Synthesis of decadeoxyribonucleotides containing 5-modified uracils and their interactions with restriction endonucleases *Bgl* II, *Sau* 3AI and *Mbo* I (Nucleosides and Nucleotides 82¹)

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Abstract

Decadeoxyribonucleotides containing uracil, 5-bromouracil, 5-cyanouracil and 5-ethyluracil in recognition sequences of restriction endonucleases *Bgl* II, *Sau* 3AI, *Mbo* I were synthesized. Decanucleotides containing 5-bromouracil in place of thymine had essentially the same susceptibility to all the restriction endonucleases. Uracil-containing decanucleotides were however very resistant to attack. Decanucleotides containing 5-cyanouracil in the recognition sequence were strongly resistant to hydrolysis by *Sau* 3AI, but were hydrolysed by *Bgl* II and *Mbo* I as well as the parent decanucleotide. Decanucleotides containing 5-ethyluracil were strongly resistant to hydrolysis by *Sau* 3AI, but were partially resistant to hydrolysis by *Bgl* II and *Mbo* I.

Introduction

How restriction endonucleases recognize their specific sequence is a subject of great interest. To elucidate this problem, DNAs and oligonucleotides containing modified bases have been prepared by biochemical and chemical means, and have been used to characterize the mode of action of some restriction enzymes.²

We have previously reported that decadeoxyribonucleotides containing 7-deazaadenine in place of adenine in the recognition sequence of *Bgl* II and *Sau* 3AI were highly resistant to hydrolysis by *Bgl* II, and partially resistant to hydrolysis by *Sau* 3AI³. Furthermore we have reported that a decanucleotide containing 3-deazaadenine was also highly resistant to hydrolysis by *Bgl* II⁴, and an oligonucleotide containing N⁶-methyladenine in place of adenine was resistant to hydrolysis by the restriction endonuclease *Mbo* I⁵.

These enzymes interact with the adenine function in an A:T pair in the recognition sequence, however whether these enzymes

also interact with and recognize the thymine function is not known. In this paper, in order to elucidate this point, we synthesized decanucleotides containing 5-modified uracils and investigate the interactions of these oligonucleotides with Bgl II (AGATCT)⁶, Sau 3AI (GATC)⁷, and Mbo I (GATC)⁸. In Fig. 1, structures of the 5-modified uracil moieties and sequences of the decanucleotides containing 5-modified uracils used in this study are listed.

Materials and Methods

Enzymes

T4 Polynucleotide kinase (E. coli B), and restriction endonucleases Bgl II and Sau 3AI, were purchased from Takara Shuzou Co., Ltd. Mbo I was purchased from Pharmacia P-L Biochemical Inc.

2'-Deoxynucleosides

2'-Deoxynucleosides for oligonucleotide synthesis were purchased from Yamasa Shoyu Co., Ltd.

Instrumental analysis

Melting points were determined on a Yanaco MP-3 micromelting point apparatus and are uncorrected. UV spectra were taken with a Shimadzu UV-260 spectrophotometer. ¹H-NMR spectra were recorded on a JEOL JNM-FX 100FT or 270FT spectrometer with tetramethylsilane as an internal standard. Chemical shifts are expressed in ppm (δ), and signals are described as s (singlet), d (doublet), dd (double doublet), t (triplet), q (quartet), m (multiplet), or br (broad).

Triethylammonium 5'-O-dimethoxytrityl-2'-deoxyuridine 3'-(o-chlorophenyl phosphate) (1) (Fig. 2)

2'-Deoxyuridine (2, 1.0g, 4.4mmol) was dried by distillation twice with anhydrous pyridine and the residue was dissolved in pyridine (10ml). Dimethoxytrityl chloride (DMTrCl, 1.6g, 1.05eq) was added to the solution and the whole was stirred at room temperature for 1 hr. MeOH (1ml) was added and the solution was concentrated in vacuo. The residue was dissolved in CHCl₃, and washed with 5%NaHCO₃ and water. The organic layer was concentrated in vacuo, and the residue was applied to a column of silica gel (20g). The column was eluted with CHCl₃-MeOH (50:1 to 20:1), the eluate concentrated to a small volume, and the concentrate

Figure 1. 5-Modified uracils and decanucleotides containing 5-modified uracils.

was added dropwise to a mixture of ether-hexane (1:1). The precipitate was collected by centrifugation to give 1.6g (3.0mmol, 68%) of 5'-O-dimethoxytrityl-2'-deoxyuridine (3). ¹H-NMR (CDCl₃): 10.93 (1H, br, H-N³), 7.93 (1H, d, H-6), 7.38-6.76 (13H, m, dimethoxytrityl), 6.30 (1H, t, H-1'), 5.61 (1H, d, H-5), 4.39 (1H, m, H-3'), 3.93 (1H, m, H-4'), 3.78 (6H, s, methoxy groups of DMTr), 3.76-3.68 (2H, m, H-5'), 2.33-2.07 (2H, m, H-2').

Figure 2. Modified nucleotide blocks.

Compound 3 (1.0g, 1.9mmol) was dried by distillation twice with anhydrous pyridine and the residue was dissolved in dioxane (5ml). A 0.2 M solution of *o*-chlorophenylphosphoroditriazolidine⁹ (in dioxane, 2eq) was added to the solution under cooling in an ice-bath, the solvent concentrated to 10ml, and the mixture was kept at room temperature for 3 hr. 90% Pyridine-water (5ml) was added to the solution under cooling in an ice bath and the solvent was concentrated in vacuo. The residue was dissolved in CHCl_3 , and the solution was washed with 0.1M triethylammonium bicarbonate buffer (TEAB buffer) and with water. The organic layer was separated, passed through a Whatman 1PS filter paper, and the filtrate was concentrated in vacuo. The residue was triturated in ether-hexane to give the title compound (1, 1.2g, 1.4mmol, 77%). ¹H-NMR (CDCl_3): 11.77 (1H, br, onium proton of triethylammonium), 8.63 (1H, br, H-N³), 7.84 (1H, d, H-6), 7.55-6.78 (17H, m, DMTr and *o*-chlorophenyl), 6.18 (1H, t, H-1'), 5.68 (1H, d, H-5), 5.06 (1H, m, H-3'), 4.12 (1H, m, H-4'), 3.93-3.76 (8H, m, H-5' and methoxy groups of DMTr), 3.04 (6H, m, methylene groups of triethylammonium), 2.50 (1H, m, H-2'), 2.42 (1H, m, H-2'), 1.29 (9H, t, methyl groups of triethylammonium).

Triethylammonium N³-anisoyl-5-bromo-5'-O-dimethoxytrityl-2'-deoxyuridine 3'-(*o*-chlorophenyl phosphate) (4) (Fig. 2)

5-Bromo-2'-deoxyuridine (5, 3.0g, 9.8mmol) was dried by distillation twice with anhydrous pyridine and the residue was dissolved in pyridine (30ml). DMTrCl (3.5g, 1.1eq) was added to the solution and the whole was stirred at room temperature for 1.5hr. MeOH (5ml) was added to the solution and the solution was concentrated in vacuo. The residue was taken up in CHCl_3 , and washed with 5%NaHCO₃ and water. The organic layer was concentrated in vacuo and the residue was applied to a column of silica gel (100g). The product was eluted with CHCl_3 -MeOH (50:1) and the eluate was concentrated in vacuo. The residue was taken up in benzene and the separated crystals were collected to give 5.2g of a yellow solid. These crystals were recrystallized from benzene to give 4.5g (5.4mmol, 54%) of 5'-O-dimethoxytrityl-5-bromo-2'-deoxyuridine (6) co-crystallized with benzene (1:3), mp 110.5-113.5°C. Anal. Calcd for $\text{C}_{30}\text{H}_{29}\text{O}_7\text{N}_2\text{Br} \cdot 3\text{C}_6\text{H}_6$ (843.77): C, 68.33; H, 5.61; N, 3.32; Br, 9.47. Found: C, 68.31; H, 5.61; N, 3.32; Br,

9.64. $^1\text{H-NMR}$ (DMSO-d_6): 8.08 (1H, s, H-6), 7.41-6.85 (31H, m, protons of dimethoxytrityl group and benzene), 6.31 (1H, t, H-1'), 4.60 (1H, m, H-3'), 4.10 (1H, m, H-4'), 3.80 (6H, s, methoxy groups of DMTr), 3.15 (2H, d, H-5'), 2.50 (2H, m, H-2').

Compound 6 (2.0g, 2.4mmol) was converted to 5-bromo-5'-O-dimethoxytrityl-2'-deoxyuridine 3'-(o-chlorophenyl phosphate) (7, 2.3g, 2.5mmol, 100%) by the procedure described previously.

Compound 7 (60mg, 0.067mmol) was dried by distillation twice with anhydrous pyridine and the residue was dissolved in pyridine (2ml). Anisoyl chloride (114mg, 10eq) was added to the solution and the whole was stirred at room temperature for 6 hr. The mixture was concentrated in vacuo, the residue was dissolved in CHCl_3 , and washed with 0.1M TEAB buffer and with water. The organic layer was separated through a Whatman 1PS filter paper, then the filtrate was concentrated in vacuo. The residue was triturated in ether-hexane (1:1) and the precipitate was collected by centrifugation. The residue was retrituated and centrifuged three times and the title compound (4) was obtained as a syrup (60mg, 0.058mmol, 87%). $^1\text{H-NMR}$ (DMSO-d_6): 9.37 (1H, br, onium proton of triethylammonium), 8.16-7.98 (3H, m, H-6 and o-protons of anisoyl), 7.55-6.90 (19H, m, DMTr and o-chlorophenyl and m-protons of anisoyl), 6.10 (1H, m, H-1'), 4.91 (1H, m, H-3'), 4.21 (1H, m, H-4'), 3.89 (3H, s, methoxy group of anisoyl), 3.73 (6H, s, methoxy groups of DMTr), 3.60-3.00 (10H, m, methylene groups of triethylammonium and H-5'), 2.60-2.15 (2H, m, H-2'), 1.15 (9H, t, methyl groups of triethylammonium).

Triethylammonium N³-anisoyl-5-cyano-5'-O-dimethoxytrityl-2'-deoxyuridine 3'-(o-chlorophenyl phosphate)(8) (Fig. 2)

5-Cyano-2'-deoxyuridine (9) was prepared by the reported method.¹⁰ Compound 9 (0.75g, 3.0mmol) was dried by distillation twice with anhydrous pyridine and the residue was dissolved in pyridine (10ml). DMTrCl (1.1g, 1.1eq) was added and the solution was stirred at room temperature for 2hr. MeOH (2ml) was added and the solution was concentrated in vacuo. The residue was taken up in CHCl_3 , and washed with 5% NaHCO_3 and water. The organic layer was concentrated in vacuo, the residue dissolved in toluene and the solution was concentrated. The residue was applied to a column of silica gel (22g). The product was eluted with CHCl_3 -

MeOH (20:1) and concentrated to a small volume which was added dropwise to a mixture of ether-hexane (2:1). The precipitate was collected by centrifugation to give 1.0g (1.8mmol, 62%) of 5-cyano-5'-O-dimethoxytrityl-2'-deoxyuridine (10). $^1\text{H-NMR}$ (CDCl_3): 8.30 (1H, s, H-6), 7.40-6.85 (13H, m, DMTr), 6.16 (1H, t, H-1'), 4.60 (1H, m, H-3'), 4.19 (1H, m, H-4'), 3.77 (6H, s, methoxy groups of DMTr), 3.47 (2H, m, H-5'), 2.82-2.20 (2H, m, H-2').

Compound 10 (0.50g, 0.90mmol) was dried by distillation twice with anhydrous pyridine and the residue was dissolved in dioxane (5ml). o-Chlorophenylphosphoroditriazolidide (0.2M in dioxane, 6.3ml, 1.3mmol) was added and the solution was stirred at room temperature for 45 min. Then 90% pyridine-water (2.5ml) was added under cooling, and the solution was stirred at room temperature for 10 min. The solution was then concentrated in vacuo, the residue taken up in CHCl_3 and washed with 0.1 M triethylammonium bicarbonate buffer and water. The organic layer was dehydrated by passing it through a Whatman 1-PS filter paper and concentrated to a small volume. The residue was added dropwise to a mixture of ether-hexane (1:2). The precipitate was collected by centrifugation to give 0.69g (0.81mmol, 90%) of triethylammonium 5-cyano-5'-O-dimethoxytrityl-2'-deoxyuridine 3'-(o-chlorophenyl phosphate) (11). $^1\text{H-NMR}$ (DMSO-d_6): 12.08 (1H, br, onium proton of triethylammonium), 9.45 (1H, br, H-N³), 8.40 (1H, s, H-6), 7.54-6.84 (17H, m, DMTr and o-chlorophenyl), 6.04 (1H, dd, H-1'), 4.78 (1H, m, H-3'), 4.19 (1H, m, H-4'), 3.73 (6H, s, methoxy groups of DMTr), 3.57 (2H, m, H-5'), 3.06 (6H, q, methylene groups of triethylammonium), 2.50-2.37 (2H, m, H-2'), 1.16 (9H, t, methyl groups of triethylammonium).

To a solution of 11 (300mg, 0.36mmol) in pyridine (5ml), anisoyl chloride (612mg, 10eq) was added and the solution was stirred at room temperature for 6hr. The solution was then concentrated to a small volume, the residue taken up in CHCl_3 , washed with 0.1 M triethylammonium bicarbonate, and the organic layer was concentrated in vacuo. The residue was dissolved in a small portion of pyridine and the solution was added dropwise to ether (150ml). The precipitate was collected by centrifugation to give 335mg (0.34mmol, 94%) of 8. $^1\text{H-NMR}$ (DMSO-d_6): 9.13 (1H, br, onium proton of triethylammonium), 8.08-6.85 (21H, m, anisoyl, o-chlo-

rophenyl, and DMTr), 6.06 (1H, t, H-1'), 4.83 (1H, m, H-3'), 4.25 (1H, m, H-4'), 3.89 (3H, s, methoxy group of anisoyl), 3.73 (6H, s, methoxy group of DMTr), 3.38-3.05 (8H, q, methylene group of triethylammonium and H-5'), 2.50 (2H, m, H-2'), 1.16 (9H, t, methyl groups of of triethylammonium).

Triethylammonium 5'-O-dimethoxytrityl-5-ethyl-2'-deoxyuridine 3'-(o-chlorophenyl phosphate) (12) (Fig. 2)

5-Ethyl-2'-deoxyuridine (13) was prepared by the reported method.¹¹ Compound 13 (200mg, 0.77mmol) was dried by distillation twice with anhydrous pyridine and the residue was dissolved in pyridine (5ml). DMTrCl (260mg, 1.1eq) was added and the solution was stirred at room temperature for 2.5hr. Then MeOH (5ml) was added and the solution was concentrated in vacuo. The residue was taken up in CHCl₃, washed with 5%NaHCO₃ and water, and the organic layer was passed through a Whatman 1PS filter paper, then concentrated to a small volume. The residue was dissolved in toluene and again concentrated. The residue was then applied to a silica gel column (20g), and the product was eluted with CHCl₃-MeOH (30:1). The eluate was concentrated to a small volume which was added dropwise to hexane. The precipitate was collected by centrifugation to give 340mg (0.60mmol, 78%) of 5'-O-dimethoxytrityl-5-ethyl-2'-deoxyuridine (14). ¹H-NMR (CDCl₃): 8.18 (1H, br, H-N³), 7.66 (1H, s, H-6), 7.42-6.81 (13H, m, DMTr), 6.38 (1H, dd, H-1'), 4.55 (1H, m, H-3'), 4.04 (1H, m, H-4'), 3.79 (6H, d, methoxy groups of DMTr), 3.48-3.36 (2H, m, H-5'), 2.52-2.29 (3H, m, H-2'a and methylene group of 5-ethyl), 2.00-1.92 (2H, m, H-2'b and 3'-OH), 0.86 (3H, t, methyl group of 5-ethyl).

Compound 14 (260mg, 0.46mmol) was dried by distillation twice with anhydrous pyridine and the residue was dissolved in dioxane (3ml). o-Chlorophenylphosphoroditriazolidine (0.2M dioxane solution, 1.4eq) was added and the solution was concentrated to 2ml, and stirred at room temperature for 2hr. Then 90% pyridine-water (3ml) was added under cooling on an ice bath, and the whole was stirred at room temperature for 10 min. The solution was then concentrated and the residue was taken up in CHCl₃, washed with 0.1M triethylammonium bicarbonate and water, and the organic layer was concentrated to a small volume. The residue was applied to a column (C-18, Waters 4x5) and the product was eluted with

acetone-water (45:55-55:45). The eluate was concentrated to half its volume, and the product was extracted with CHCl_3 . The organic layer was concentrated to a small volume which was added dropwise to hexane and the precipitate was collected by centrifugation to give 300mg (0.35mmol, 76%) of **12**. $^1\text{H-NMR}$ (CDCl_3): 11.81 (1H, br, onium proton of triethylammonium), 8.40 (1H, br, H-N^3), 7.52 (1H, s, H-6), 7.58-6.78 (17H, m, DMTr and o-chlorophenyl), 6.18 (1H, t, H-6), 5.05 (1H, m, H-3'), 4.10 (1H, m, H-4'), 3.94-3.74 (8H, m, H-5' and methoxy groups of DMTr), 3.02 (6H, m, methylene groups of triethylammonium), 2.51-2.22 (4H, m, H-2' and methylene group of 5-ethyl), 1.29 (9H, t, methyl groups of triethylammonium), 1.11 (3H, t, methyl group of 5-ethyl).

Synthesis of decadeoxyribonucleotides

The decanucleotides listed in Table 1 were prepared by the solid phase phosphotriester method¹². The protected oligomers on the polymer support were released from the resin and deprotected with conc. NH_4OH at room temperature for 15 hr then heated successively at 50°C for 5 hr, then with 80% acetic acid at room temperature for 15 min. All the decamers were purified by anion exchange column chromatography followed by reversed phase HPLC to a purity of over 99%.

The sequence of purified materials was confirmed by 5'-phosphate labelling followed by partial digestion with snake venom phosphodiesterase¹³ and mobility-shift-analysis¹⁴ of the respective partial hydrolyzates. Some typical examples of the autoradiograms are shown in Fig. 3.

Measurement of CD spectra and melting temperatures

The CD spectra of the decanucleotides were measured on a JASCO J-500A spectropolarimeter in 0.1M NaCl, 0.01M sodium cacodylate (pH 7.5) at 18°C . 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.) in same buffer.

5'-Labelling of decanucleotides

The oligonucleotides (1 nmol) to be labelled were incubated at 37°C for 1 hr with $\gamma\text{-}^{32}\text{P-ATP}$ 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 15 μl . The product

Figure 3. Mobility shift analysis of decanucleotides.

was adsorbed on to Whatman DE 50 (bicarbonate form) and washed with 0.4 M triethylammonium bicarbonate, eluted with 2M triethylammonium bicarbonate and the eluate was concentrated *in vacuo* to leave the product.

Digestion of decanucleotides

Method A) The 5'-³²P-labelled oligonucleotide (50000cpm) and cold oligonucleotide (final concentrations, 0.25μM-5.0μM) were incubated with restriction endonucleases (units for each enzyme were: 18units for Bgl II, 20 units for Sau 3AI and 20units for Mbo I) in an appropriate buffer (10mM Tris-HCl, pH7.5, 7mM MgCl₂, 100mM NaCl, 7mM 2-mercaptoethanol for Bgl II, 10mM Tris-HCl, pH7.5, 7mM MgCl₂, 100mM NaCl for Sau 3AI and 50mM Tris-HCl, pH8.0, 10mM MgCl₂, 50mM NaCl for Mbo I) at 20°C in a total volume of 20ul. An aliquot of the reaction mixture was removed and adsorbed on to a DEAE cellulose plate (Polygram Cel 300 Macherey-

Nagel) at appropriate times (0.5min-60min) after incubation to determine the initial velocities.

Method B) 5'-³²P-labelled GGAGATCTCC (50000cpm) together with the unlabelled decamer (final concentrations of 0.6μM, 0.8μM, 1.25μM, 2.0μM, and 4.0μM) were incubated with Bgl II (18-units) in the presence of GGAGAUCTCC in a concentration of 0.2μM, 0.5μM or 1.0μM, under the same conditions as described in Method A. An aliquot of incubation mixture was removed and adsorbed on to a DEAE cellulose plate at 0.5, 1.0, 1.5, 2.0 and 3.0 min after incubation.

Method C) 5'-³²P-labelled GGAGATCTCC (50000cpm) together with the unlabelled decamer (final concentrations of 0.5μM, 0.7μM, 1.0μM, 1.25μM, or 2.0μM) were incubated with Sau 3AI (20-units) in the presence of the decamer containing uracil or in the presence of decamer containing 5-cyanouracil or 5-ethyluracil in concentrations of 0.5μM, 1.0μM, or 2.0μM under the same conditions as described in Method A. An aliquot of the incubation mixture was removed and adsorbed onto a DEAE cellulose plate.

All digests in the above methods were developed by homochromatography¹⁵ and the radioactive spots were excised and counted with a toluene based scintillator on a Beckman LS-230 liquid scintillation counter.

K_m, V_{max} and K_i values were determined by Lineweaver-Burk plots.

Results and Discussion

Protection of the N³-position in 5-substituted uracils

The protection at the N³-position of 5-substituted uracils¹⁶ was necessary for oligonucleotide synthesis by the solid-phase phosphotriester method, especially when the 5-position of uracil was substituted by electron-attracting groups such as Br or CN, because the 4-carbonyl function of 5-bromouracil and 5-cyanouracil are easily phosphorylated by treatment of the nucleotide blocks containing such bases with MSNT in pyridine (data not shown). When the normally protected nucleotide blocks of uracil derivatives were treated with anisoyl chloride in pyridine, the anisoyl group was easily introduced into the N³ position of the

Table 1. Melting temperatures of synthetic decanucleotides.

T _m (°C)		T _m (°C)	
d(GGAGATCTCC)	42		
d(GGAGATCUCC)	42	d(GGAGAUCTCC)	40
d(GGAGATCU ^{Br} CC)	43	d(GGAGAU ^{Br} CTCC)	43
d(GGAGATCU ^{CN} CC)	39	d(GGAGAU ^{CN} CTCC)	37
d(GGAGATCU ^{Et} CC)	38	d(GGAGAU ^{Et} CTCC)	38

All samples were dissolved in 0.01M Na cacodylate buffered at pH7.5 containing 0.1M NaCl.

uracil derivatives (Fig. 2). This method is suitable for preparation of N³-protected nucleotide blocks of uracil derivatives.

Structure of the decanucleotides containing 5-modified uracils

Melting temperatures (T_ms) of the self-complementary decanucleotides are shown in Table 1. The T_ms of decanucleotides containing uracil or 5-bromouracil were similar to the parent decamer GGAGATCTCC. Decanucleotides containing 5-cyanouracil or 5-ethyluracil showed rather lower T_ms than that of the parent decamer. The CD spectra of all decamers containing uracil derivatives, like that of parent decamer, showed the usual pattern of a B-type DNA duplex (Fig. 4).

Interactions of decanucleotides with restriction endonucleases

K_m values and relative V_{max} for the reaction with synthetic oligonucleotides and restriction enzymes are shown in Table 2. Decanucleotides containing uracil in place of thymine in the recognition sequence were strongly resistant to hydrolysis by Mbo I, and perfectly resistant to Sau 3AI. Mbo I hydrolysed the decanucleotide containing uracil in position 6 (from the 5' end) with a K_m value which is similar to that for this enzyme with the parent decamer and with a very small relative V_{max} value. The interaction of Bgl II with decanucleotides containing uracil was dependent upon the position of the uracil residue. The decanucleotides containing uracil in position 6 (from the 5' end) was perfectly resistant, and the decanucleotide containing uracil in position 8 (from the 5' end) was strongly resistant to hydrolysis by Bgl II. The decanucleotide containing uracil just outside the recognition sequence was hydrolyzed by Sau 3AI and Mbo I under

All samples were dissolved in 0.01M Na cacodylate buffered at pH 7.5 containing 0.01M NaCl.

Figure 4. CD spectra of synthetic decanucleotides.

same conditions as the parent decamer.

5-Bromouracil introduced in the recognition sequence had no significant effect on hydrolysis by the three enzymes. Like 5-bromouracil, 5-cyanouracil introduced into the recognition sequence had no significant effect upon hydrolysis by Bgl II and Mbo I, but Sau 3AI did not hydrolyze the decamer containing 5-cyanouracil.

The decanucleotide containing 5-ethyluracil in the recognition sequence was completely resistant to hydrolysis by Sau 3AI, and strongly resistant to hydrolysis by Mbo I. Like the effect of uracil, the effect of 5-ethyluracil on Bgl II was dependent upon the position in which 5-ethyluracil was introduced. The decanucleotide containing 5-ethyluracil in position 8 (from the 5'end) was strongly resistant, but the decanucleotide containing

Table 2. Km values and relative Vmax values for the reactions with synthetic oligonucleotides and Bgl II or Sau3AI or Mbo I. The conditions are described in Method A.

	<u>Bgl</u> II (-AGATCT-)		<u>Sau</u> 3AI (-GATC-)		<u>Mbo</u> I (-GATC-)	
	Km(μ M)	rel.Vmax*	Km(μ M)	rel.Vmax	Km(μ M)	rel.Vmax
d(GGAGATCTCC)	2.0	100	54	100	0.38	100
d(GGAGATCUCC)	0.5	0.6	14	35	0.48	200
d(GGAGAUCTCC)	- - *	- -	- -	- -	1.4	3
d(GGAGATCU ^{Br} CC)	5.0	65	50	39	0.25	124
d(GGAGAU ^{Br} CTCC)	2.2	35	25	48	0.19	171
d(GGAGATCU ^{CN} CC)	0.9	34	67	60	0.38	281
d(GGAGAU ^{CN} CTCC)	1.2	44	- -	- -	0.86	152
d(GGAGATCU ^{Et} CC)	1.0	4	30	35	0.20	105
d(GGAGAU ^{Et} CTCC)	0.2	44	- -	- -	1.3	9

* Relative Vmax have been normalized relative to GGAGATCTCC.

**--, no cleavage detectable.

5-ethyluracil in position 6 was partially resistant to hydrolysis by Bgl II.

These results show that the three restriction enzymes are sensitive to substitution at the 5-position of thymine. In Table 3, the steric parameters and the hydrophobic parameters¹⁷ for substituents of uracil derivatives are shown. When compared with the methyl group, the hydrogen atom is very small, and it has a low hydrophobic parameter. These differences in the two parameters are sufficient to effect hydrolysis by the restriction

Table 3. Aromatic substituent constants.

	MR (Steric parameter)	(Hydrophobic parameter)
-CH ₃	5.63	0.56
-H	1.03	0
-Br	8.88	0.86
-CN	6.33	-0.57
-C ₂ H ₅	10.30	1.02

enzymes. Conversely, the bromine atom, which was not so effective in preventing hydrolysis by the restriction enzymes when it is introduced in the recognition sequence, is rather bigger than the methyl group, and has a positive hydrophobic parameter like that of the methyl group. The ethyl group, which has a positive hydrophobic parameter like that of the methyl group or bromine atom, is too large to constitute a normal recognition complex with the restriction enzymes.

When compared with the other substituents, the cyano group has a unique property. The steric parameter of the cyano group is similar to that of the methyl group, but the cyano group has a negative hydrophobicity when compared with the other substituents. This small difference in the steric parameters of the methyl group and the cyano group has no effect on the rate of hydrolysis by the restriction enzymes, as shown by the bromine atom which is larger than the cyano group but is hydrolyzed under the condition as the parent decamer. Thus, Sau 3AI could not hydrolyze the oligonucleotide containing 5-cyanouracil because this enzyme is sensitive to significant differences in the hydrophobicity of the methyl group and the cyano group. In contrast to Sau 3AI, Bgl II and Mbo I are not sensitive to this difference in hydrophobicity. These results show that for these enzymes, at least two recognition mechanisms exist to recognize the methyl group of thymine. To one belongs type Sau 3AI and to the other belongs Bgl II and Mbo I; one system is sensitive to hydrophobicity and the other is not sensitive.

Table 4. KI values of three decanucleotides to Bgl II and Sau3AI. The conditions are described in Method B and C.

Enzyme Inhibitor	KI(μM)	
	<u>Bgl</u> II	<u>Sau</u> 3AI
d(GGAGAUCTCC)	1.0	6.0
d(GGAGAU ^{CN} CTCC)	--	27
d(GGAGAU ^{Et} CTCC)	--	6.5

-- not detected.

This hypothesis is also confirmed by the inhibition experiment (Table 4). All decanucleotides which were completely resistant to hydrolysis show a competitive inhibition mode. Comparing the K_i values of the three decanucleotides to Sau 3AI, the K_i value of the decanucleotide containing 5-cyanouracil was larger than that of the other decanucleotides containing uracil or 5-ethyluracil. This result shows that Sau 3AI is more sensitive to hydrophobicity at the 5-position of thymine than it is to a steric effect at this position.

The K_i values of decanucleotides containing uracil or 5-ethyluracil to Sau 3AI were smaller than the K_m value of the parent decamer. Similarly to Sau 3AI, the K_i value of GGAGAUCTCC to Bgl II was smaller than the K_m value of the parent decamer. These results show that these enzymes are bound to the oligonucleotides containing uracil derivatives having hydrophobic substituents at the 5-position. However, the enzyme cannot hydrolyze these oligomers because this binding is somewhat unusual due to the steric nature of the substituents in the uracil derivatives which are different from the methyl group in thymine.

Previously we have reported that oligonucleotides containing 7-deazaadenine in place of adenine in the recognition sequences of Bgl II and Sau 3AI were completely or partially resistant to hydrolysis by these enzymes.⁴ Oligonucleotides containing 3-deazaadenine were completely resistant to Bgl II and partially resistant to Sau 3AI and Mbo I, and oligonucleotides containing N⁶-methyladenine in the 5'-side of the recognition sequence were resistant to Bgl II.⁵ It was already known from biological experiments that recognition sequences containing N⁶-methyladenine are resistant to the hydrolysis by Mbo I.⁶ From these results and the results reported in this paper, it is suggested that these enzymes recognize A:T pairs by interacting with almost all base functions in the A:T pair, such as the proton acceptor groups of hydrogen bonding (3- and 7- nitrogen of the adenine base), the proton donor group (the amino group at the 6-position in the adenine base), and the hydrophobic group (the 5-position of thymine). This phenomenon is very interesting and we are currently investigating whether other restriction endonucleases have such a complex recognition mode.

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