Palindromic Octa- and Dodecanucleotides Containing 2′-Deoxytubercidin: Synthesis, Hairpin Formation, and Recognition by the Endodeoxyribonuclease *Eco*RI†

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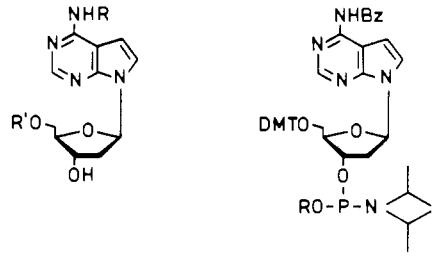
ABSTRACT: Octa- and dodecanucleotides containing 2′-deoxytubericidin within the endodeoxyribonuclease *Eco*RI recognition fragment d(GAATTC) have been prepared by solid-phase synthesis. Whereas octamers as well as dodecamers with a "random" flanking region formed duplexes in aqueous solution, the dodecamer d(CGCGAATTCGCG) and isosterically modified oligomers thereof showed a strong tendency of hairpin formation. Due to this, cleavage with the endodeoxyribonuclease *Eco*RI was strongly decreased. In contrast, d(GTAGAATTCTAC) was easily cleaved by the enzyme. Single replacement of one of the dA residues by 2′-deoxytubericidin within the recognition sequence decreased the cleavage velocity but retained specificity. Twofold modification prevents cleavage of the oligomer. This implies that both N-7 purine nitrogens are proton acceptor sites for the endodeoxyribonuclease *Eco*RI.

Type II endodeoxyribonucleases are highly specific enzymes that recognize short stretches of palindromic DNA fragments. As a result, they are ideal probes for studying protein–nucleic acid interactions (Modrich & Roberts, 1982). One of the most investigated systems is that of the endodeoxyribonuclease *Eco*RI with cognate DNA fragments containing the recognition sequence d(GAATTC) (Greene et al., 1974; Modrich & Zabel, 1976). In a previous paper we have reported on the synthesis and enzymatic recognition of d(GGAATTCC) octamers containing 7-deaza-2′-deoxyguanosine instead of dG[1] at the cleavage site (Seela & Driller, 1985). These oligomers have an isosterically altered recognition sequence and showed only extremely weak substrate properties toward the *Eco*RI enzyme. From these findings it was concluded that guanine N-7, located at the major groove of the DNA duplex, is important for the recognition of the particular DNA fragment by the enzyme. As a consequence, this position can be discussed to be a proton acceptor site for the enzyme, as suggested by X-ray data of Rosenberg and co-workers (Frederick et al., 1984). From the same author it has also been concluded that the 7-nitrogens of both 2′-deoxyadenosine residues within the recognition sequence display proton acceptors for a particular amino acid of the enzyme (Rosenberg et al., 1985).

Recently, we have reported on the synthesis of 7-deaza-2′-deoxyadenosine (**1a**; Chart I) (Seela & Kehne, 1983), which is the 2′-deoxy derivative of the naturally occurring rare nucleoside tubercidin (Anzai et al., 1957). This deoxynucleoside has been converted into the appropriately protected phosphoramidite **2a** (Seela & Kehne, 1985a) and was incorporated into oligomers with alternating thymidine and 2′-deoxytubercidin (Seela & Kehne, 1985b). The same nucleoside has now been used as a substitute for the first or second dA residue within the recognition sequence d(GAATTC) of the endodeoxyribonuclease *Eco*RI. Oligomers containing this particular structure lack potential N-7 binding sites, and their processing by the endodeoxyribonuclease *Eco*RI should give information about the DNA recognition by this enzyme.

Chart I



1a R = H; R′ = H
b R = Bz; R′ = DMT

2a R = CH_3
b R = CH_2CH_2CN

d(GGAATTCC)	3	d(CGCGc^{7}Ac^{7}ATTCGCG)	10
d(GGc^{7}AATTCC)	4	d(GTAGAATTCTAC)	11
d(GGAc^{7}ATTCC)	5	d(GTAGc^{7}AATTCTAC)	12
d(GGc^{7}Ac^{7}ATTCC)	6	d(GTAGAc^{7}ATTCTAC)	13
d(CGCGAATTCGCG)	7	d(GTAGc^{7}Ac^{7}ATTCTAC)	14
d(CGCGc^{7}AATTCGCG)	8	d(GTc^{7}AGAATTCTAC)	15
d(CGCGAc^{7}ATTCGCG)	9	d(GTAGAATTCTc^{7}AC)	16

Experimental Procedures

NMR spectra were recorded on a Bruker WM 250 spectrometer; δ values are in ppm relative to external 85% phosphoric acid (^{31}P). Chemical shifts are positive when downfield to the external standard. Thin-layer chromatography (TLC) was performed on silica gel SIL G-25 UV_{254} plates (Macherey-Nagel, West Germany). Flash chromatography was performed with silica gel 60H (Merck, West Germany) at 0.5 bar N_2.

The endodeoxyribonuclease *Eco*RI (EC 3.1.23.13), snake venom phosphodiesterase (EC 3.1.16.1, *Crotallus durissus*), and alkaline phosphatase (EC 3.1.3.1, *Escherichia coli*) were products of Boehringer Mannheim (West Germany). A

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[1] Abbreviations: dc^{7}A, 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; Tris, tris(hydroxymethyl)aminomethane; Bz, benzoyl; DMT, 4,4′-dimethoxytrityl; τ/2, half-rate time; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; T_m, temperature of melting; EDTA, ethylenediaminetetraacetic acid.

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sample of the endodeoxyribonuclease *Eco*RI with high activity was a gift of Dr. A. Pingoud (Institut für Biophysikalische Chemie, Zentrum für Biochemie, Medizinische Hochschule Hannover).

Melting Curves. The melting curves were measured in Teflon-stoppered cuvettes with a 10- or 2-mm light path length in a thermostatically controlled cell holder with a Shimadzu 210-A recording spectrometer connected with a Kipp & Zonen BD 90 recorder. The increase of absorbance at the appropriate wavelengths was recorded while the temperature of the solution was increased linearly with time at a rate of 20 deg on a Lauda PM-351 programmer and a Lauda RCS 6 bath equipped with an R 22 unit (MWG Lauda, West Germany). The actual temperature was measured in the measuring cell with a Pt-100 resistor.

HPLC Separation. High-performance liquid chromatography for preparative scale was carried out on a 250 × 4 mm (7 μm) RP-18 LiChrosorb column (Merck, West Germany) connected with a 25 × 4 mm RP-18 precolumn by a Merck-Hitachi HPLC with one pump (Model 655-12) connected with a proportioning valve, a variable-wavelength monitor (Model 655A), a LC controller (Model L-5000), and an integrator (Model D-2000). Analytical-scale HPLC was performed on a LKB system with two pumps (Model 2150), a variable-wavelength monitor (Model 2152), and a controller (Model 2151) connected with a Merck-Hitachi integrator (Model D-2000). Solvent systems contained acetonitrile (5%) in 0.1 M triethylammonium acetate, pH 7.0 (A), and acetonitrile (B). They were used in the following order: solvent system I, 100% A; solvent system II, 3 min (15% B), 7 min (15–40% B); solvent system III, 15 min (5–20% B); solvent system IV, 5% B; solvent system V, 15 min (5–15% B). For desalting of the oligomers on a 25 × 4 mm RP-18 cartridge, loaded with the particular oligomer, the following procedure was employed: eluation of salt with water for 5 min; eluation of the oligomer with 0–60% MeOH in water for 2 min. The flow rate of all experiments was 1 mL/min.

Enzymatic Analysis of the Oligonucleotides **3–16.** The oligomers (about 0.2 A_{260} unit) were dissolved in 0.1 M Tris-HCl buffer, pH 8.5 (100 μL), and incubated with snake venom phosphodiesterase (2 μg) for 45 min at 30 °C. After further incubation with alkaline phosphatase (2 μg) at 30 °C for 15 min, the mixture was analyzed by HPLC with solvent system I as eluent. Quantification of the material was made on the basis of the peak areas, which were divided by the extinction coefficients of the nucleoside constituents (ϵ_{260}: dA, 15.4; dC, 7.3; dG, 11.4; T, 8.8; dc^7A, 10.6).

*4-(Benzoylamino)-7-[3-O-[(N,N-diisopropylamino)(β-cyanoethoxy)phosphanyl]-5-O-(4,4′-dimethoxytrityl)-2-deoxy-β-*D*-erythro-pentofuranosyl]-7H-pyrrolo[2,3-d]pyrimidine* (**2b**). Compound **1b** (180 mg, 0.27 mmol) (Seela & Kehne, 1985a; Ono et al., 1984) was dissolved in anhydrous tetrahydrofuran (1.5 mL). Diisopropylethylamine (200 μL) was added. (β-Cyanoethoxy)chloro(*N,N*-diisopropylamino)-phosphane (200 μL, 0.6 mmol) (Sinha et al., 1984) was added dropwise over a period of 30 min. The reaction mixture was stirred for another 15 min and was evaporated to a yellowish foam. It was dissolved in argon-saturated ethyl acetate (2 mL) and purified by flash chromatography (silica gel 60H, 10 × 2 cm column). The main zone was collected and evaporated to give a colorless foam (120 mg, 52%): ^{31}P NMR ($CDCl_3$) δ 147.1, 147.3; TLC (ethyl acetate, silica gel) R_f 0.9.

Oligonucleotide Synthesis. The oligomers **3–16** were synthesized on two different DNA synthesizers: Model 380 B (Applied Biosystems) employing methoxyphosphoramidite chemistry (Beaucage & Caruthers, 1981) and the Gene Assembler of Pharmacia (Sweden) employing (cyanoethyl)-phosphoramidite chemistry (Sinha et al., 1984). The synthesis was carried out on solid support in columns with 1 μmol of immobilized 2′-deoxynucleoside on CPG (Applied Biosystems) or Mono Beads (Pharmacia, Sweden). The synthesis followed the reaction cycle of detritylation, coupling, oxidation, and capping. To cleave the OCH_3 protecting group, the thiophenol reaction was carried out on solid support. The cleavage of the oligomer from the solid support with concentrated ammonia for 15 min at room temperature on the stage of the 5′-protected oligomers also removed the cyanoethyl protecting group. Cleavage of the nucleobase protecting groups by concentrated ammonia at 60 °C was accomplished within 24 h in the case of the nonmodified oligomers **3**, **7**, and **11** and within 72 h in the case of the modified oligomers **4–6**, **8–10**, and **12–16**. The 5′-protected oligomers were purified by HPLC (RP-18, solvent system II), and detritylated was by the action of 80% acetic acid for 5 min. After removal of the acid the oligomer was dissolved in water (5 mL), and the tritylmethanol was extracted with diethyl ether. The oligomers were then purified by reverse-phase HPLC with solvent system III or V. Samples of the main zone were lyophilized and desalted by reverse-phase HPLC on a 25 × 4 mm RP-18 cartridge. The fractions were collected and lyophilized from water and methanol. The oligomers were dissolved in water (100 μL) and stored frozen at −20 °C. Oligomers were obtained in amounts between 20 and 40 A_{260} units. Table I shows the retention times of the oligomers **3–16**.

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

compd	oligomer	retention time (min)	solvent system
3	d(GGAATTCC)	9.2	III
4	d(GGc^{7}AATTCC)	9.0	III
5	d(GGAc^{7}ATTCC)	9.0	III
6	d(GGc^{7}Ac^{7}ATTCC)	8.6	III
7	d(CGCGAATTCGCG)	16.2	V
8	d(CGCGc^{7}AATTCGCG)	15.9	V
9	d(CGCGAc^{7}ATTCGCG)	16.0	V
10	d(CGCGc^{7}Ac^{7}ATTCGCG)	15.6	V
11	d(GTAGAATTCTAC)	11.2	III
12	d(GTAGc^{7}AATTCTAC)	10.5	III
13	d(GTAGAc^{7}ATTCTAC)	10.7	III
14	d(GTAGc^{7}Ac^{7}ATTCTAC)	9.5	III
15	d(GTc^{7}AGAATTCTAC)	9.8	III
16	d(GTAGAATTCTc^{7}AC)	9.2	III

Kinetics of Oligomer Hydrolysis by the Endodeoxyribonuclease EcoRI. The oligomers (about 0.25 A_{260} unit, 1.8 nmol) in 10 mM Tris-HCl buffer, pH 7.5 (100 μL), containing 80 mM NaCl and 20 mM $MgCl_2$ were incubated with the enzyme (73 pmol) at 30 °C. Portions of 10 μL of the incubation mixture were analyzed on a HPLC column (RP-18, 250 × 4 mm, connected with a 25 × 4 mm RP-18 precolumn) at different intervals of time with solvent system III as eluent. The cleavage products were collected and lyophilized. Analysis of the nucleoside composition was carried out by cleavage with snake venom phosphodiesterase followed by treatment with alkaline phosphatase according to the procedure described before.

Results and Discussion

Solid-Phase Synthesis of the Octamers **3–6** *and Dodecamers* **7–16** *Employing the Phosphoramidites* **2a** *and* **2b**. As reported in a previous publication (Seela & Driller, 1985), the

Table II: T_m Values of the Oligomers **3–16** under *Eco*RI Cleavage Conditions

compd	T_m (°C)[a]	compd	T_m (°C)[a]
d(GGAATTCC) (**3**)	30	d(GTAGAATTCTAC) (**11**)	44
d(GGc^{7}AATTCC) (**4**)	28	d(GTAGc^{7}AATTCTAC) (**12**)	43
d(GGAc^{7}ATTCC) (**5**)	29	d(GTAGAc^{7}ATTCTAC) (**13**)	44
d(GGc^{7}Ac^{7}ATTCC) (**6**)	24	d(GTAGc^{7}Ac^{7}ATTCTAC) (**14**)	39
		d(GTc^{7}AGAATTCTAC) (**15**)	42
		d(GTAGAATTCTc^{7}Ac) (**16**)	43

[a] The T_m values at 260 nm were determined in 10 mM Tris-HCl buffer, pH 7.5, containing 80 mM NaCl, 20 mM $MgCl_2$, and an oligomer concentration of 4 μM single strands.

octamer d(GGAATTCC) (Connolly et al., 1984) is a useful DNA fragment for studying the influence of isosteric bases on the interaction of the particular recognition sequence d(GAATTC) with the endodeoxyribonuclease *Eco*RI. It exhibits a melting temperature of 30 °C, which is in a temperature range where the endodeoxyribonuclease shows enough activity to cleave duplexes in a suitable time for kinetic measurements. The isosterically modified octamer d(Gc^{7}GAATTCC) (Seela & Driller, 1985) shows a T_m value that is similar to that of the parent oligomer.

Because of these findings, octamers were synthesized, which contained 2′-deoxytubercidin (**1a**) (Seela & Kehne, 1983) instead of 2′-deoxyadenosine within the recognition sequence d(GAATTC). In particular, the following oligomers were prepared: d(GGc^{7}AATTCC) (**4**), d(GGAc^{7}ATTCC) (**5**), and d(GGc^{7}Ac^{7}ATTCC) (**6**), lacking potential N-7 binding sites for the enzyme.

Recently, it has been shown that flanking sequences at the 3′- as well as the 5′-end of the recognition sequence can influence the cleavage rate (Alves et al., 1984). Therefore, we synthesized two sets of dodecamers, namely, the compounds **7–10** and **11–16** with the parent sequences d(CGCGAATTCGCG) (**7**) (Wing et al., 1980; Drew et al., 1981; Dickerson & Drew, 1981) and d(GTAGAATTCTAC) (**11**). The former set of dodecamers contain flanking GC base pairs, which form regions of high stability. The second set of dodecamers (**11–16**) exhibit a rather random base composition at the flanking sequences, which should lead to decreased stability of the duplex.

We have shown earlier the utility of the methyl-protected phosphoramidite **2a** in oligonucleotide synthesis on solid support (Seela & Kehne, 1985a,b). Employing the phosphoramidite method of Caruthers and co-workers (Beaucage & Caruthers, 1981; Matteuci & Caruthers, 1981; McBride & Caruthers, 1983), shorter oligonucleotides were obtained in good yields. According to findings in other laboratories, (β-cyanoethyl)phosphoramidites are more convenient to use in the synthesis of oligomers of increased chain lengths (Sinha et al., 1984; Zon et al., 1985). Since it has been reported that the application of (β-cyanoethyl)phosphoramidites also resulted in cleaner reaction products (Zon et al., 1985), we synthesized the phosphoramidite **2b**.

Appropriately protected 2′-deoxytubercidin (**1b**) (Seela & Kehne, 1985; Ono et al., 1984) was derivatized with (β-cyanoethoxy)chloro(*N*,*N*-diisopropylamino)phosphane according to the procedure of Köster (Sinha et al., 1984). According to the low-scale preparation, a simplified work-up procedure was employed. The ^{31}P NMR spectrum showed two signals at 147.1 and 147.3 ppm, which corresponded to the diastereoisomers of compound **2b**. Employing this phosphoramidite in solid-phase synthesis, a high coupling efficiency was attained yielding to pure oligomeric products.

The synthesis and purification of the oligomers **3–16** were carried out on solid support according to the procedure described under Experimental Procedures. Anomalous chromatographic properties were observed during HPLC of the dodecamers **8–10** as well as of the parent oligomer d(CGCGAATTCGCG) (**7**). A sample of the purified and desalted oligomer **7** showed a two-peak pattern on reverse-phase HPLC, where the ratio of the peak areas varied with the concentration of the injected sample, whereby a low concentration increased the first peak. On the other hand, separation of the zones followed by HPLC analysis of each peak content gave again rise to the two-peak pattern observed before. This problem was overcome by chromatography at elevated temperature (50 °C), employing the isocratic solvent system IV. Analysis of nucleoside content was achieved by HPLC analysis after digestion of the oligomers with snake venom phosphodiesterase followed by treatment with alkaline phosphatase.

Melting Profiles and Structure of the Oligomers **3–16**. Due to the fact that the recognition process of the endodeoxyribonuclease *Eco*RI requires double-stranded DNA, structural data of the oligomers, described under Oligonucleotide Synthesis, had to be collected.

Temperature-dependent UV measurements at 260 nm of the octamers **3–6** (Table II) in 10 mM Tris-HCl buffer, pH 7.5, containing 20 mM $MgCl_2$, 80 mM NaCl, and an oligomer concentration of 4 μM gave cooperative melting profiles. According to Table II, the T_m value of the octamer d(GGAATTCC) (**3**) (Connolly et al., 1984) (T_m = 30 °C) was only slightly decreased in the case of d(GGc^{7}AATTCC) (**4**) (T_m = 28 °C) and of d(GGAc^{7}ATTCC) (**5**) (T_m = 29 °C). The replacement of both dA residues by 2′-deoxytubercidin (**1a**) decreased the T_m further to 24 °C. This implied that the exchange of one adenine base by a 7-deazaadenine residue had a minor influence on the tertiary structure of the DNA fragments. Previous findings (Seela et al., 1986, 1987), which reported a T_m value below 15 °C for compound **4**, have to be corrected and were due to the fact that the sample contained trace amounts of heavy metal ion impurities. The correct T_m value of the oligomer **4** was obtained after an additional purification step and was found to be 28 °C as reported in Table II.

Earlier investigations in our laboratory and by others (Seela & Driller, 1985; Ohtsuka et al., 1984; Connolly et al., 1984) had shown that enzymatic hydrolysis of **3** by the *Eco*RI enzyme requires temperatures around 20 °C. This temperature ensured duplex formation under cleavage conditions. However, the enzymatic activity of the endodeoxyribonuclease was fairly low at that point, resulting in time-consuming experiments for the determination of kinetic data. As a result, a dodecamer with a high content of d(GC) base pairs was chosen for further experiments.

Single-crystal X-ray analysis on the dodecamer d(CGCGAATTCGCG) (**7**) (Wing et al., 1980; Drew et al., 1981; Dickerson & Drew, 1981) illustrated that the oligomer exhibits a complete turn of a right-handed B-DNA structure in the solid state. Melting experiments carried out in 1 mM phosphate buffer solution, pH 7.0, containing 1 mM EDTA

Table III: T_m Values of the Dodecamers **7–10** Containing 2′-Deoxytubercidin (**1a**) Instead of 2′-Deoxyadenosine at 260 and 280 nm at Various Salt Concentrations

	T_m (°C)[a]					
	1 mM NaCl		10 mM NaCl		100 mM NaCl	
compd	260 nm	280 nm	260 nm	280 nm	260 nm	280 nm
d(CGCGAATTCGCG) (**7**)	34 (62)[b]	59	36 (62)	60	52	65
d(CGCGc^{7}AATTCGCG) (**8**)	31 (63)	61	33 (63)	62	48	60
d(CGCGAc^{7}ATTCGCG) (**9**)	32 (62)	61	33 (63)	60	53	60
d(CGCGc^{7}Ac^{7}ATTCGCG) (**10**)					46	58

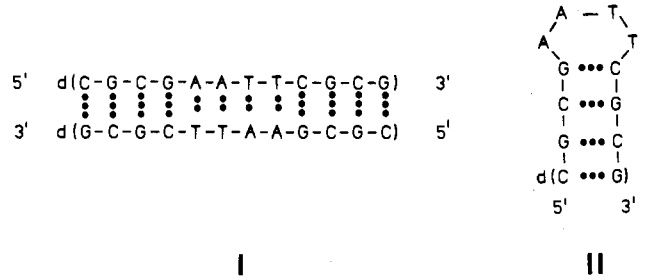
[a] In 1 mM phosphate buffer, pH 7.0, containing 1 mM EDTA at an oligomer concentration of 4 μM. [b] T_m value of the second transition.

and various amounts of NaCl resulted in completely different melting profiles at 260 and 280 nm (Table III). In 1 mM NaCl a low T_m at 34 °C with an indistinct second transition at 62 °C was found at 260 nm. However, at 280 nm only a monophasic melting with a T_m of 59 °C was observed. Similar results were obtained at a sodium chloride concentration of 10 mM, whereas in 100 mM NaCl only monophasic melting curves were observed at 280 nm as well as at 260 nm but still with different T_m values.

The melting of d(AT) and d(GC) base pairs can be observed at 260 nm, whereas at 280 nm the melting of d(GC) base pairs can be followed. This is due to the fact that in DNA at 260 nm the $\Delta\Delta\epsilon$ of a d(AT) base pair is about 4 times larger than that of a d(GC) base pair, whereas at 280 nm the $\Delta\epsilon$ of d(GC) is approximately 8 times larger than that of d(AT) (dA-dT, $\Delta\epsilon_{260} = 3059$, $\Delta\epsilon_{282} = 204$; dG-dC, $\Delta\epsilon_{260} = 709$, $\Delta\epsilon_{282} = 1697$) (Blake & Hydorn, 1985). Applying these data to the findings of Table III, it was considered that the low T_m value belonged to a melting process mainly depending on d(AT) denaturation, whereas the T_m at the higher temperature corresponded to a melting of d(GC) base pairs. Owing to the structure of the oligomer **7**, a simple explanation would result from the fact that the low-temperature transition represented the conversion of a duplex into a hairpin, while the high-temperature transition showed the melting of the hairpin into single strands. This interpretation was underlined by the fact that the T_m value at 280 nm was nearly independent of the salt concentration (Table III).

In order to confirm these findings, the T_m values at 260 and 280 nm were determined at different oligomer concentrations. From the graph of Figure 1 it can be seen that the process observed at 260 nm depended strongly on the oligomer concentration, whereas that at 280 nm was relatively independent of the strand concentration. Again these findings supported a biphasic transition from a duplex into two hairpins as a concentration-dependent process and the denaturation of hairpins into single strands, which is independent of concentration.

As can be seen from the duplex I and the hairpin structure II, the duplex–hairpin transition affords only the melting of



I II

d(AT) base pairs, while the sum of d(GC) base pairs is constant. Only intermolecular G-C hydrogen bonds are replaced by intramolecular ones. We believe that this reaction follows a unimolecular process with a first-order rate constant. The

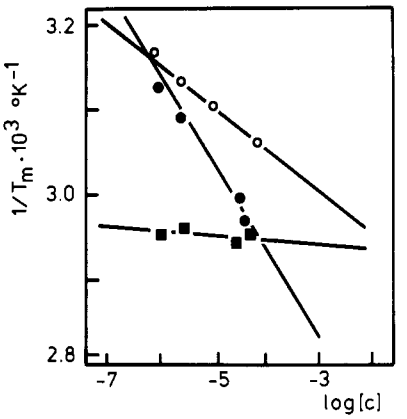


FIGURE 1: Dependence of melting behavior of d(CGCGAATTCGCG) (**7**) from the oligomer concentration at 260 (●) and 280 nm (■) measured in 1 mM phosphate buffer, pH 7.0, containing 100 mM NaCl and 1 mM EDTA and of the oligomer d(GTAGAATTCTAC) (**11**) (○) measured in 10 mM Tris-HCl buffer, pH 7.5, containing 20 mM $MgCl_2$ and 80 mM NaCl (260 nm).

process may be initiated by a premelting of the d(AT) region within the duplex of **7** followed by the rearrangement of the looped-out duplex into two hairpins.

The transition observed at 280 nm represents the melting of d(GC) base pairs. The d(GC) melting could also be observed at 260 nm at low salt concentrations as a second transition. Data (Table III) are given in parentheses.

The formation of hairpin structures of the dodecamer **7** at low salt concentration has already been reported by Breslauer and co-workers (Marky et al., 1983). Due to the fact that the biphasic nature of the melting profile disappeared at higher salt (100 mM NaCl), they concluded that under these conditions a transition from a duplex into single strands took place. In contrast, our data, which showed different T_m values at 260 and 280 nm at 100 mM NaCl concentration (Table III), clearly demonstrated that hairpins as well as duplexes were present at that salt concentration. The monophasic profile found at that high salt conditions was simply due to the very similar T_m values of hairpins and duplexes, which were not resolved in the melting profile. This was in agreement with earlier observations showing that the T_m value of a hairpin is much less salt dependent than that of a duplex (Marky et al., 1983), which is shown in Table III.

Repeating the experiments of Breslauer and co-workers at 1 mM NaCl, a biphasic transition was observed only during the first heating process. As Figure 2 shows, renaturation was not complete upon cooling. From the absorbance value obtained in the renaturation profile it could be concluded that hairpin melting was reversible, whereas melting of the duplex was not under those conditions. The profile taken at 280 nm confirmed that the sum of d(GC) base pairs stayed constant upon melting.

Additionally, the comparison of calculated thermodynamic data with the experimental values of the melting process of

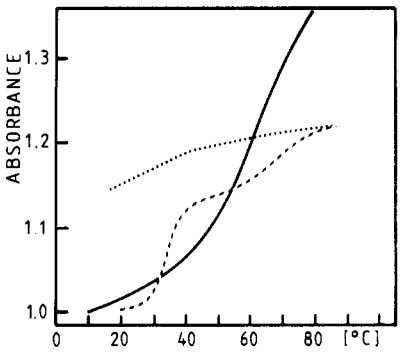


FIGURE 2: Melting profiles of d(CGCGAATTCGCG) (**7**) at 260 (---) and 280 nm (—) measured in 1 mM phosphate buffer, pH 7.0, containing 10 mM NaCl and 1 mM EDTA. The dotted line shows the reassociation process at 260 nm.

Table IV: T_m Values of the Oligomers **11–16** at 260 and 280 nm

compd	T_m (°C)[a] 260 nm	280 nm
d(GTAGAATTCTAC) (**11**)	36	37
d(GTAGc^{7}AATTCTAC) (**12**)	32	33
d(GTAGAc^{7}ATTCTAC) (**13**)	36	37
d(GTAGc^{7}Ac^{7}ATTCTAC) (**14**)	29	31
d(GTc^{7}AGAATTCTAC) (**15**)	32	33
d(GTAGAATTCTc^{7}AC) (**16**)	31	33

[a] T_m values were determined in 1 mM phosphate buffer, pH 7.0, containing 100 mM NaCl and 1 mM EDTA.

the oligomers **7** and **11** gave evidence that two different processes took place upon melting. From the plot $1/T_m$ vs. log $c_{oligomer}$ the enthalpy and entropy changes could be calculated at completely identical conditions [1 mM Tris-HCl, pH 7.0, 100 mM NaCl, 1 mM EDTA (Figure 1)]. For the oligomer **11**, values of ΔH = −80 kcal/mol and ΔS = −220 cal/(mol·deg) were attained. On the other hand, the enthalpy and entropy changes for the oligomer **7** gave values of ΔH = −60 kcal/mol and ΔS = −160 cal/(mol·deg). Regarding the higher content of G-C base pairs within the oligomer d(CGCGAATTCGCG) (**7**), the ΔH value of the melting of the oligomer **7** is far too small, if one would assume a transition from a duplex into two single strands. This result underlined that d(CGCGAATTCGCG) shows a high tendency of hairpin formation at low oligomer concentration. Nevertheless, at high oligomer concentration duplexes are formed preferentially, which can be derived from X-ray (Frederick et al., 1984) and calorimetric data (Marky et al., 1983).

In contrast to these findings, the oligomer d(GTAGAATTCTAC) (**11**) with a random composition of the 3′- and 5′-flanking nucleotides exhibited almost identical T_m values at 260 and 280 nm and no biphasic transition (Table IV). This demonstrated that the melting of the oligomers **11–16** was represented by a simple conversion of a duplex into single strands, omitting hairpin structures.

From the data of Tables II–IV it could be concluded that the replacement of dA residues by 2′-deoxytubercidin (**1a**) had only a minor effect on the T_m values. In general, T_m values were decreased, which implied that the modified nucleoside **1a** destablilized base pairing and/or stacking. If the T_m values of the oligomers with dc^{7}A (**1a**) at the cleavage site of *Eco*RI (**4**, **8**, and **12**), obtained in the absence of Mg^{2+} (Tables III and IV), were compared to those of oligomers having the dA residue replaced next to thymidine (**5**, **9**, and **13**), it became apparent that the modification at the cleavage site destabilized the duplex structure more strongly than a replacement next to thymidine. Replacement of two dA residues within the recognition site d(GAATTC) decreased the T_m values further. Finally, the modification of 2′-deoxyadenosine within the flanking regions also resulted in decreased duplex stability (**15** and **16**) (Tables III and IV).

Oligomer Cleavage by the Endodeoxyribonuclease EcoRI and Recognition of d(GAATTC) Sequences Containing 2′-Deoxytubercidin (**1a**). As reported in the introduction, the aim of the enzymatic studies was the detection of N-7 binding sites of the two 2′-deoxyadenosine residues within the recognition sequence d(GAATTC) toward the endodeoxyribonuclease *Eco*RI. For this purpose, the oligomers **3–16** should be investigated with respect to enzymatic cleavage.

Cleavage experiments were carried out in 10 mM Tris-HCl buffer, pH 7.5, containing 80 mM NaCl and 20 mM $MgCl_2$. The concentration of the oligomers was about 10 μM and that of the dimeric enzyme was 0.7 μM. With respect to the T_m value around 30 °C, the experiment with the octamer **3** was carried out at 16 °C. The dodecamers **7–16** were incubated at 30 °C.

Earlier experiments have shown that the nonmodified oligomer **3** was cleaved by the endodeoxyribonuclease *Eco*RI even at a low temperature of 16 °C (Connolly et al., 1984). Cleavage was followed by HPLC analysis (Seela & Driller, 1985), and half-rates of cleavage of more than 10 h were found. Since it was difficult to collect kinetic data with octamers of type **3** containing 2′-deoxytubercidin (**1a**), which might even show slower cleavage rates, we chose higher melting dodecamers for kinetic investigations. Since the type II endodeoxyribonucleases recognize only duplex structures, it was expected that the oligomers **7–10**, which showed a high tendency of hairpin formation, were only slowly cleaved by the *Eco*RI enzyme. Cleavage experiments with the commercially available enzyme supported this idea; the oligomer **7** was cleaved only with low velocity to the tetramer d(CGCG) and the octamer d(pAATTCGCG).

This finding was fairly unexpected since Rosenberg and co-workers (Frederick et al., 1984; Rosenberg et al., 1985) have reported that the endodeoxyribonuclease *Eco*RI recognized a similar oligomer, d(TCGCGAATTCGCG), in the crystalline state. From the X-ray data it was clear that duplex formation occurred under those conditions. However, as shown in the previous section, hairpin structures of the very similar dodecamer d(CGCGAATTCGCG) (**7**) were formed in dilute solution, which cannot be recognized by the *Eco*RI enzyme. A comparison of cleavage velocities of both nonmodified oligomers **7** and **11** showed that d(GTAGAATTCTAC) (**11**) with a random flanking sequence was cleaved by the endodeoxyribonuclease *Eco*RI (Boehringer, low enzyme concentration) within 7 h. The oligomer d(CGCGAATTCGCG) (**7**), however, required 24 h to be cleaved to an amount of less than 45% presumably due to the presence of hairpins.

As a consequence, we focused our interest toward dodecamers having a random composition of the flanking regions. As the target oligomer, compound **11** was chosen, which showed a normal melting behavior from duplex to single strands without the occurrence of hairpin structures in the assay solution. The almost identical T_m values—determined at 260 and 280 nm—of the dodecamers **12–16** containing 2′-deoxytubercidin (**1a**) instead of 2′-deoxyadenosine at all possible positions (Table IV) indicated that also these oligomers formed only two species—duplexes and single strands—in solution.

At a temperature of 30 °C and with the *Eco*RI enzyme at high concentration, the cleavage of the parent oligomer d(GTAGAATTCTAC) (**11**) was complete within 55 min. The reaction was monitored by HPLC on a RP-18 column where

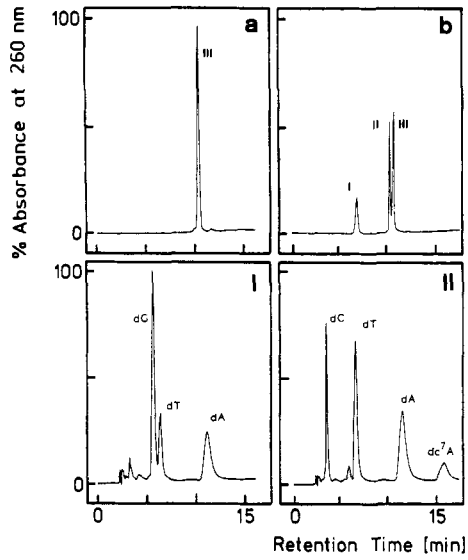


FIGURE 3: HPLC profiles from the cleavage of d-(GTAGc⁷AATTCTAC) (**12**) by the endodeoxyribonuclease *Eco*RI under the conditions described under Experimental Procedures. Part a shows the HPLC profile of the reaction mixture at the beginning and part b the reaction after 30 min. Solvent system III was used as eluent. HPLC patterns of the cleavage products I and II were taken after cleavage with snake venom phosphodiesterase and treatment with alkaline phosphatase under solvent system I and are shown in the lower part of the figure.

Table V: Half-Rates of Cleavage of the Oligomers **11–16** Catalyzed by the Endodeoxyribonuclease *Eco*RI[a]

compd	$\tau/2$ (min)
d(GTAGAATTCTAC) (**11**)	21
d(GTAGc⁷AATTCTAC) (**12**)	66
d(GTAGAc⁷ATTCTAC) (**13**)	61
d(GTAGc⁷Ac⁷ATTCTAC) (**14**)	
d(GTc⁷AGAATTCTAC) (**15**)	14
d(GTAGAATTCTc⁷AC) (**16**)	13

[a] The incubation mixture contained 10 mM Tris-HCl buffer, pH 7.5, 80 mM NaCl, 20 mM $MgCl_2$, an oligomer concentration of 10 μM double strands, and 0.7 μM dimeric enzyme at a temperature of 30 °C. The reaction was followed by HPLC analysis on a RP-18 column with solvent system III as eluent.

the starting oligomer was separated from the cleavage products of the tetramer d(GTAG) and the octamer d(pAATTCTAC). The reaction products were identified by total hydrolysis of the peak contents into the nucleosides by snake venom phosphodiesterase followed by treatment with alkaline phosphatase.

Next the oligomers **12–16** were incubated under the same conditions. The cleavage of the oligomers **12** and **15** followed by enzymatic analysis of the peak contents is shown in Figures 3 and 4. Kinetic data of the cleavage experiments are given in Figure 5 and Table V.

As one can see, the incorporation of dc⁷A (**1a**) at the cleavage site (**12**) increased the $\tau/2$ value from 21 to 66 min. A similar decrease of the cleavage velocity was observed when the other dA residue within the recognition sequence was replaced by dc⁷A (oligomer **13**). However, if both dA residues were exchanged (**14**), no cleavage took place by the enzyme. On the other hand, if a dA residue within the 3′- or 5′-flanking region was introduced (**15** and **16**), even a slightly faster reaction was observed. From the data described above the following conclusions can be drawn.

(i) Recent findings on solid state (Frederick et al., 1984; Rosenberg et al., 1985), which suggested that the purine 7-nitrogens of both dA residues within the sequence d-(GAATTC) are proton acceptor sites for the enzyme, are supported by our experiments carried out in solution. However, a structural alteration of the duplex by the modified nucleoside **1a** could not be excluded.

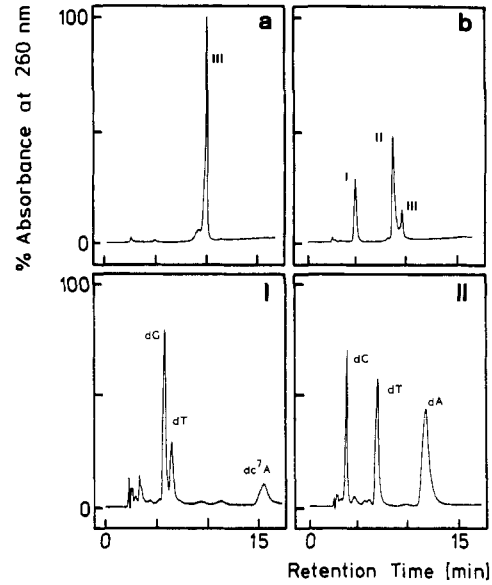


FIGURE 4: HPLC profiles obtained from the cleavage of the oligonucleotide **15** [d(GTAGAATTCTc⁷AC)] with the endodeoxyribonuclease *Eco*RI, as described in Figure 3.

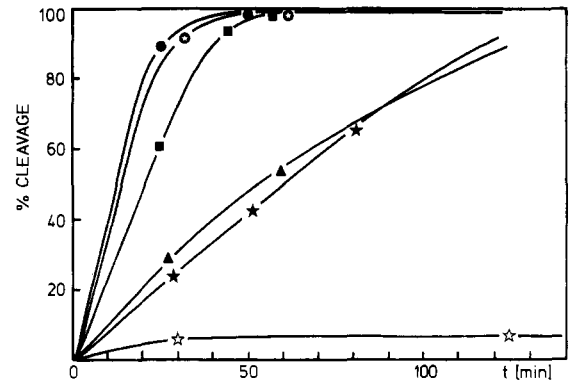


FIGURE 5: Cleavage of the oligomers **11–16** with the endodeoxyribonuclease *Eco*RI in 10 mM Tris-HCl buffer, pH 7.5, containing 80 mM NaCl, 20 mM $MgCl_2$, 10 μM dimeric oligomer, and 0.7 μM dimeric enzyme. Incubation temperature was 30 °C. The reaction was monitored by RP-18 HPLC. The percentage of cleavage was calculated on the basis of the uncleaved oligomer. **11** (■); **12** (▲); **13** (★); **14** (☆); **15** (open star in circle); **16** (●).

(ii) The loss of one purine acceptor site can be accommodated by the enzyme, which was demonstrated by a slower, but still specific cleavage of the oligomers **12** and **13**. Only twofold modification as represented in oligomer **14** abolished enzymatic cleavage. This indicates that enzyme binding to one of the two adenine bases is not a prerequisite for enzyme specificity but improves the processing of the substrate.

(iii) If short oligomers are used as models for high molecular weight DNA, one has to consider the special features of such short DNA fragments, e.g., hairpin formation, which may obscure the interpretation of enzymatic data.

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Registry No. **1b**, 90335-57-2; **2a**, 98921-82-5; **2b**, 107134-59-8; **3**, 70755-49-6; **4**, 107115-02-6; **5**, 107115-03-7; **6**, 107115-04-8; **7**, 77889-82-8; **8**, 107115-05-9; **9**, 107115-06-0; **10**, 107115-07-1; **11**, 107115-08-2; **12**, 107115-09-3; **13**, 107115-10-6; **14**, 107115-11-7;

15, 107115-12-8; **16**, 107115-13-9; EC 3.1.23.13, 80498-17-5; NC-$(CH_2)_2OP(Cl)N(Pr\text{-}i)_2$, 89992-70-1.

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Catalysis by Human Leukocyte Elastase. Aminolysis of Acyl-Enzymes by Amino Acid Amides and Peptides†

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ABSTRACT: Acyl-enzymes of human leukocyte elastase (HLE) were generated in situ during the hydrolysis of peptide thiobenzyl esters and served as substrates for aminolysis by a variety of amino acid amides and short peptide nucleophiles. For amino acid amides, there is a positive correlation between nucleophilic reactivity toward *N*-methoxysuccinyl (MeOSuc)-Ala-Ala-Pro-Val-HLE and the hydrophobicity of the side chain. For peptides, nucleophilicity toward MeOSuc-Ala-Ala-Pro-Val-HLE decreases dramatically with increasing chain length. Combined, these results suggest that (i) substrate specificity for the P_1' residue may be more dependent on side chain hydrophobicity than on specific, structural features of the side chain and (ii) there may be no important binding interactions available past S_1'. Kinetic parameters were also determined for the nucleophilic reactions of $PheNH_2$ and $TyrNH_2$ with MeOSuc-Pro-Val-HLE, MeOSuc-Ala-Pro-Val-HLE, MeOSuc-Ala-Ala-Pro-Val-HLE, and MeOSuc-Ala-Ala-Pro-Ala-HLE. Reactivity of these acyl-enzymes toward nucleophilic attack displays no dependence on peptide chain length but does increase significantly for the substrate with Ala at P_1. This same correlation between reactivity and acyl-enzyme structure is also seen for nucleophilic attack by water.

Serine proteases catalyze the hydrolyses of esters and amides by a mechanism involving the intermediacy of an acyl-enzyme. An important consequence of this mechanism is that it allows nucleophiles, of appropriate structure, to compete with water for the acyl-enzyme and provides a second pathway for deacylation. This mechanism is illustrated in Scheme I. According to this mechanism, the acyl-enzyme, formed from reaction within the Michaelis complex, can react with either

† For part 7 of this series, see Stein (1987b).

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