Synthesis of deoxyoligonucleotides containing 7-deazaadenine: recognition and cleavage by restriction endonuclease *Bgl* II and *Sau* 3AI (Nucleosides and Nucleotides Part 55¹)

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ABSTRACT

Deoxydecanucleotides having a recognition sequence of *Bgl* II and *Sau* 3AI, and their 7-deazaadenine analogs were synthesized. The decanucleotides containing 7-deazaadenine in place of adenine were partially resistant to the hydrolysis by *Sau* 3AI and strongly resistant to that by *Bgl* II. A new hypothesis on the mode of recognition and cleavage of specific nucleotide sequences by *Bgl* II, recognizing one strand and cleaving the other strand, is presented.

INTRODUCTION

Current results of studies on the mode of recognition of nucleotide sequences by restriction endonucleases have suggested the importance of the 7-azomethine functions of purine bases for the recognition^{2,3}. To confirm this point we undertook the synthesis of deoxydecanucleotides containing 7-deazaadenine in place of adenine in the recognition sequences of *Bgl* II (AGATCT)⁴ and *Sau* 3AI (GATC)⁵. As one would expect that, since 7-deazaadenine differs from adenine only at the 7-position where the 7-azomethine group of adenine was replaced by a methine group, the usual base pairing is not restricted in the duplex formation in the oligomers. The conformation around the glycosyl bond is also expected to be quite similar to that of 2'-deoxyadenosine.

This paper describes the synthesis and some properties of deoxyoligonucleotides containing 7-deazaadenine in or nearby the recognition sequences of *Bgl* II and *Sau* 3AI.

MATERIALS AND METHODS

5'-O-Dimethoxytrityl-N⁶-benzoyl-7-deaza-2'-deoxyadenosine (3)

7-Deaza-2'-deoxyadenosine (2) was prepared from tubercidin

(1) by recently established procedure⁶. The physical constants of 2 were identical with those reported by Seela and Kehne⁷, and Robins and co-workers⁸. To the solution of compound 2 (150 mg) in 4 ml of pyridine was added benzoyl chloride (0.42 ml, 6 eq.) in an ice bath under stirring, and the solution was kept at room temperature for 25 min, then cooled. To the solution was added a mixture of CHCl_3 (6 ml), 5% NaHCO_3 solution (6 ml), and ice (1.5 g) under stirring. The organic layer was separated and concentrated in vacuo to leave a residue. The residue was dissolved in tetrahydrofuran (4 ml) and 10 ml of 1 N NaOH in 50 % EtOH was added under stirring in an ice bath. After standing at room temperature for 30 min, the solution was neutralized with 2 N HCl, and the solvent was evaporated. The residue was passed through a reversed phase silica gel column (C_{18} , Waters, 4x 7 cm) with acetone-0.1 N triethylammonium acetate as the solvent. The fractions containing the product (N^6 -benzoyl-2'-deoxy-7-deazaadenosine) were collected, the solvent evaporated, and the residue was crystallized from EtOH-EtOAc to give 150 mg (0.53 mmol, 88%). This compound (133 mg) was dissolved in 2 ml of pyridine and 1.1 eq. of dimethoxytrityl chloride was added under stirring at room temperature. After 1 hr, MeOH (1 ml) was added and the solvent was removed in vacuo, and the residue was taken up in $\text{CHCl}_3\text{-H}_2\text{O}$. The organic layer was separated and passed through a silica gel column (Kieselgel H, Typ 60, short column method⁹). The residue from the eluate was suspended in ether-hexane and the product (3) was collected by centrifugation to give 278 mg of 3 (0.42 mmol, 80%). mp 100-101° C. Anal. Calcd for $\text{C}_{39}\text{H}_{36}\text{N}_4\text{O}_6$: C, 71.33; H, 5.52; N, 8.53. Found: C, 71.58; H, 5.53; N, 8.57.

Triethylammonium salt of 5'-O-Dimethoxytrityl-2'-deoxy-N⁶-benzoyl-7-deazaadenosine 3'-(o-chlorophenyl phosphate) (4)

Compound 3 (150 mg, 0.23 mmol) was dehydrated by distillation with anhydrous pyridine twice and the residue was dissolved in 2 ml of dioxane. A solution of o-chlorophenylphosphoroditriazolidine in dioxane (0.69 mmol, in 4 ml) was added under cooling and the solution was concentrated to a volume of 3 ml. After stirring the solution at room temperature for 1 hr, 1 ml of 10% pyridine- H_2O was added in an ice bath. The solvent was removed and the residue was taken up in 30 ml of CHCl_3 , which was washed

three times with 0.1 M triethylammonium bicarbonate (10 ml each). The organic layer was separated, dried over MgSO_4 , the solvent was concentrated, and the residue was suspended in ether-hexane. Centrifugation of the suspension gave the product 4 (176 mg, 0.19 mmol, 83%).

Deoxyoligonucleotides containing 7-deazaadenine

Compound 4 was converted to the dimer blocks, $\text{d}(\text{DMTr})\text{N}^{\text{bz}}\text{pG}^{\text{ib}}\text{p}$ and $\text{d}(\text{DMTr})\text{N}^{\text{bz}}\text{pTp}$, and following deoxydecanucleotides were prepared by the solid phase phosphotriester method of Itakura et al.¹⁰: $\text{d}(\text{GGAGNTCTCC})$, $\text{d}(\text{GGNGATCTCC})$, $\text{d}(\text{CCAGNTCTCC})$, and $\text{d}(\text{CCNGATCTCC})$, where N denotes 7-deazaadenine. The following oligomers of natural sequences have also been prepared by the same procedure: $\text{d}(\text{GGAGATCTCC})$, $\text{d}(\text{GGAGATCTGG})$, $\text{d}(\text{CCAGATCTCC})$, $\text{d}(\text{CCGATCGG})$, and $\text{d}(\text{AGACCGGTCT})$. These protected oligomers on the polymer supports were released from the resin and deprotected with tetramethylguanidinium pyridine-2-aldoximate¹¹ followed by treatment with ammonia and then with 80% acetic acid. The decamers were purified through anion exchange column chromatography followed by reversed phase HPLC to the purity of over 99%, respectively. The sequences of these nucleotides were confirmed by the 5'-phosphate labeling and successive partial digestion with nuclease P1 and the mobility shift analysis¹² of the respective digests. A typical example of the scintigram is shown for $\text{d}(\text{GGAGATCTCC})$ and $\text{d}(\text{GGAGNTCTCC})$ in Fig. 1.

Enzymes

Polynucleotide kinase, Bgl II, and Sau 3AI were purchased from Takara Shuzo Co. Ltd.

5'-Labeling of nucleotides

The oligonucleotide (1 nmole) to be labeled was incubated with γ - ^{32}P -ATP and polynucleotide kinase (1 unit) in 50 mM Tris-HCl (pH 9.6), 10 mM MgCl_2 , 10 mM DTT, 2 mM spermine, and 100 mM KCl in a total volume of 5 μl at 37° for 1 hr. The product was isolated by a gel filtration on a column (0.7x37 cm) of Sephadex G-50 (fine) equilibrated with 100 mM triethylammonium bicarbonate (pH 7.5).

Digestion of oligonucleotides with Bgl II or Sau 3AI

Method A) ^{32}P -Labeled oligonucleotide (10000 to 30000 cpm) was incubated at 20°C for 48 hr in 10 mM Tris-HCl (pH 7.5), 7 mM

Fig.1 The scintigram for d(GGAGATCTCC) and (GGAGNTCTCC)

MgCl₂, 60 mM NaCl, and 7 mM 2-mercaptoethanol with 1.0 OD unit of the same unlabeled oligonucleotide as a carrier with 15 units of Bgl II in a total volume of 10 μ l.

Method B) A ³²P-labeled oligonucleotide (0.005 OD unit) and 0.20 OD unit of appropriate oligodeoxynucleotide were incubated at 20° C for 24 hr in 10 mM Tris-HCl (pH 7.5), 7 mM MgCl₂, 100 mM NaCl, and 7 mM 2-mercaptoethanol in a total volume of 20 μ l.

Method C) A ³²P labeled oligonucleotide diluted with 1 OD unit of the same unlabeled one was incubated at 20° for 48 hr in 10 mM Tris-HCl (pH 7.5), 7 mM MgCl₂, 100 mM NaCl with 25 units of Sau 3AI in a total volume of 10 μ l.

All digests were developed on homochromatography and the spots having radioactivities were excised and counted with toluene scintillater on a Beckman LS-230 liquid scintillation counter.

Melting temperature determination

Melting temperature was measured by a Shimadzu UV-240 spectrophotometer equipped with a temperature controller (140/SPR-5, Shimadzu) and a thermometer (Model 2542, Yokokawa electric Co.)

CD spectra measurements

The CD spectra of decamers were measured on a JASCO J-40

Table I. Melting Temperatures of Selected Oligonucleotide Duplexes.

Nucleotide	T _m (°C)
GGAGATCTCC CCTCTCGAGG	39
GGAGNTCTCC CCTCTNGAGG	35
GGNGATCTCC CCTCTAGNGG	36
CCAGNTCTCC GGGTCTAGAGGG	43
CCGATCGG GGCTAGCC	39

T_m's were measured in a buffer of 0.01 M sodium cacodylate containing 0.1 M NaCl at pH 7.5.

spectropolarimeter in 0.1 M NaCl containing 0.01 M sodium cacodylate (pH 7.5) at 18°C.

High performance liquid chromatography (HPLC)

HPLC was performed by a Liquid chromatograph LC-3A (Shimadzu) and Chromatopac C-R1A (Shimadzu). The column used was TSK-GEL LS 410AK (Toyo Soda).

RESULTS AND DISCUSSIONS

The oligodeoxynucleotides containing 7-deazaadenine showed reasonable melting temperatures as expected for the usual decanucleotides (Table I). Therefore the digestion experiment was performed at 20°C in order to maintain the duplex structure of the respective decamers in the digestion experiments. The CD spectra of the decamers containing 7-deazaadenine also showed the regular CD patterns of the B-type of DNA. For example, the CD spectra of d(GGAGNTCTCC) is shown in Fig. 2 along with its natural sequence nucleotide.

The rates of hydrolysis of the oligomers by Bgl II digestion are summarized in Table II. After 48 hr incubation in the conditions of digestion (Method A) the natural oligomer d(pGGAGATCTCC) was completely hydrolyzed to afford ³²pGGA (Table 2, row 1). However, the duplexes containing 7-deazaadenine in positions 5 or

Fig.2 The CD spectra of d(GGAGATCTCC) and d(GGAGNTCTCC)

3 from the 5'-end in both strands were strongly resistant (Table 2, rows 2 and 3). The oligonucleotide duplexes containing 7-deazaadenine in one strand were prepared and ^{32}P -labeling was also performed in one strand. The results of Bgl II digestions with these oligomers are shown in Table II, rows 4 to 7 for substitution at the middle adenines and Table II, rows 8 to 11 for substitution at the 5'-side adenines. It is clear that the substitution of single adenine moiety to 7-deazaadenine greatly reduced the extent of the hydrolysis of either strands. In addition, it should be emphasized that, especially in the case of replacement of the adenine at the 5'-end sides, the rates of hydrolysis of the natural sequences are slower as compared to those of the modified strands (Table II, rows 8 versus 9, and 10 versus 11).

It is conceivable that the modification of the base part of the nucleotide sequences may cause either the loss (or decrease) of the binding of enzymes to the specific sequences, and/or the inability of cleavage of the phosphodiester linkage due to alteration of the specific base moiety. To clarify this point we carried out the "inhibition experiment" by adding a large excess of unlabeled substrates or other nucleotides of different sequence (Method B). The results are shown in Table III. In the conditions where the labeled decamer was cleaved in 68% (row 2),

Table II. Cleavage of Deoxyoligonucleotides Containing 7-Deazaadenine by Restriction Endonuclease Bgl II.

Row	Nucleotides	Rate of Hydrolysis (%)
1	pG G A G A T C T C C C C T C T A G A G Gp	100
2	pG G A G N T C T C C C C T C T N G A G Gp	0
3	pG G N G A T C T C C C C T C T A G N G Gp	2
4	pC C A G N T C T C C G G T C T A G A G G	3
5	C C A G N T C T C C G G T C T A G A G Gp	4
6	pC C A G N T C T C C G G G T C T A G A G G G	4
7	C C A G N T C T C C G G G T C T C G A G G Gp	2
8	pC C N G A T C T C C G G T C T A G A G Gp	5
9	C C N G A T C T C C G G T C T A G A G Gp	1
10	pC C N G A T C T C C G G G T C T A G A G G G	4
11	C C N G A T C T C C G G G T C T A G A G G Gp	0

The conditions for digestion are described in Method A. N denotes for 7-deazaadenine and p denotes for labeled phosphate.

addition of 0.2 OD unit of unlabeled decamer showed decrease of the rate to 2% (row 1), which is quite reasonable by simply accounting the dilution effect of the substrate. On the other hand, addition of the non-substrates (rows 3 and 4) did not affect the rate of cleavage, showing that these nucleotides did not bind to the enzyme. However, addition of the decamers having the same sequences except one substitution of the adenine to the 7-deazaadenine greatly reduced the hydrolysis rates (rows 5 and 6). This may be explained by the competitive binding of the modified substrates to the enzyme. Due to a substitution of the 7-

Table III. "Inhibition Experiment" of Bgl II Digestion.

Row	Nucleotides	OD Unit	Counts* (c/r, cpm)	Hydrolysis (%)
1	GGAGATCTCC CCTCTAGAGG	0.2		
	pGGAGATCTCC CCTCTAGAGGp	0.005	6/385	2
2	pGGAGATCTCC CCTCTAGAGGp	0.005	310/143	68
3	CCGATCGG GGCTAGCC	0.2		
	pGGAGATCTCC CCTCTAGAGGp	0.005	144/67	68
4	AGACCGGTCT TCTGGCCAGA	0.2		
	pGGAGATCTCC CCTCTAGAGGp	0.005	184/142	56
5	GGAGNTCTCC CCTCTNGAGG	0.2		
	pGGAGATCTCC CCTCTAGAGGp	0.005	22/256	8
6	GGNGATCTCC CCTCTAGNGG	0.2		
	pGGAGATCTCC CCTCTAGAGGp	0.005	46/225	17

The conditions for the digestion are described in Method B.

*The counts determined for c (cleaved) and r (remained intact).

azomethine part of either adenines to the methine group in the modified substrates, the enzyme cannot cleave the specific phosphodiester bond to release the hydrolyzates. It seems that, for the binding of Bgl II to the AGATCT sequence, the adenine at the 5'-end is more crucial which would reflect in the difference of the inhibition rates shown in the rows 5 and 6 of Table III. It is of interest to recall that the position of the cleavage of the phosphoester linkage is at the 3'-side of the 5'-end adenine nucleotide of the recognition sequence.

As described so far, the substitution of only one 7-azomethine function out of four adenines in the recognition duplexes

Table IV. Cleavage of Deoxyoligonucleotides Containing 7-Deazaadenine by Restriction Endonuclease Sau 3AI.

Row	Nucleotides	Counts (c/r, cpm)*	Rate of Hydrolysis (%)
1	p G G A G A T C T C C C C T C T A G A G G p	16000/0	100
2	p G G A G N T C T C C C C T C T N G A G G p	6000/9000	40
3	p G G N G A T C T C C C C T C T A G N G G p	15000/0	100
4	p C C A G N T C T C C G G T C T A G A G G p	5200/5100	50
5	C C A G N T C T C C G G T C T A G A G G p	22000/0	100
6	p C C N G A T C T C C G G T C T A G A G G	26000/800	97
7	C C N G A T C T C C G G T C T A G A G G p	19000/600	97

The conditions for the digestion are described in Method C.

*The counts for c (cleaved) and r (remained intact).

containing AGATCT resulted in a drastic reduction of the rate of hydrolysis by Bgl II, and in that case the cleavage (although it is very slow) occurred in the modified sequence rather than in the natural sequence.

To explain this interesting results we present following assumption. Since the 7-azomethine moiety of the adenine residues is located in the major groove of the DNA helix, it is clear that the restriction enzymes attach to the major groove. It has been reported¹³ that Bgl II can form the dimer, and the dimer is an active species in the endonuclease reaction³. The assumption, that each enzyme recognizes and cleaves the hexanucleotide sequence (AGATCT) in each strand, does not satisfy the present results. The alternate assumption that each enzyme recognizes the trimer duplex AGA:TCT and cleaves the phosphoester bond is also not suitable to account for the results.

Closer investigation of the tertiary structure of B-DNA helix model shows that, although the phosphorus of phosphodiester

group to be cleaved by Bgl II is located at the end of the hexanucleotide primary sequence, it does occupy the location with roughly equal distances from each six nucleobases of the other strand. Therefore, we present a new assumption that each enzyme binds to the hexanucleotide sequence of one strand of the duplex and cleaves the phosphoester of the other strand. This assumption would explain the results shown in Table II, rows 8,9 and 10,11. However, this does not fully explain the striking reduction of the rate of cleavage of specific sequence by only one atom alteration. Therefore it is conceivable that the deletion of one hydrogen bond acceptor at the N⁷ of the adenine moiety may alter the mode of binding in one enzyme which will affect the binding mode of the second enzyme to the other strand, resulting in the great decrease of extent of the cleavage of phosphoester linkage.

Further studies on the mode of recognition are necessary with other restriction endonucleases recognizing six-base sequences to confirm this type of enzyme action.

After completion of our work Bodnar and co-workers have reported¹⁴ the effect of substitution of nucleobases including 7-deazaadenine in the DNA to the cleavage of several endonucleases recognizing four to five nucleotide sequences, showing variable results with the enzymes.

While the 7-azomethine function of adenine moieties in the recognition sequence is important in the step of cleavage by Bgl II, this is not prerequisite in the case of Sau 3AI cleavage as shown in Table IV. The replacement of the adenine in the recognition sequence reduced the rate to about one half (rows 2 and 4) but the natural sequence pairing to the modified one was cleaved as well (row 5). The alteration of the adenine moiety outside of the recognition sequence had no effect at all (rows 3, 6 and 7). Therefore it may be concluded that the mode of binding and cleavage of Sau 3AI is completely different to those of the enzymes recognizing six-base sequence. Thus, Sau 3AI binds to the nucleotide sequence d(GATC) and cleaves the same strand.

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