.58, 149.53.

RESULTS AND DISCUSSION

Monomers

The isopropoxy nucleosides **4a**,**b**, which have been described previously (19), served as precursor molecules for synthesis of the 8-aza-7-deazapurin-2,6-diamine (pyrazolo[3,4-*d*]pyrimidin- 4,6-diamine) nucleosides **2a**,**b** (Scheme 2). Treatment of the alkoxy derivatives with 25% aqueous NH_3 for 4 days at 70°C furnished the diamino compounds **2a**,**b**. Nucleosides **2a**,**b** showed differences in chemical enzymatic stability in comparison to the purin-2,6-diamine nucleoside **1**. The half-life of the *N*-glycosylic bond of compound **2a** (τ = 91 min, 0.5 N HCl, 20°C) indicated an ~15-fold higher glycosylic bond stability than the parent 2-amino-2′-deoxyadenosine (**1**) (τ = 6 min, 0.5 N HCl, 20°C). The glycosylic bond of the bromo derivative **2b** was stable under these conditions; stronger acidic conditions (2 N HCl) were necessary to hydrolyze this molecule (τ = 87 min). A similar stabilization of the glycosylic bond has been reported for other 7-bromo-8-aza-7-deazapurine 2′-deoxyribonucleosides (13,15). Treatment of compound **2a** with adenosine deaminase resulted in formation of 8-aza-7-deaza-2′-deoxyguanosine (**4c**). The reaction was followed by UV spectrophotometry. Deamination of **2a** occurred much more slowly than that of purine nucleoside **1**. The 7-bromo derivative **2b** was stable against deamination even at high enzyme concentrations (23).

The stability of oligonucleotides is influenced by the conformation of the sugar–phosphate backbone and a modified nucleobase can alter the N↔S pseudorotational equilibrium of the

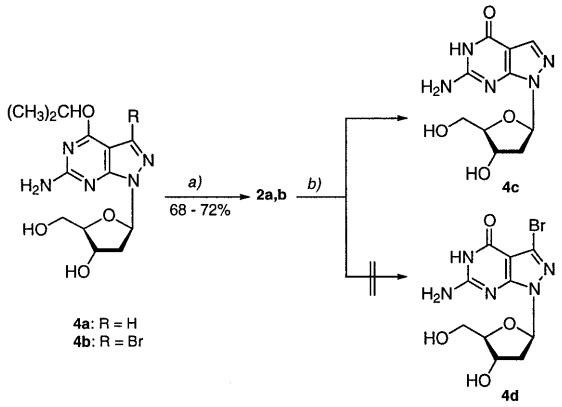
Table 2. $^3J_{H,H}$ coupling constants of the sugar moieties and conformer populations (N/S) of 2′-deoxyribonucleosides **1**–**3** at 303 K[a]

	$^3J_{H,H}$ (Hz)							Conformation				
	1′,2′	1′,2″	2′,3′	2″,3′	3′,4′	4′,5′	4′,5″	N (%)	S (%)	$\gamma^{(+)g}$	γ^{t}	$\gamma^{(-)g}$
1	7.30	6.10	7.00	3.10	3.40	3.20	4.30	31	69	62	25	13
2a	6.60	6.80	6.90	3.70	3.60	4.00	5.80	37	63	36	42	22
2b	6.60	6.70	6.80	3.70	3.90	4.30	5.90	37	63	32	43	25
3	7.90	6.40	6.20	3.10	3.00	3.87	4.82	25	75	48	32	20

[a]Solvent D_2O; r.m.s. < 0.4 Hz; $|\Delta J_{max}| < 0.4$ Hz.

sugar moiety significantly. The ^{1}H NMR spectra of nucleosides **1**–**3** were measured in D_2O and ^{1}H,^{1}H NMR coupling constants were determined. Analysis with respect to the sugar ring conformation was then performed using the program PSEUROT (24). According to Table 2 8-aza-7-deazapurin-2,6-diamine nucleosides **2a**,**b** show a more pronounced N conformer population than the corresponding purine nucleoside **1**, while the N-type population of the related 7-deazapurine nucleoside **3** is decreased. The conformation around the C(4′)–C(5′) bond indicates that 8-aza-7-deazapurin-2,6-diamine nucleosides **2a**,**b**, as for 8-aza-7-deaza-2′-deoxyguanosines (25), prefer the γ^t-(–sc) rotamer population, while for the regular purine nucleosides the $\gamma^{(+)g}$-(+sc) conformation is predominant (26). This means that in both cases the nucleobase and the CH_2OH group undergo a disrotatory motion so that the Coulomb repulsion between N(8) and O(5′) and O(4′) is minimized. These observations indicate that the conformational parameters of nucleosides **2a**,**b** are similar but not the same as those found for purin-2,6-diamine 2′-deoxyribonucleoside **1**. These differences can affect base pairing as well as stacking interactions within a DNA duplex.

Next, the two amino groups of nucleosides **2a**,**b** were protected. This can be done only with difficulty, as already recognized in the case of purin-2,6-diamine nucleoside **1**. In contrast to guanine, the 2-amino group of purin-2,6-diamine is rather basic. As a result, benzoyl protection at that position leads to stable amides, which are difficult to hydrolyze. If a more labile protecting group is chosen for both amino functions, that attached to the 6-amino group readily dissociates. This has already been demonstrated in the case of benzoyl groups (1) and phenoxyacetyl residues (21,27,28). Apart from those difficulties, the acylated derivatives show an increased tendency to depurinate (1). Fortunately, the *N*-glycosylic bond of nucleosides **2a**,**b** is significantly more stable than that of purine nucleoside **1**. Thus, phenoxyacetyl as well as benzoyl groups were successfully introduced into compound **2a** when 2,4,5-trichlorophenyl phenoxyacetate (21) or benzoyl chloride were used and the transient protection protocol was employed (29) (**2a**→**5a**,**b**). The yield of the bis-phenoxyacylated derivative **5a** was rather low (30%) while the bis-benzoylated nucleoside **5b** was isolated in 63% yield (Scheme 3). Both compounds **5a**,**b** were converted into the DMT derivatives **6a**,**b** using standard reaction conditions. Phosphitylation with 2-cyanoethyl diisopropylphosphoramido chloridite afforded the phosphoramidites **7a**,**b** (30). As the DMT derivatives are poorly soluble, a THF-containing solution had to be used for phosphitylation. The yield of the phosphoramidite, which is normally ~80%, was only 67%.



Scheme 2. (**a**) Aqueous 25% NH_3, 60°C, 4 days. (**b**) Adenosine deaminase from calf intestine mucosa (EC 3.5.4.4), Tris–HCl buffer, pH 7.0, 20°C.

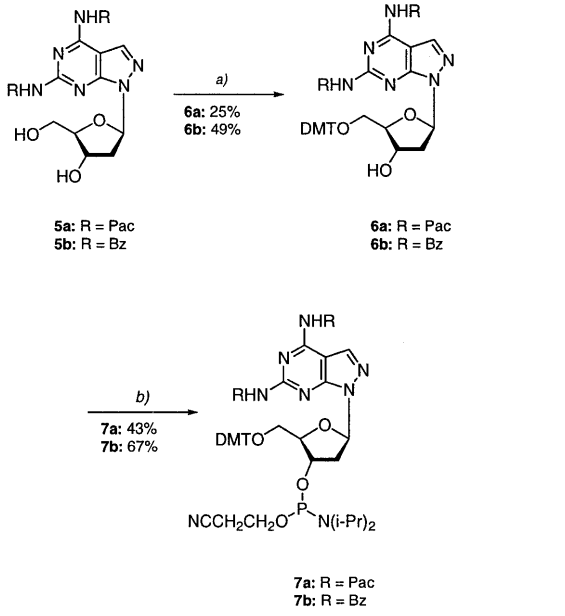
Deprotection of compound **5a** (25% aqueous NH_3, 40°C, HPLC monitoring) showed that removal of the pac groups is fast and complete deprotection occurs in <1 h. Complete removal of the two benzoyl groups of compound **5b** (25% aqueous NH_3, 40°C, HPLC monitoring) required 10 h while deprotection of bis-benzoylated purine nucleoside **1** took several days. From this point of view the benzoyl-protected phosphoramidite **7b** represents a useful building block for incorporation of compound **2a** into oligonucleotides. Nevertheless, the low solubility of the intermediate **6b** represents a problem. In spite of this fact, *N*,*N*-dialkylaminomethylidene groups were considered for protection. Early attempts to introduce *N*,*N*-dimethylaminomethylidene residues into purine nucleoside **1** failed due to the instability of the protecting groups. Our laboratory has used the *N*,*N*-di-*n*-butylaminomethylidene group (22) for protection of the amino function of 8-aza-7-deaza-2′-deoxyisoguanosine and 5-aza-7-deaza-2′-deoxyguanosine (31). This group was now introduced into nucleosides **2a**,**b**. The bis-amidines **8a**,**b** were obtained as the major products (50% yield) while the mono adducts **9a**,**b** were formed as minor components (17%). For the protected nucleosides **8a**,**b** the time required for complete deprotection (concentrated ammonia, 40°C, HPLC monitoring at 260 nm) was determined to be 440 min for **8a** and 450 min for **8b**. A half-life value for the particular residues was not determined.

Subsequently, the 5′-hydroxyls were protected with 4,4′-dimethoxytrityl chloride. After the work-up followed by silica gel flash chromatography one *N*,*N*′-dibutylaminomethylidene residue was hydrolyzed to yield the formyl group (Scheme 4).

Table 3. ^{13}C NMR chemical shifts of pyrazolo[3,4-*d*]pyrimidine 2′-deoxyribonucleosides[a]

	C(2)[b,d] C(6)[c]	C(4)[d] C(7a)	C(5) C(3a)	C(6)[d] C(4)	C(7) C(3)	C=O/=CH	C=O/=CH	C(1′)	C(2′)	C(3′)	C(4′)	C(5′)
2a	156.9	158.3	95.5	162.7	133.3	–	–	83.3	38.0	71.3	87.4	62.7
2b	157.4	157.6	94.5	162.7	119.2	–	–	83.0	37.5	70.9	87.3	62.4
5a	152.2	154.8	100.5	156.1	136.1	168.5	169.1	83.6	37.7	71.1	87.7	62.5
5b	153.3	155.2	102.2	156.1	134.0	165.6	166.3	83.5	37.6	70.9	87.5	62.3
6a	152.2	154.8	100.5	155.9	135.9	168.5	169.2	83.7	38.1	70.8	85.6	64.3
6b	153.3	155.3	102.3	156.1	132.2	165.7	166.4	83.7	38.0	70.9	85.6	64.4
8a[f]	157.1	157.6	106.0	158.9	134.9	164.1	166.2	86.1	41.2	73.7	89.1	64.3
8b	156.1	156.1	103.6	157.9	121.4	162.1	164.4	83.3	37.5	70.8	87.5	62.2
8b[f]	157.1	157.7	104.7	159.2	122.9	163.4	166.7	86.2	41.0	73.8	89.0	64.2
9a	156.6	157.3	102.4	162.3	133.7	162.9	–	83.1	37.8	71.2	87.3	62.6
10a[f]	155.5	155.5	107.1	156.8	134.9	161.8	163.7	84.2	38.3	73.6	86.7	64.9
10b	156.0	156.1	103.8	157.8	121.3	162.1	164.4	83.5	–[e]	70.5	85.3	64.1

[a]Measured in (D_6)DMSO at 298 K.
[b]Purine numbering.
[c]Systematic numbering.
[d]Tentative.
[e]Superimposed by (D_6)DMSO.
[f]Measured in $CDCl_3$.



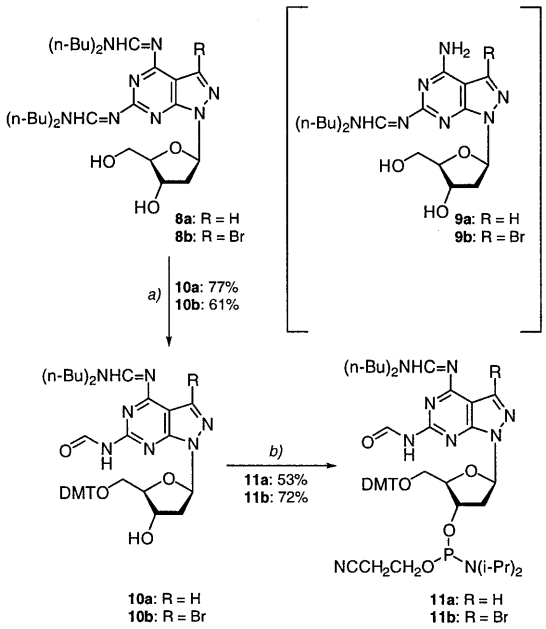
Scheme 3. (**a**) Pyridine/$(MeO)_2$TrCl, r.t., 4 h. (**b**) THF, 2-cyanoethyl diisopropylphosphoramido chloridite, r.t., 30 min.

This group was protecting the 2-amino function, while the 6-amino group carried the dibutylaminomethylidene residue. The structures of compounds **10a**,**b** were established on the basis of NMR spectra (Table 3). Phosphitylation of the DMT derivatives of **10a**,**b** was performed in THF in the presence of 2-cyanoethyl diisopropylphosphoramido chloridite, furnishing the phosphoramidites **11a**,**b** (Scheme 3). These phosphoramidites (**11a**,**b**), as well as the corresponding building block **7b** with benzoyl protection, were efficiently used in solid phase oligonucleotide synthesis with coupling yields >90%. All compounds were characterized by ^{1}H, ^{13}C and ^{31}P NMR spectra and by elemental analysis (Table 3 and Materials and Methods). The ^{13}C NMR data are particularly useful for monomer assignment as the heterocyclic bases contain only very few protons, making structural characterization from ^{1}H NMR spectra difficult.

Oligonucleotides

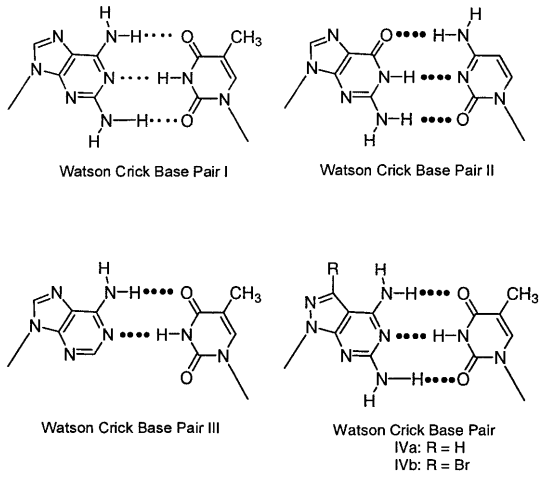
Synthesis and characterization. Automated solid phase synthesis of oligonucleotides **12**–**26** (Tables 4 and 5) was performed using standard phosphoramidite chemistry. The oligonucleotides were detritylated and purified on oligonucleotide purification cartridges or by reverse phase HPLC (for conditions of purification see Materials and Methods). The homogeneity of the compounds was proven by ion exchange chromatography. The composition of the oligonucleotides was determined by RP-18 HPLC after tandem hydrolysis with snake venom phosphodiesterase and alkaline phosphatase as described (17). 8-Aza-7-deazapurin-2,6-diamine nucleoside **2a** migrates slightly slower than dA. The bromo compound **2b** is much more hydrophobic, as can be seen from the elution profile (Fig. 1A and B). MALDI-TOF mass spectra were measured for the modified oligonucleotides (Table 1 and Materials and Methods). The correct masses were found in all cases, which underlines that all protecting groups were split off during a 10 h ammonia treatment at 60°C.

Base pairing and oligonucleotide duplex stability. Because of the presence of three hydrogen bonds the **1**-dT base pair (I) is expected to show the same or a similar stability to a dG-dC pair (II) (Scheme 5). Experimental data obtained from DNA melting curves indicate that the thermodynamic stability of duplexes containing a **1**-dT base pair is indeed somewhat



Scheme 4. (**a**) Pyridine/$(MeO)_2$TrCl, r.t., 4 h. (**b**) THF, 2-cyanoethyl diisopropylphosphoramido chloridite, r.t., 30 min.

higher than that of a dA-dT pair (III) but still remains far below that of a dG-dC pair. As the pyrazolo[3,4-*d*]pyrimidine nucleosides **2a**,**b** can form similar tridentate base pairs as purine compound **1** (motifs IVa,b) it was of interest to investigate the stability of oligonucleotides containing the modified base pairs IVa and IVb.



Scheme 5.

Various studies have been performed to shed some light on the stability differences of a 2,6-diaminopurine-dT pair versus a dG-dC pair (2–6). A rather detailed examination of this matter has been undertaken by Sági *et al*. (8). The authors compared the thermal stabilities of bidentate base pairs represented by the motifs dA-dU, dI-dC, dA-dT and dI-m^5C_d with those of tridentate pairs such as n^2A_d-dU, dG-dC, n^2A_d-dT and dG-m^5C_d. As long as a 2-amino group was absent (bidentate base pairs) the various duplexes all showed very similar stabilities, independently of the particular base structure. On insertion of a 2-amino group, which leads to a tridentate base pair, this similarity disappears. The dG-dC base pair (II) is now much more stable than the n^2A_d-dT pair (I). We were interested to compare the base pair stabilities of 8-aza-7-deazapurin-2,6-diamine nucleosides **2a**,**b** with dT. Purine nucleoside **1** was evaluated for comparison. All three compounds were incorporated into the non-self-complementary duplex 5′-d(TAGGTCAATACT) (**12**), 5′-d(AGTATTGACCTA) (**13**). This duplex is used as a standard in our laboratory to study the influence of modified nucleosides on the thermal stability and structural behavior of helical formation. The T_m value of **12**·**13** is 47°C (0.1 M NaCl, 10 mM $MgCl_2$, 10 mM Na cacodylate).

Table 4. T_m values and thermodynamic data for oligonucleotide duplexes containing purin-2,6-diamine 2′-deoxyribonucleoside **1** and pyrazolo[3,4-*d*]pyrimidine analogs **2a**,**b**[a]

	T_m (°C)	ΔG^0_{310} (kcal/mol)
5′-d(TAG GTC AAT ACT) (**12**) 3′-d(ATC CAG TTA TGA) (**13**)	47	–10.6
5′-d(T**1**G GTC **11**T **1**CT) (**14**) 3′-d(ATC C**1**G TT**1** TGA) (**15**)	50[b]	–
5′-d(TAG GTC **2a2a**T ACT) (**16**) 3′-d(ATC C**2a**G TT**2a** TGA) (**17**)	51	–12.3
5′-d(TAG GTC AAT ACT) (**12**) 3′-d(ATC C**1**G TT**1** TGA) (**15**)	49	–10.9
5′-d(TAG GTC AAT ACT) (**12**) 3′-d(ATC C**2a**G TT**2a** TGA) (**17**)	50	–12.1
5′-d(TAG GTC AAT ACT) (**12**) 3′-d(ATC C**2b**G TT**2b** TGA) (**18**)	59	–13.9
5′-d(TAG GTC **2b2b**T ACT) (**21**) 3′-d(ATC CAG TT A TGA) (**13**)	56	–13.4
5′-d(TAG GTC **2b2b**T ACT) (**21**) 3′-d(ATC C**2b**G TT **2b**TGA) (**18**)	67	–17.0
5′-d(T**2b**GGTC**2b2b**T**2b**CT) (**19**) 3′-d(A TC CAG T T ATGA) (**13**)	64	–15.8
5′-d(TAG GTC AAT ACT) (**12**) 3′-(AUC CAG UUA UGA) (**20**)	45	–10.2
5′-d(T**1**G GTC **11**T **1**CT) (**14**) 3′-(AUC CAG UUA UGA) (**20**)	48	–10.2
5′-d(TAG GTC **2a2a**T ACT) (**16**) 3′-(AUC CAG UUA UGA) (**20**)	48	–[c]
5′-d(TAG GTC **2b2b**T ACT) (**21**) 3′-(AUC CAG UUA UGA) (**20**)	53	–12.7

[a]Measured at 260 nm in 0.1 M NaCl, 10 mM $MgCl_2$ and 10 mM Na cacodylate buffer, pH 7.0, with 5 µM + 5 µM single strand concentrations.
[b]Low cooperativity.
[c]Not measured.

Table 5. T_m values and thermodynamic data for oligonucleotide duplexes containing dG-dC base pairs, **2b**-dT pairs and mismatches

	T_m (°C)	ΔG^0_{310} (kcal/mol)
5′-d(TAG GTC AAT ACT) (**12**) 3′-d(ATC CAG TTA TGA) (**13**)	47[a]	–10.6
5′-d(TAG GCC **GG**C ACT) (**22**) 3′-d(ATC C**G**G CC**G** TGA) (**23**)	66[a]	–16.0
5′-d(TAG GTC **2b2b**T ACT) (**21**) 3′-d(ATC C**2b**G TT**2b** TGA) (**18**)	67[a]	–17.0
5′-d(TAG G**T**C AAT ACT) (**12**) 3′-d(ATC C**2b**G TT**2b** TGA) (**18**)	59[a]	–13.9
5′-d(TAG G**A**C AAT ACT) (**24**) 3′-d(ATC C**2b**G TT**2b** TGA) (**18**)	47[a]	–10.4
5′-d(TAG G**G**C AAT ACT) (**25**) 3′-d(ATC C**2b**G TT**2b** TGA) (**18**)	51[a]	–12.3
5′-d(TAG G**C**C AAT ACT) (**26**) 3′-d(ATC C**2b**G TT**2b** TGA) (**18**)	48[a]	–11.1
5′-d(TAG G**T**C AAT ACT) (**12**) 3′-d(ATC C**A**G TTA TGA) (**13**)	50[b]	–12.0
5′-d(TAG G**A**C AAT ACT) (**24**) 3′-d(ATC C**A**G TTA TGA) (**13**)	40[b]	–8.4
5′-d(TAG G**G**C AAT ACT) (**25**) 3′-d(ATC C**A**G TTA TGA) (**13**)	48[b]	–10.5
5′-d(TAG G**C**C AAT ACT) (**26**) 3′-d(ATC C**A**G TTA TGA) (**13**)	38[b]	–8.0

[a]Measured at 260 nm in 0.1 M NaCl, 10 mM $MgCl_2$ and 10 mM Na cacodylate, pH 7.0, with 5 µM + 5 µM single strand concentrations.
[b]Measured at 260 nm in 1 M NaCl, 100 mM $MgCl_2$ and 60 mM Na cacodylate, pH 7.0, with 5 µM + 5µM single strand concentrations.

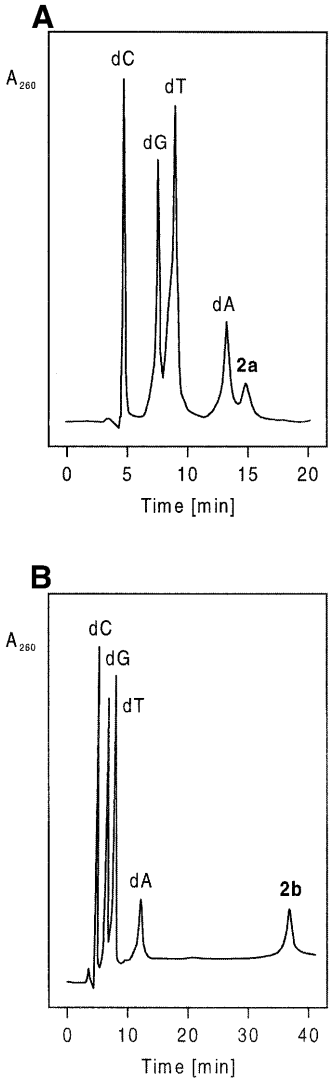


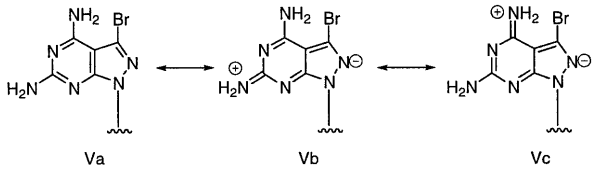
Figure 1. HPLC profiles of the reaction products obtained after enzymatic hydrolysis of oligomers **17** (**A**) and **18** (**B**) with snake venom phosphodiesterase and alkaline phosphatase in 0.1 M Tris–HCl buffer (pH 8.0) at 37°C. Column, RP-18 (200 × 10 mm); gradient: 0–20 min 100% B, 20–50 min 0–15% A in B.

The incorporation of six compound **1** residues instead of six dA residues increased the T_m value by only 3°C (see duplex **14·15**, Table 4). This corresponds to a 0.5°C T_m increase per residue. Similar findings have been reported from experiments performed in other laboratories (3–8). When only four 8-aza-7-deazapurin-2,6-diamine nucleosides (**2a**) replaced dA residues an increase in the T_m value from 47 to 51°C was measured, which corresponds to a 1.0°C increase per modified residue (see duplex **12·13** versus **16·17**). Thus, the pyrazolo[3,4-*d*]pyrimidine nucleoside **2a** forms a more stable tridentate base pair with dT (motif IVa) than purine nucleoside **1** (motif I).

Earlier our laboratory has shown that 7-substituents of 7-deazapurines as well as of 8-aza-7-deazapurines are well accommodated in the major groove of DNA (12–15). In particular, halogen substituents show favorable properties with regard to duplex stability. This prompted us to replace dA residues by the 7-bromo derivative **2b**. According to Table 4 duplex **12·18** carrying only two **2b** residues is significantly more stable (T_m = 59°C) than the non-brominated duplex **12·17** (T_m = 50°C). In this case duplex stability was strengthened by 4.5°C per modified base. Likewise, duplex **18·21** was 16°C more stable than duplex **16·17** (T_m = 67°C), which corresponds to a T_m increase of 4°C per modified residue. Also, duplex **12·18**, incorporating only two nucleoside **2b** residues, was significantly more stable than duplex **14·15**, containing six diaminopurine nucleosides (**1**) (T_m = 59 versus 50°C). A comparison of the stability of duplex **18·21** with the parent hybrid **12·13** shows that replacement of a dA residue by the base-modified nucleoside **2b** increases the T_m value by 5°C per residue. Oligonucleotides containing nucleosides **1** or **2a**,**b** were also hybridized with the complementary oligoribonucleotide **20**. According to Table 4 DNA–RNA hybrids **14·20** and **16·20** show identical T_m stabilizations for compounds **1** and **2a** over that of dA (duplex **12·20**). A stronger stabilization was observed when the bromo compound **2b** replaced **2a**. Nevertheless, stabilization of the DNA–RNA hybrid by **2b** was lower than that of DNA–DNA

duplexes. The ΔG values shown in the Tables 4 and 5 were calculated at 310 K from the ΔH and ΔS values obtained by curve shape analysis of the melting profiles using the program Meltwin 3.0 (18). The free energies correlate well with the T_m values and correspond to those determined from the concentration dependence of the T_m values within 15%.

From the data in Table 4 it is apparent that the contribution of compound **2b** to DNA–DNA duplex stability is extraordinarily high. Thus, it was of interest to prove whether the modified **2b**-dT base pair approaches the stability of the canonical dG-dC pair. For this purpose oligonucleotides **22** and **23** were synthesized and hybridized. Duplex **22·23** is derived from duplex **12·13** by replacing four dA-dT base pairs by four dG-dC pairs. Then the duplex stability of **21·18** containing four **2b**-dT base pairs was compared with that of **22·23**. From Table 5 it is apparent that both duplexes display very similar T_m values, indicating that the modified dA-dT base pair shows the same stability as a dG-dC pair. An explanation for this observation can be given on the basis of two effects. (i) The more favorable proton donor properties of 8-aza-7-deazapurin-2,6-diamine nucleosides **2a**,**b** compared to those of purin-2,6-diamine nucleoside **1.** This is supported by the decreased stability of acyl groups protecting the 2-amino group of **2a**,**b** compared to compound **1.** The acylated derivatives of **2a**,**b** are much more easily hydrolyzed than those of nucleoside **1**. The mesomeric structures Va–c (Scheme 6) with a positive charge on the amino group and a negative one at the pyrazole N2 underline this observation. (ii) The second effect is caused by the 7-bromo substituent. It reduces the electron density of the heterocycle by the –I effect of the halogen and increases the hydrophobic character of the nucleoside molecule (see Fig. 1A and B). When the bromo nucleoside **2b** is present in a DNA duplex water molecules or alkali cations can be expelled from the major groove, resulting in increased stacking interactions of the nucleobases.



Scheme 6.

Next, the base pair discrimination of compound **2b** was studied in comparison to dA. Oligonucleotide duplexes containing this nucleoside in one strand and adenine, guanine, thymine and cytosine in the second strand opposite to it were hybridized and the T_m values were determined (Table 5). Almost selective base pairing with dT was observed, as is known for 2′-deoxyadenosine. Discrimination of the modified nucleoside **2b** against dG was even more pronounced than for dA. This demonstrates that replacement of dA residues by **2b** not only stabilizes the base pair with dT but also shows a selective base recognition for dT. Regarding enzymatic incorporation of 7-bromopyrazolo[3,4-*d*]pyrimidine 2′-deoxyribonucleoside triphosphates into a growing DNA chain, the Klenow fragment of DNA polymerase has been used successfully (data not shown). Such incorporation is difficult to

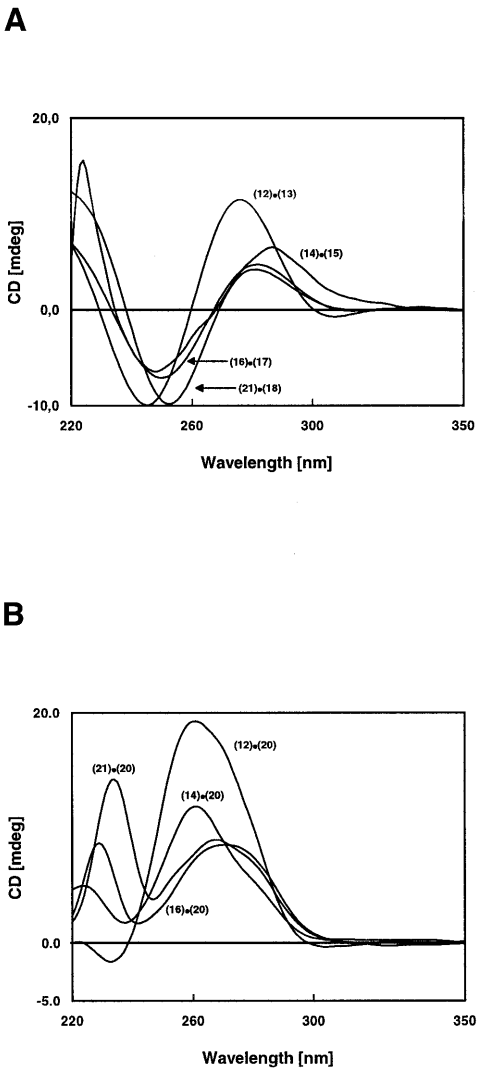


Figure 2. CD spectra of (**A**) the DNA–DNA duplexes and (**B**) the DNA–RNA duplexes measured at 20°C in 0.1 M NaCl containing 10 mM $MgCl_2$ and 10 mM Na cacodylate, pH 7.0, at 5 µM + 5 µM single strand concentrations.

achieve when duplex-stabilizing nucleoside triphosphates modified in the sugar moiety, such as hexitol nucleotides (32) or sugar locked nucleotides (33), are used.

In order to identify the helical structure of the duplexes CD spectra of **12·13**, **14·15**, **16·17** and **21·18** were measured (Fig. 2A). The CD spectra of all duplexes indicate a B-like DNA structure, with a positive Cotton effect around 270–290 nm and a negative lobe around 250 nm. The CD spectra of the DNA–RNA hybrids (Fig. 2B) adopt the A-DNA form. Nevertheless, purin-2,6-diamine nucleoside **1** as well as the 8-aza-7-deazapurine derivatives **2a**,**b** induce spectral changes which are the result of conformational differences between purine and 8-aza-7-deazapurine nucleosides (25).

CONCLUSIONS

Diaminopurine nucleoside **1** (34), which can form three hydrogen bonds with dT, stabilizes the dA-dT pair only very little, in contrast to the canonical tridentate dG-dC pair. Oligonucleotides

incorporating the pyrazolo[3,4-*d*]pyrimidin-4,6-diamine nucleoside **2a** instead of **1** form more stable duplex structures. Nevertheless, the **2a**-dT base pair is still considerably less stable than the dG-dC pair. The 'dA-dT' base pair reaches the stability of a dG-dC pair when the bromo nucleoside **2b** replaces dA. Furthermore, compound **2b** is rather stable against acid-catalyzed depurination and resistant to adenosine deaminase compared to the labile purine nucleoside **1**. The base pair-stabilizing effect of **2b** adjusts the stability of a 'dA-dT' pair to that of a dG-dC pair, thereby showing a similar base discrimination and maintaining the sequence specificity of an oligonucleotide. Application of this phenomenon to sequence-specific hybridization in solution or on oligonucleotides immobilized in DNA arrays is currently under investigation.

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