Bending of Oligonucleotides Containing an Isosteric Nucleobase: 7-Deaza-2′-deoxyadenosine Replacing dA within $d(A)_6$ Tracts†

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ABSTRACT: Decanucleotide duplexes of the parent sequence $d(GGCA_6C)\cdot d(CCGT_6G)$ containing various numbers of 2′-deoxytubercidin (c^7A_d) in place of 2′-deoxyadenosine have been synthesized. Phosphoramidites of protected c^7A_d (**3a**,**b**) were used in automated solid-phase synthesis together with those of regular nucleosides. Upon enzymic 5′-phosphorylation and ligation, multimers of **5** and **7**–**11** were analyzed by polyacrylamide gel electrophoresis and compared with regard to intrinsic, sequence-directed bending. Replacement of dA by c^7A_d within the oligomers decreased bending, but the extent depends strongly on the position of incorporation: strong bending was still observed if the 3′- and 5′-terminal dA residues of the dA tract were replaced while the interruption of the $d(A)_6$ tract by c^7A_d reduced bending strongly.

DNA sequences containing repeated $d(A)_n\cdot d(T)_n$ tracts in phase with the B-DNA helical repeat (10.4 bp per turn) show strongly decreased mobility in polyacrylamide gel electrophoresis.[1] This phenomenon is suggested to result from intrinsic, sequence-directed bending of the DNA and was first observed in trypanosome kinetoplast DNA and later visualized by electron microscopy (Marini et al., 1982; Trifonov, 1985; Lilley, 1986; Griffith et al., 1986; Diekmann, 1987; Coll et al., 1987; Nelson et al., 1987; Gupta et al., 1988).

Essential features for DNA bending are the following: (i) the $d(A)_n\cdot d(T)_n$ tract has to be 4–6 bp long (Koo et al., 1986); (ii) the bending elements must be repeated in phase with the helix pitch in order to add coherently (Koo et al., 1986; Hagerman, 1985). Moreover, it has been shown that interruption of a $d(A)_5\cdot d(T)_5$ tract by a (dG)·(dC) bp or a (nR_d)·(dT) bp (nR_d: 2-aminopurine 2′-deoxyribofuranoside) eliminates DNA bending while substitution of a central (dA)·(dT) bp by (dI)·(dC) leads only to a slight reduction of the electrophoretic migration anomaly (Diekmann et al., 1987; Koo & Crothers, 1987). This latter finding is also true for an oligonucleotide sequence containing a $d(A)_5\cdot d(U)_5$ tract (Koo & Crothers, 1987). All these results demonstrate that (i) the 2-amino group on purines is an interfering factor and (ii) the 5-methyl group on pyrimidines is not essential for bending (Diekmann et al., 1987; Koo & Crothers, 1987).

In order to evaluate to which extent an altered π-electron system of a nucleobase influences DNA bending, we introduced different numbers of 7-deaza-2′-deoxyadenosine residues [2′-deoxytubercidin, c^7A_d (**1a**; see Chart I) (Seela & Kehne, 1983)] into oligonucleotides with an $d(A)_6\cdot d(T)_6$ tract and analyzed resulting multimers with regard to their electrophoretic mobility. Herewith, for the first time dA of a bending element is replaced by an isosteric molecule having an altered nitrogen pattern.

Experimental Procedures

NMR spectra were recorded on a AC-250 spectrometer with a BVT-1000 temperature control unit (Bruker, Karlsruhe, West Germany); δ values are relative to internal tetramethylsilane for ^1H and ^{13}C nuclei and to external 85% phosphoric acid for ^{31}P. Chemical shifts are positive when downfield to the external standard. UV spectra were recorded on a Hitachi 150-20 spectrophotometer (Hitachi, Tokyo, Japan). Melting curves of the oligonucleotides were measured in Teflon-stoppered cuvettes (10-mm light path length) in a thermostatically controlled cell holder with a Shimadzu 210-A recording spectrophotometer (wavelength 260 nm) connected with a Lauda RCS-6 bath, a Lauda PM 351 programmer and an R 22 unit (MWG-Lauda, Lauda, West Germany).

Lyophilization was performed with a Speed-Vac concentrator (Savant Instruments, Farmingdale, NY). Thin-layer chromatography (TLC) was performed with silica gel SIL G-25 UV_{254} plates (Macherey-Nagel, Düren, West Germany). Flash chromatography was performed with silica gel 60H (Merck, Darmstadt, West Germany) at 0.5 bar. Solvent systems used for TLC and flash chromatography are (A) CH_2Cl_2–MeOH (9:1), (B) CH_2Cl_2–EtOAc–NEt_3 (45:45:10), (C) CH_2Cl_2–EtOAc (1:1), (D) CH_2Cl_2–MeOH (93:7), (E) CH_2Cl_2–MeOH (92:8), (F) CH_2Cl_2–MeOH (99:1), and (G) $CHCl_3$–MeOH (8:2). HPLC separation of the oligonucleotides was carried out on a 250 × 4 mm (7 μm) RP-18 LiChrosorb column (Merck, Darmstadt, West Germany) connected with a 25 × 4 mm RP-18 precolumn according to Seela and Kehne (1987). HPLC solvent systems were (A) acetonitrile (5%) in 0.1 M triethylammonium acetate, pH 7.0, and (B) acetonitrile. Solvent system I was 3 min (15% B in A), 7 min (15–40% B in A), and 5 min (40% B in A); solvent system II was 15 min (5–20% B in A); solvent system III was 100% A. Desalting of the oligonucleotides was performed on a 25 × 4 mm RP-18 cartridge by elution of salt with water (7 min); elution of the oligomers was accomplished with methanol–water (3:2, 5 min). Dichloromethane and aceto-

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[1] Abbreviations: 2′dTu, 2′-deoxytubercidin, = c^7A_d, 7-deaza-2′-deoxyadenosine, = 4-amino-7-(2′-deoxy-β-D-ribofuranosyl)-7*H*-pyrrolo-[2,3-*d*]pyrimidine; dA, 2′-deoxyadenosine; dG, 2′-deoxyguanosine; dC, 2′-deoxycytidine; dT, thymidine; nR_d, 2-aminopurine 2′-deoxyribofuranoside; Tris, tris(hydroxymethyl)aminomethane; $\tau/2$, half-rate time; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; T_m, temperature of melting; R_L, ratio of apparent length to real length according to Koo et al. (1986); bp, base pair.

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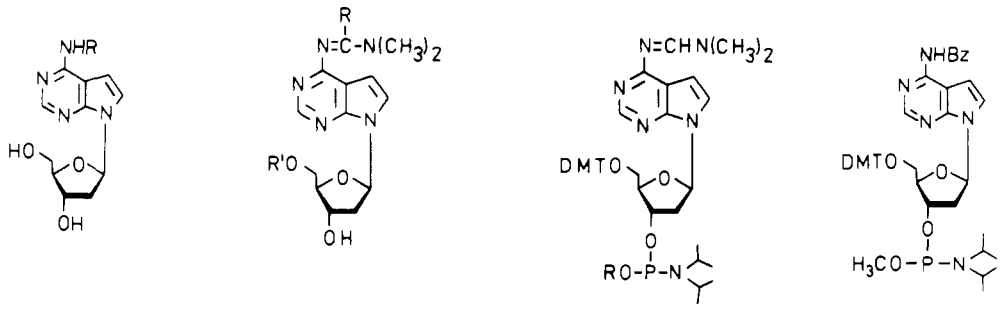
Chart I



nitrile were predried with P_4O_{10} and redestilled from CaH_2.

Polynucleotide kinase (EC 2.7.1.78), T4 DNA ligase (EC 6.5.1.1), snake venom phosphodiesterase (EC 3.1.4.1, *Crotallus durissus*), alkaline phosphatase (EC 3.1.3.1), and the DNA molecular weight marker V (digestion products from a cleavage of plasmid pBR 322-DNA with endodeoxyribonuclease *Hae*III) were products of Boehringer Mannheim (Mannheim, West Germany). Fractosil (30–70 μmol of 2′-deoxynucleoside/g of solid support) was a product of Biosyntech (Hamburg, West Germany). Methyl phosphoramidites of regular 2′-deoxynucleosides were from Applied Biosystems (Weiterstadt, West Germany).

4-(Benzoylamino)-7-[2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl]-7*H*-pyrrolo[2,3-*d*]pyrimidine 3′-(methyl *N*,*N*-diisopropylphosphoramidite) (**4**) was prepared according to Seela and Kehne (1985). Chloro(2-cyanoethoxy)(diisopropylamino)phosphine was synthesized according to Sinha et al. (1984).

7-(2′-Deoxy-β-D-erythro-pentofuranosyl)-4-[[(dimethylamino)methylidene]amino]-7H-pyrrolo[2,3-d]pyrimidine (**2a**). Compound **1a** (Seela & Kehne, 1983) (450 mg, 1.8 mmol), dissolved in dry DMF (8 mL), was treated with *N*,*N*-dimethylformamide diethyl acetal (3.4 mL, 19.3 mmol). After a 1-h stirring at 50 °C (Ar atmosphere) the solvent was evaporated. Residual solvent was removed by repeated coevaporation with toluene. The residue was chromatographed on silica gel 60H (column: 13 × 5 cm, solvent D, 0.5 bar). From the main zone colorless, amorphous **2a** (400 mg, 73%) was obtained upon evaporation: TLC (silica gel, solvent A) R_f 0.35; UV (MeOH) λ_{max} 223, 259, 312 nm (ε 17 900, 11 200, 23 300); ^{1}H NMR (Me_2SO-d_6) δ 2.19 (m, H_α-2′), 2.58 (m, H_β-2′), 3.11, 3.17 (s, 2 CH_3), 3.56 (m, H_2-5′), 3.84 (m, H-4′), 4.37 (m, H-3′), 5.11 (t, 5′-OH, J = 5.6 Hz), 5.29 (d, 3′-OH, J = 4.0 Hz), 6.55 (d, H-5, J = 3.5 Hz), 6.58 (m, H-1′), 7.52 (d, H-6, J = 3.7 Hz), 8.34 (s, H-2), 8.83 (s, N=CH); ^{13}C NMR (Me_2SO-d_6) δ 151.4 (C-2), 156.9 (C-4), 111.3 (C-4a), 100.3 (C-5), 123.7 (C-6), 151.0 (C-7a), 83.2 (C-1′), 39.8 (C-2′), 71.3 (C-3′), 87.4 (C-4′), 62.2 (C-5′), 160.5 (N=CR), 34.6 [$N(CH_3)_2$]. Anal. Calcd for $C_{14}H_{19}N_5O_3$: C, 55.07; H, 6.27; N, 22.94. Found: C, 54.99; H, 6.40; N, 22.88.

7-(2′-Deoxy-β-D-erythro-pentofuranosyl)-4-[[1-(dimethylamino)ethylidene]amino]-7H-pyrrolo[2,3-d]pyrimidine (**2b**). Compound **2b** was prepared from **1a** (270 mg, 1.08 mmol) as described for **2a** but with *N*,*N*-dimethylacetamide dimethyl acetal (0.4 mL, 2.7 mmol); yield was 250 mg (73%) of colorless amorphous **2b**: TLC (silica gel, solvent A) R_f 0.4; UV (MeOH) λ_{max} 217, 302 nm (ε 23 000, 15 500); ^{1}H NMR (Me_2SO-d_6) δ 2.06 (s, C–CH_3), 2.18 (m, H_α-2′), 2.57 (m, H_β-2′), 3.08 [6 H, s, $N(CH_3)_2$], 3.54 (m, H_2-5′), 3.84 (m, H-4′), 4.36 (m, H-3′), 5.09 (t, 5′-OH, J = 5.6 Hz), 5.28 (d, 3′-OH, J = 4.0 Hz), 6.38 (d, H-5, J = 3.6 Hz), 6.57 (m, H-1′), 7.50 (d, H-6, J = 3.6 Hz), 8.35 (s, H-2); ^{13}C NMR (Me_2SO-d_6) δ 151.3 (C-2), 160.4 (C-4), 111.4 (C-4a), 100.3 (C-5), 123.6 (C-6), 150.9 (C-7a), 83.3 (C-1′), 39.8 (C-2′), 71.2 (C-3′), 87.4 (C-4′),62.2 (C-5′), 161.5 (N=CR), 37.8 [$N(C-H_3)_2$], 16.5 (C–CH_3). Anal. Calcd for $C_{15}H_{21}N_5O_3$: C, 56.41; H, 6.63; N, 21.93. Found: C, 56.56; H, 6.63; N, 21.78.

7-[2′-Deoxy-5′-O-(4,4′-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-4-[[(dimethylamino)methylidene]amino]-7H-pyrrolo[2,3-d]pyrimidine (**2c**). To a stirred solution of 4,4′-dimethoxytriphenylmethyl chloride (541 mg, 1.6 mmol) and *N*-ethyldiisopropylamine (0.3 mL, 1.7 mmol) in anhydrous pyridine (5 mL) was added compound **2a** (250 mg, 0.82 mmol). Stirring was continued for 2 h at room temperature. After addition of aqueous $NaHCO_3$ (5%, 8 mL), the solution was extracted twice with CH_2Cl_2 (20 mL each). The combined organic layers were dried over Na_2SO_4 and filtered, and the solvent was evaporated. The residue was chromatographed on silica gel 60H (column: 8 × 5 cm, solvent F, 0.5 bar). Isolation of the content of the main zone and repeated coevaporation with acetone yielded colorless, amorphous **2c** (400 mg, 80%): TLC (silica gel, solvent A) R_f 0.6; UV (MeOH) λ_{max} 230, 312 nm (ε 35 500, 21 800); ^{1}H NMR (Me_2SO-d_6) δ 2.28, (m, H_α-2′), 2.61 (m, H_β-2′), 3.15 (m, H_2-5′), 3.11, 3.17 [2 s, $N(CH_3)_2$], 3.72 (6 H, s, 2 OCH_3), 3.96 (m, H-4′), 4.40 (m, H-3′), 5.36 (d, 3′-OH, J = 4.5 Hz), 6.53 (d, H-5, J = 3.5 Hz), 6.61 (t, H-1′, J = 6.8 Hz), 6.81–7.36 (13 H, m, aromatic H), 7.40 (H-6), 8.34 (s, H-2), 8.83 (s, N=CH); ^{13}C NMR (Me_2SO-d_6) δ 151.6 (C-2), 156.8 (C-4), 111.3 (C-4a), 100.5 (C-5), 123.3 (C-6), 151.2 (C-7a), 82.7 (C-1′), 39.9 (C-2′), 71.0 (C-3′), 85.3 (C-4′), 64.4 (C-5′), 160.5 (N=CH), 34.5 [$N-(CH_3)_2$], 55.1 (OCH_3), 85.5 (Tol.–CO). Anal. Calcd for $C_{35}H_{37}N_5O_5$: C, 69.17; H, 6.13; N, 11.52. Found: C, 69.05; H, 6.27; N, 11.32.

7-[2′-Deoxy-5′-O-(4,4′-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-4-[[(dimethylamino)methylidene]amino]-7H-pyrrolo[2,3-d]pyrimidine 3′-(Methyl N,N-diisopropylphosphoramidite) (**3a**). To a stirred solution of compound **2c** (250 mg, 0.41 mmol) in dry MeCN (3 mL) were added *N*-ethyldiisopropylamine (205 μL, 1.2 mmol) and chloro(diisopropylamino)methoxyphosphine (85 μL, 0.43 mmol) at room temperature under Ar atmosphere. Stirring was continued for 25 min, and then aqueous $NaHCO_3$ (5%, 6 mL) was added. The resultant was extracted twice with CH_2Cl_2, the combined organic layers were dried over sodium sulfate and filtered, and the solvent was evaporated. The residue was chromatographed on silica gel 60H (column: 6 × 5 cm, solvent B, 0.5 bar). Evaporation of the main zone followed by repeated coevaporation with acetone gave colorless amorphous **3a** (230 mg, 73%): TLC (silica gel, solvent B) R_f 0.6; ^{1}H NMR

($CDCl_3$) δ 1.05–1.28 [m, $[CH(CH_3)_2]_2$], 2.51 (m, H_α-2'), 2.60 (m, H_β-2'), 3.14, 3.17 [2 s, $N(CH_3)_2$], 3.75 (6 H, s, 2 OCH_3), 3.74 (s, P–OCH_3), 4.23 (m, H-4'), 4.69 (m, H-3'), 6.60 (d, H-5, J = 3.7 Hz), 6.62 (m, H-1'), 7.42–6.74 (14 H, H-6 and aromatic H), 8.45 (s, H-2), 8.76 (s, CH); ^{31}P NMR ($CDCl_3$) δ 149.37, 149.30.

7-[2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-4-[[(dimethylamino)methylidene]amino]-7H-pyrrolo[2,3-d]pyrimidine 3'-(2-Cyanoethyl N,N-diisopropylphosphoramidite) (**3b**). To a stirred solution of compound **2c** (140 mg, 0.23 mmol) in dry dichloromethane (2 mL) were added *N*-ethyldiisopropylamine (130 μL, 0.76 mmol) and chloro(2-cyanoethoxy)(diisopropylamino)phosphine (60 μL, 0.27 mmol) at room temperature under Ar atmosphere. After being stirred for 15 min the reaction was worked up as described for **3a**. Yield was 90 mg (49%) of colorless amorphous **3b**: TLC (silica gel, solvent C) R_f 0.36, 0.45; ^{1}H NMR ($CDCl_3$) δ 1.06 [d, $CH(CH_3)_2$, J = 6.8 Hz], 1.26–1.12 [2 m, $CH(CH_3)_2$], 2.41 (t, $CNCH_2CH_2$, J = 6.5 Hz), 2.5 (m, H_α-2'), 2.60 (m, H_β-2'), 2.72 (t, $CNCH_2$–CH_2, J = 6.2 Hz), 3.13, 3.17 [6 H, 2 s, $N(CH_3)_2$], 3.75 (6 H, s, 2 OCH_3), 4.19 (m, H-4'), 4.70 (m, H-3'), 6.61 (d, H-5, J = 3.6 Hz), 6.79–6.74 (m, H-1' and 4 aromatic H), 7.43–7.21 (m, 9 aromatic H), 7.23 (d, H-6, J = 3.7 Hz), 8.45 (s, H-2), 8.76 (s, CH); ^{31}P NMR ($CDCl_3$) δ 146.04, 146.85.

Solid-Phase Synthesis of the Oligonucleotides **5–11**. The synthesis of the oligonucleotides was performed on a 1-μmol scale with the methyl phosphoramidites of $[(MeO)_2Tr]T_d$, $[(MeO)_2Tr]bz^6A_d$, $[(MeO)_2Tr]ib^2G_d$, and $[(MeO)_2Tr]bz^4C_d$, as well as **3a** or **4** (*User Manual*, 1986; Beaucage & Caruthers, 1981). Cleavage of the nucleobase protecting groups by concentrated ammonia at 60 °C was accomplished within 20 h in the case of the nonmodified oligomers **5**, **6**, and **11** and within 70 h in the case of the modified oligomers **7–10** by applying a 4-benzoyl-protected methyl phophoramidite of 2'-deoxytubercidin (**4**) (Seela & Kehne, 1985). Application of compound **3a** reduced the time for deprotection to 24 h. The 5'-(dimethoxytrityl)-protected oligomers were purified by HPLC (RP-18, solvent system I) and detritylated by the action of 80% acetic acid for 5 min. After removal of the acid, the oligomers were purified by reverse-phase HPLC with solvent system II. Desalting and lyophilization gave colorless foams which were stored frozen at −20 °C. See Table II for retention times. All oligonucleotides were analyzed by tandem hydrolysis with snake venom phosphodiesterase followed by alkaline phosphatase according to Seela and Kehne (1987).

Enzymatic Phosphorylation and Condensation of the Oligomers **5–11**. The single-stranded oligonucleotides **5–11** (6 μg, 2 nmol each) were 5'-phosphorylated with polynucleotide kinase (10 units) in a reaction mixture containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), and 1 mM ATP (total volume 30 μL) at 37 °C for 3 h. The solution of **6** was mixed with that of **5** or **7–10**, heated for 10 min to 55 °C, and cooled slowly overnight to 0 °C to form hybrids. The duplexes were then self-ligated by adding T4 DNA ligase (3 μL, 5 units) to half of the reaction mixture, thus leading to a total volume of 30 μL containing 20 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, and 0.6 mM ATP, pH 7.6 (total volume approximately 30 μL). The mixtures were then incubated at 15 °C. Except for the blunt-end duplex of oligomer **11**, which was allowed to ligate for a period of 2 h, from all the other mixtures aliquots were taken at 20 min, 1 h, and 2 h. The reactions were quenched by quick freezing the samples in liquid nitrogen. In order to get a homogeneous multimer distribution, the probes taken at different incubation times were mixed and evaporated in a Speed-Vac concentrator.

Table I: HPLC Retention Times of Oligonucleotides on a 250 × 4 mm RP-18 Reverse-Phase Column Connected with a 25 × 4 mm RP-18 Precolumn (Solvent System II)

compd	oligomer	retention time (min)
5	d(GGCAAAAAAC)	10.4
6	d(CCGTTTTTTG)	9.8
7	d(GGCAATuAAAG)	8.8
8	d(GGCAATuTuAAG)	8.4
9	d(GGCTuAAAATuG)	8.6
10	d(GGCATuATuATuG)	9.8
11	d(CGGGATCCCG)	10.4

Electrophoresis. The multimers of **5** and **7–10** [with **6** as (−) strand] as well as **11** were taken up in Tris–borate–EDTA buffer (TBE, 5×, 10 μL) and mixed with gel loading buffer (0.25% xylene cyanol, 0.25% bromophenol blue, and 15% Ficoll in H_2O, 3 μL). After a prerun of 1 h the samples (6 μL per lane) were run on nondenaturing 8% polyacrylamide gels [ratio monoacrylamide vs bis(acrylamide) 29:1; 90 mM Tris–borate, 2 mM EDTA, pH 8.3; gel thickness 1 mm] at either gel temperature (25° C) or 5 °C until the bromophenol blue dye had migrated approximately 14 cm. The constant power was 10 W (350 V). After electrophoresis the gel was stained for 45 min with ethidium bromide (0.5 μg/mL in 1× TBE buffer) and photographed during UV illumination at 302 nm (CAM-AG-Transilluminator; Polaroid MP 4 Landcamera, visible blocking filter). Ligated products of 10-bp *Bam*HI linkers as well as a *Hae*III digest of pBR 322 were used as size standards. The apparent length of each multimer is defined as the length of the nonbended marker DNA having the same mobility. The ratio R_L of apparent length to real length indicated the extent of anomaly of electrophoretic mobility, which is assumed to be an increasing function of bending (Koo et al., 1986).

Results and Discussion

Oligonucleotide Synthesis. The oligonucleotide duplexes **7–10** [with **6** as (−) strand]—derived from **5** but containing c^7A_d (**1a**) instead of dA—were synthesized by use of P(III) chemistry on solid support (Letsinger & Lunsford, 1976) (Table I). For this purpose the phosphoramidite **3a**,**b** or **4** (Seela & Kehne, 1985) was required.

Compound **4** has already been successfully employed in oligonucleotide synthesis, but it became apparent that debenzoylation of 6-NH_2-protected 2'-deoxytubercidin (c^7A_d) residues was more difficult as in the case of 6-NH_2-protected dA. Therefore as an alternative amidine protection was considered. [(Dimethylamino)alkylidene]amino residues have already successfully been used for protection of regular 2'-deoxynucleosides during oligonucleotide synthesis (McBride et al., 1986). They offer several advantages: (i) The synthesis of base-protected nucleosides does not require transient protection of sugar hydroxyls; (ii) amidine-functionalized 2'-deoxyadenosine is approximately 20-fold more resistant to depurination than bz^6A_d under conditions of DNA synthesis (Froehler & Matteucci, 1983); (iii) the amidine can be hydrolyzed in some cases more easily with 25% aqueous ammonia than the amide groups. In order to test scope and limitations of amidine protecting groups in the case of 2'-deoxytubercidin, compound **1a** was converted into the formamidine **2a** as well as into the acetamidine **2b** (Zemlicka & Holy, 1967; McBride et al., 1986). Both compounds (**2a**,**b**) were obtained in more than 70% yield. Their structure was confirmed by elemental analyses and ^{1}H, as well as ^{13}C, NMR spectroscopy.

As the 6-NH_2 protecting group of **1a** has to be stable during oligonucleotide synthesis but removable during deprotection

Table II: $\tau/2$ and k Values of NH_2-Protected 2′-Deoxytubercidin and 2′-Deoxyadenosine in 25% Aqueous NH_3 at 60 °C

compd	k (min^{-1})	$\tau/2$ (min)
bz^6A_d	1.9×10^{-2}	36
2a	6.9×10^{-2}	10
2b	2.1×10^{-3}	330
1b	1.1×10^{-2}	65

with 25% aqueous ammonia, we have carried out hydrolysis experiments at 60 °C on compounds **2a,b**, **1b**, and bz^6A_d. The kinetics were followed UV spectrophotometrically at 312 (**2a,b**), 304 (**1b**), or 295 nm (bz^6A_d), respectively, and are all of pseudo first order. As Table II shows, base-catalyzed hydrolysis of the formamidine **2a** is 6.5 times faster than debenzoylation of **1b** while the latter occurs at a 5 times faster rate as the hydrolysis of the acetamidine **2b**. As a result, compound **2b** seems not to be suitable for application in oligonucleotide synthesis while **2a** is a suitable alternative to the benzoylated compound **1b** as it can be hydrolyzed at a similar rate as bz^6A_d (Table I). As the formamidine **2a** is more labile against acid than the benzamide **1b**, problems may arise during oligonucleotide synthesis. Therefore, we tested the stability of compound **2c** in 3% trichloroacetic acid in dichloromethane, which is generally used for detritylation reaction. TLC monitoring (silica gel, solvent G) of the reaction mixture showed that no 2′-deoxytubercidin was formed within 1 h.

During ^1H NMR analysis it became apparent that **2a** exhibits two separate singlets for the methyl resonances at rt (298 K) which coincide at ca. 355 K whereas compound **2b** showed only one singlet at rt. The latter begins to separate below 283 K. These findings confirm a higher rotation barrier at the $C-N(CH_3)_2$ bond in case of compound **2a** compared to **2b** and imply that at rt **2a** is better represented by a dipolar charged structure in contrast to **2b**. In line with findings on other nucleoside amidines the compound with the lowest rotation barrier (**2b**) is the most stable one against base-catalyzed hydrolysis (Seela & Driller, 1988).

Next, the 5′-OH group of **2a** was protected with a 4,4′-dimethoxytriphenylmethyl residue. The reaction was carried out analogously as described for 2′-deoxyadenosine. 5′-*O*-Protection (**2c**) was confirmed by its ^{13}C NMR spectrum, which shows a downfield shift of C-5′ ($\Delta\delta$ = 2 ppm). The conversion of **2c** into its methyl phosphoramidite followed a protocol developed by Caruthers and co-workers (Beaucage & Caruthers, 1981; Matteucci & Caruthers, 1981) for regular nucleosides and gave **3a** in 73% yield. The cyanoethyl phosphoramidite **3b** was prepared in 49% yield according to the method of Köster and co-workers (Sinha et al., 1984). Both compounds were characterized by ^1H and ^{31}P NMR spectroscopy. The phosphoramidites **3a,b** and **4** together with those of dA, dG, dC, and dT were then employed in automated DNA synthesis on solid support with an automated DNA synthesizer.

Bending of the Oligomers **5** *and* **7–11**. The single-stranded oligonucleotides were 5′-phosphorylated with polynucleotide kinase, hybridized to its complementary strand (**6**) with a two base pair overhang at the 5′ end of each strand, and then self-ligated to multimers with T4 DNA ligase. The sticky ends on each side of the strands ensure exclusive ligation in one direction so that the dA_6 tract or its modified version is repeated only in one strand of the double helix. The condensation products were analyzed on nondenaturating 8% polyacrylamide gels either at ambient temperature or at 5 °C (Table III).

Figure 1 displays a typical gel representing the multiple repeats of the original complementary decamers. When

Table III: Relative DNA Bending at 25 and 5 °C and Melting Points of Oligonucleotide Duplexes[a]

compd	relative bending (%) 25 °C	relative bending (%) 5 °C	T_m (°C)[b]
5·6	100	100	31
7·6	81	85	30
8·6	54	61	28
9·6	92	95	29
10·6	43	52	27
11·11	0	0	55

[a] Except the *Bam*HI linker (**11**) all duplexes have a two base pair overhang at the 5′ end of each strand. Percentage of bending was calculated at the R_L factor for the corresponding 120-bp multimer. The R_L value of a 120-bp multimer of **12** was arbitrarily set as 100%. [b] 50 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT, pH 7.5; 0.15 A_{260} unit of each single strand/mL (1.3 μM); 260 nm.

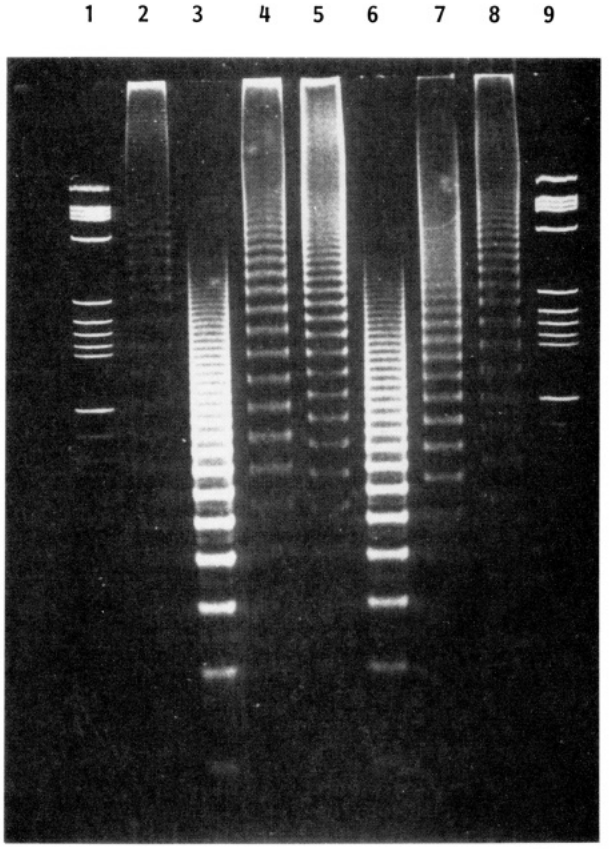


FIGURE 1: Ligation ladders and length markers. Lanes: (1) Endodeoxyribonuclease *Hae*III digest of plasmid pBR 322; (2) multimers of **5**; (3) multimers of **11** (*Bam*HI length marker); (4) multimers of **7**; (5) multimers of **8**; (6) same as lane 3; (7 multimers of **10**; (8) multimers of **9**; (9) same as lane 1. For details, see Experimental Procedures.

compared with the *Bam*HI standard (**11**) multimers of known numbers of base pairs, anomalous gel migration can be visualized in lanes 2, 4, 5, 7, and 8. Figure 2 shows a plot of the R_L^{rt} factor versus the number of repeats of the oligonucleotides **5** and **7–10** presented in Table III. Up to a 50-bp length, all oligonucleotides exhibit normal migration behavior, which is probably due to the insensitivity of the 8% polyacrylamide gels to small degress of bending (Diekmann et al., 1987). Between an oligonucleotide length of 50 and 150 bp, R_L^{rt} is increasing almost linearily. Above 150 bp all curves begin slowly to flatten, indicating independence of R_L^{rt} from the oligonucleotide length. This might result from an imperfect phase relation between the sequence and helix repeat. As the helical pitch is presumably dependent on the number of c^7A_d residues in the dA_n tract, the point at which the corresponding curve

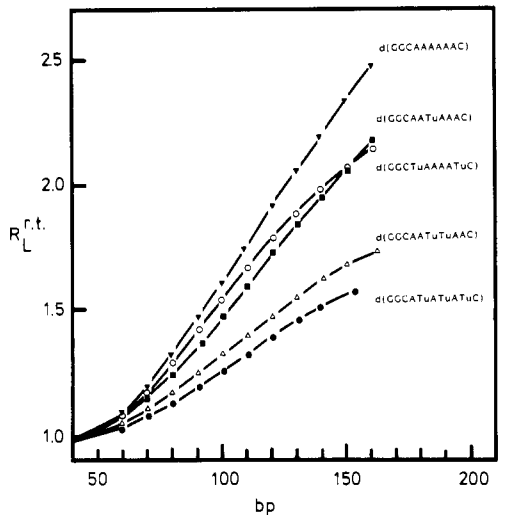


FIGURE 2: Graph of R_L^{rt} factor versus number of repeats of the oligonucleotides **5** and **7–10**. The ratio R_L of apparent length to real length indicates the extent of gel electrophoretic mobility and is an increasing function of bending extent.

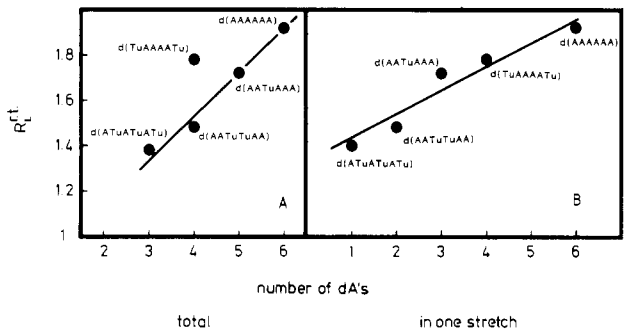


FIGURE 3: (A) Graph of the R_L^{rt} factor versus the total number of dA residues in the bending element; (B) graph of the R_L^{rt} factor versus the number of dA residues in one stretch within the bending element.

(R_L^{rt} vs actual chain length) begins to flatten varies from one oligomer to the other. For comparison of the relative curvature of the oligomers **5** and **7–10**, we therefore calculated the R_L^{rt} values of the corresponding 120-bp multimers (Table III). Moreover, Table III shows that all sequences studied exhibit a larger bending at 5 °C than at room temperature, supporting the assumption that bends are caused by the same mechanism (Koo & Crothers, 1987).

As can be seen from Table III as well as from Figure 3B, there is a rough linear correlation between the R_L^{rt} factor and the number of dA's in one stretch which reflects the influence of substitution of dA by its isostere c^7A_d on the resulting DNA bending. Figure 3A shows a correlation between R_L^{rt} and the total number of dA's in the bending element. From this figure it can be seen that the arrangement of c^7A_d along the dA_n tract is of great importance (Diekmann et al., 1987): if a consecutively packed dA_4 tract is flanked by two c^7A_d residues, the resulting curvature of a 120-bp multimer is significantly stronger than is the case when the $d(A)_6$ tract is interrupted by two central (c^7A_d)·(dT) base pairs.

Two models are discussed with respect to the bending of $d(A)_n$·$d(T)_n$ tracts: (i) According to Crothers and co-workers bending occurs at the junction between the $d(A)_n$ tract ($n > 4$) which adopts the polymorphic H-DNA form (heteronomous DNA) and adjacent B-DNA being in phase with the helical pitch (Koo et al., 1986; Koo & Crothers, 1987; Arnott et al., 1983). (ii) Bending in $d(A)_n$ tracts can also be described in terms of large wedge angles between contiguous $d(A)_2$·$d(T)_2$ dinucleotides (Trifonov, 1985; Ulanovsky et al., 1986, 1987).

Moreover, it has been reported that due to a spine of hydration in the minor groove of oligo(dA)·oligo(dT), with water bridges between N-3 of a dA and O-2 of adjacent dT, the base pairs fold into the minor groove with the consequence of bending (Chuprina, 1987; Dickerson et al., 1982; Koo & Crothers, 1987). This suggestion is in line with the finding that a chemical substitution in the minor groove of the d-$(A)_n$·$d(T)_n$ sequence, namely, the substitution of a central (dA)·(dT) base pair by (dG)·(dC), disrupting the zigzag spine of hydration destroys bending completely (Koo & Crothers, 1987). According to the model of Crothers an ordered water spine in the minor groove stabilizes the H-DNA form of the $d(A)_n$·$d(T)_n$ tract (Koo & Crothers, 1987).

The most striking difference between dA and its isostere c^7A_d is the lack of a proton acceptor site at N-7 as well as an altered π-electron system. Therefore, when c^7A_d is introduced into oligonucleotides in place of dA, altered stacking interactions are expected. Moreover, the major groove of the oligomers is hydrophobized (Diekmann & McLaughlin, 1988). As a consequence, replacement of dA by 2'-deoxytubercidin in oligonucleotides such as $d(GGAATTCC)_2$ leads to duplex destabilization demonstrated by decreased T_m values (Seela & Kehne, 1987). Also the oligonucleotides reported here show a decrease of T_m with increasing number of c^7A_d residues (Table III).

The bending decrease of multimers of the oligonucleotides **7–10** with increasing number of c^7A_d residues can therefore be referred to the following reasons:

(i) A decreasing stability of the oligomers with increasing numbers of c^7A_d residues (**5 > 7 > 9 > 8 > 10**) due to the altered π-electron system of c^7A_d compared to dA may lead to an altered helix pitch and therefore to destabilization of the H-form of the bending element. This results in a stepwise normalization of gel electrophoretic mobility with increasing number of c^7A_d residues (Figure 3B). However, an important feature is the position of the c^7A_d residues within the bending element. Helical distorsion of the bending element going along with destabilization of the H-DNA is significantly altered if two c^7A_d residues are nearest neighbors (**8**), while oligomers in which both c^7A_d residues are separated by a dA_4 tract (**9**, Figure 3A) behave similar to those with dA_6 elements.

(ii) An increasing imperfection of the sequence phasing (Hagerman, 1985) relative to the helical screw axis of oligomers containing one or more c^7A_d residues may also contribute to the decrease of bending. Nevertheless, it is unlikely that dA substitutions by c^7A_d move the phase match with the sequence repeat out of the acceptable range of 10–11 base pairs per turn. Replacement of dA residues of a bending element by other isosteric nucleosides will help to elucidate the chemical determinants of DNA bending.

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Discrimination between DNA Sequences by the *Eco*RV Restriction Endonuclease†

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ABSTRACT: The *Eco*RV restriction endonuclease cleaves not only its recognition sequence on DNA, GATATC, but also, at vastly reduced rates, a number of alternative DNA sequences. The plasmid pAT153 contains 12 alternative sites, each of which differs from the recognition sequence by one base pair. The *Eco*RV nuclease showed a marked preference for one particular site from among these alternatives. This noncognate site was located at the sequence GTTATC, and the mechanism of action of *Eco*RV at this site was analyzed. The mechanism differed from that at the cognate site in three respects. First, the affinity of the enzyme for the noncognate site was lower than that for the cognate site, but, by itself, this cannot account for the specificity of *Eco*RV as measured from the values of k_{cat}/K_m. Second, the enzyme had a lower affinity for Mg^{2+} when it was bound to the noncognate site than when it was bound to its cognate site: this appears to be a key factor in limiting the rates of DNA cleavage at alternative sites. Third, the reaction pathway at the noncognate site differed from that at the cognate site. At the former, the *Eco*RV enzyme cleaved first one strand of the DNA and then the other while at the latter, both strands were cut in one concerted reaction. The difference in reaction pathway allows DNA ligase to proofread the activity of *Eco*RV by selective repair of single-strand breaks at noncognate sites, as opposed to double-strand breaks at the cognate site. The addition of DNA ligase to reactions with *Eco*RV made no difference to product formation at the cognate site, but products from reactions at noncognate sites were no longer detected.

The mechanism for the interaction of a protein with a specific DNA sequence must involve two processes, the recognition of the target sequence and the discrimination against alternative sequences. In principle, the recognition of the target sequence can be accounted for by the array of hydrogen-bonding functions along the edge of each base pair (bp)[1] (Seeman et al., 1976). Hydrogen bonds with the edges of the base pairs are now known to play major roles in the recognition of specific DNA sequences by many (though not all) DNA binding proteins (McClarin et al., 1986; Anderson et al., 1987; Otwinowski et al., 1988). However, it may be more difficult to account for the discrimination against alternative DNA sequences. If the alternative sequence differs from the recognition sequence by just 1 bp, it might be able to interact with

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[1] Abbreviations: AMP and ATP, adenosine 5′-phosphate and 5′-triphosphate, respectively; bp, base pair(s); BSA, bovine serum albumin; BME, β-mercaptoethanol; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EtdBr, ethidium bromide; K_D, equilibrium dissociation constant; M_r, relative molecular mass; NAD, nicotinamide adenine dinucleotide; R/M, restriction/modification; Tris, tris(hydroxymethyl)aminomethane.

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