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# Synthesis of decadeoxyribonucleotides containing $N^6$-methyladenine, $N^4$-methylcytosine, and 5-methylcytosine: recognition and cleavage by restriction endonucleases (nucleosides and nucleotides part 74)[1]

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Abstract

The naturally-occurring modified bases, $N^6$-methyladenine, $N^4$-methylcytosine, and 5-methylcytosine were chemically introduced in place of the adenine or cytosine in the decadeoxyribonucleotides containing recognition sequences of Bgl II, Sau 3AI, Mbo I and Mfl I. The modified oligomers bind to the enzymes but the rates of cleavage by the enzymes are variable.

## Introduction

More than 400 restriction endonucleases have been found and their recogition sites identified. However, studies on the precise mode of recognition and cleavage of these enzymes is meager.[2] DNAs and oligonucleotides containing modified bases have been prepared by biochemical and chemical means, and used to characterize the mode of action of restriction enzymes.[3] 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 resisant to the enzyme Bgl II while partially resistant to Sau 3AI, and a new mode of recognition by Bgl II was presented.[4]

In general, bacteria contain DNA methylases corresponding to their restriction endonucleases. Thus, the DNA methylases protect their own bacterial DNAs from cleavage by their restriction endonucleases. Three types of DNA methylations have been found. One is methylation on the 6-amino function of the adenine moiety, which occurs widely in bacterial cells, and sometimes found in lower eukaryotes. [5] Methylases of the 5-position of the cytosine moiety have been found both in prokaryotic and eukaryotic cells and significant roles for this methylation in eukaryotes have been recognized.[5] A recently-found methylation of the 4-amino

function of the cytosine moiety in some prokaryotes is interesting and its role is so far unknown.[6]

In this paper we report the synthesis of decadeoxyribonucleotides containing these naturally-occurring modified bases, $N^6$-methyladenine, $N^4$-methylcytosine, and 5-methylcytosine, in the recognition sequences of Bgl II (AGATCT)[7], Sau 3AI (GATC)[8], Mbo I (GATC)[9] and Mfl I (PuGATCPy)[10], and their interactions with these enzymes.

## Materials and Methods

### Enzymes

T4 polynucleotide kinase (E. coli B), Bgl II, and Sau 3AI were purchased from Takara Shuzo Co. Ltd. Mbo I was purchased from Pharmacia and Mfl I from Boehringer-Mannheim.

### Synthesis of Methylated Nucleotides

Melting points were determined on a Yanaco MP-3 micromelting point apparatus and are corrected. UV spectra were taken with a Shimadzu UV-260 spectrophotometer. $^1$H-NMR spectra were recorded on a JEOL JNM-FX270FT spectrometer with tetramethylsilane as an internal standard. Chemical shifts are expressed in ppm ($\delta$), and signals are described as s(singlet), d(doublet), dd(double doublet), t(triplet), q(quartet), m(multiplet), or br(broad). Field desorption mass spectra (FD-MS) were taken on a JEOL JMS-01SG-2 mass spectrometer.

### Triethylammonium 5'-O-dimethoxytrityl-2'-deoxy-$N^6$-methyladenosine 3'-(o-chlorophenyl phosphate)(1)[11]

2'-Deoxy-$N^6$-methyladenosine (**2**) was prepared by the reported method.[12] A solution of **2** (100 mg, 0.38 mmol) and dimethoxytrityl chloride (DMTrCl, 135 mg, 1.05 eq.) in pyridine (10 ml) was kept at room temperature for 1 hr. Methanol (1 ml) was added and the solvent was removed in vacuo, the residue was taken up in $CHCl_3$, and the $CHCl_3$ layer was washed with 5% $NaHCO_3$. The organic layer was separated, passed through a Whatman 1PS filter paper, and the filtrate was applied to a column of silica gel (Kieselgel, 10 g). Elution with $CHCl_3$-MeOH (50:1) removed dimethoxytritanol and 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 and n-hexane. The precipitate was collected by centrifugation to give

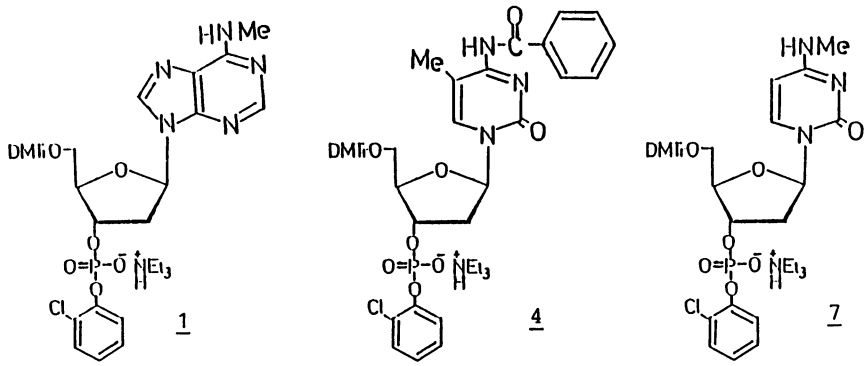


Fig. 1

150 mg (0.26 mmol, 70%) of 5'-O-dimethoxytrityl-2'-deoxy-$N^6$-methyladenosine (**3**), mp 94-96°C. UV in MeOH max 267nm, 235nm. FD-MS m/z: 567($M^+$). $^1$H-NMR($CDCl_3$): 8.33(1H, s, H-8), 7.88(1H, s, H-2), 7.40-6.76(13H, m, dimethoxytrityl group), 6.43(1H, t, H-1'), 5.89(1H, m, H-$N^6$), 4.65(1H, m, H-3'), 4.13(1H, m, H-4'), 3.77(6H, s, methoxy groups of dimethoxytrityl), 3.44(2H, m, H-5'), 3.18(3H, m, $N^6$-Me), 2.85-2.75(1H, m, H-2'a), 2.56-2.47(1H, m, H-2'b). This was used in the next step without further purification.

Compound **3** (50 mg, 0.08 mmol) was dehydrated by distillation twice with anhydrous pyridine and the residue was dissolved in 1 ml of dioxane. A 0.2 M solution of o-chlorophenyl phosphoroditriazolide (1.5 eq)[13] was added to the solution under cooling in an ice-bath and the whole was kept at room temperature for 1 hr. Pyridine-$H_2O$ (1ml) was then added, and the solvent was removed *in vacuo*. The residue was taken up in $CHCl_3$, washed with 0.1 M triethylammonium bicarbonate, and the organic layer was separated through a Whatman 1PS filter paper, then the solvent was removed *in vacuo*. The residue was triturated in ether-n-hexane to give 48 mg (0.06 mmol, 75%) of **1** (Fig. 1). UV in MeOH max 266nm, 235nm. FD-MS m/z: 859($M^+$), 870($M^+$+11). $^1$H-NMR($CDCl_3$): 11.89(1H, br, $Et_3N^+H$), 8.49(1H, s, H-8), 7.96(1H, s, H-2), 7.65-6.80(17H, m, dimethoxytrityl group and o-chlorophenyl group), 6.30-6.25(1H, dd, H-1'), 5.27(1H, m, H-3'), 4.39(1H, m, H-4'), 3.85-3.76(11H, m, H-5', methoxy groups of dimethoxytrityl and

methyl group of $N^6$), 3.10-2.30(8H, m, H-2', and methylene groups of triethylamine), 1.31(9H, t, methyl groups of triethylamine).

Triethylammonium 5'-O-dimethoxytrityl-2'-deoxy-$N^4$-benzoyl-5-methylcytidine 3'-(o-chlorophenyl phosphate) (**4**)

The following procedure for the preparation of 2'-deoxy-5-methylcytidine is similar to the reported method.[14] A mixture of thymidine (2.4 g, 10 mmol) and acetic anhydride (2.8 ml, 1.5 eq.) in pyridine (5 ml) was kept at room temperature for 2 hr. The solvent was removed in vacuo and the residue was taken up in $CHCl_3$. After washing with $H_2O$ the organic layer was concentrated to give the di-O-acetylthymidine which was dehydrated by distillation twice with anhydrous pyridine and dissolved in acetonitrile (30 ml). A 0.25 M solution of tri(1H-1,2,4-triazol-1-yl)phosphine oxide in acetonitrile (20 mmol, 2 eq.)[15] was added to the solution under stirring and the whole was kept at room temperature for 1 hr. The solution was cooled in an ice-bath, a mixture of $H_2O$ (2 ml) and triethylamine (9 ml) was added, and then kept at room temperature for 10 min. The solvent was removed in vacuo, the residue was taken up in $CHCl_3$, washed with 5% $NaHCO_3$, and the separated organic layer was concentrated. The residue was dissolved in a mixture of dioxane (40 ml) and conc.$NH_4OH$ (5 ml). After 2.5 hr, the solvent was evaporated to give the residue, 2'-deoxy-5-methylcytidine.

This was dehydrated by distillation twice with anhydrous pyridine and the residue was dissolved in pyridine (5 ml). Benzoyl chloride (1.2 ml, 10 mmol) was added and the solution was kept at room temperature for 1 hr. The mixture was cooled in an ice-bath and $CHCl_3$ and 5% $NaHCO_3$ were added with stirring. The organic layer was separated and the solvent was removed in vacuo. The residue was dissolved in EtOH (40 ml) and dioxane (20 ml), then 1 N NaOH (in 50% EtOH, 10 ml) was added with cooling in an ice-bath. After 5 min, the solution was neutralized by addition of 1 N HCl (in 50% EtOH, 10 ml). The solution was concentrated to a volume of 30 ml and diluted with $H_2O$ (30 ml). The solution was extracted with ether and the aqueous layer was kept at room temperature. The separated crystals were collected to give 1.7 g (4.9 mmol, 65%) of $N^4$-benzoyl-2'-deoxy-5-methylcytidine (**5**), mp 179-181°C. An aliquot was recrystallized from EtOH for analysis.

Anal. Calcd for $C_{17}H_{19}N_3O_5$ (345.36): C, 59.13; H, 5.54; N, 12.16. Found: C, 59.09; H, 5.45; N, 12.28.

Compound **5** (95mg, 0.28mmol) was dehydrated by distillation twice with anhydrous pyridine and the residue was dissolved in 10ml of pyridine. DMTrCl( 104mg, 1.1eq) was added to the solution and the whole was kept at room temperature for 10 hr. MeOH (0.5ml) was added and the solution was removed in vacuo. The residue was taken up in $CHCl_3$, washed with 5%$NaHCO_3$, and the organic layer was separated through Whatman 1PS filter paper, then the solvent was removed in vacuo. The residue was triturated in ether-n-hexane to give 150 mg (0.23mmol,83%) of 5'-O-dimethoxytrityl-$N^4$-benzoyl-2'-deoxy-5-methylcytidine (**6**). FD-MS m/z: 648 ($M^+$) $^1$H-NMR($CDCl_3$): 13.28(1H, br, H-$N^4$), 8.31-6.81(18H, m, dimethoxytrityl group and benzoyl group), 7.78(1H, s, H-6), 6.46(1H, t, H-1'), 4.59(1H, m, H-3'), 4.08(1H, m, H-4'), 3.79(6H, s, methoxy groups of dimethoxytrityl), 3.54-3.36(2H, m, H-5'), 2.50-2.29(2H, m, H-2'), 1.67(3H, s, 5-methyl group).

Compound **6** (860 mg, 2.5 mmol) was converted to the title compound **4** (1.53 g, 1.7 mmol, 68%, Fig. 1) by the procedure described previously. UV in MeOH max 330 nm. FD-MS m/z: 939($M^+$), 1041($M^+$+102). $^1$H-NMR($CDCl_3$): 11.75(1H, br, H-$N^4$), 8.33-6.80(22H, m, $N^4$-benzoyl group, dimethoxytrityl group and o-chlorophenyl group), 7.79(1H, s, H-6), 6.18(1H, t, H-1'), 5.03(1H, m, H-3'), 4.10(1H, m, H-4'), 3.93-3.88(2H, m, H-5'), 3.80(5H, s, methoxy groups of dimethoxytrityl), 3.03(6H, q, methylene groups of triethylamine), 2.30-2.28(2H, m, H-2'), 2.11(3H, s, 5-methyl group), 1.30(9H, t, methyl groups of triethylamine).

Triethylammonium 5'-O-dimethoxytrityl-2'-deoxy-$N^4$-methylcytidine 3'-(o-chlorophenyl phosphate) (**7**)

2'-Deoxy-$N^4$-methylcytidine(**8**) was prepared by the similar method reported.[16] 3',5'-Di-O-acetyl-2'-deoxyuridine (5.4 g, 1.7 mmol) in acetonitrile (60 ml) was treated with tri(1H-1,2,4-triazol-1yl)phosphine oxide (0.2 M in acetonitrile, 34 ml, 2 eq.). The solution was stirred for 45 min, then cooled in an ice-bath. $H_2O$ (4 ml) and triethylamine (18 ml) were added under stirring, and after 10 min, the solvent was removed in vacuo. The residue was dissolved in $CHCl_3$ (150 ml), washed with 5% $NaHCO_3$, then with $H_2O$, and the organic layer was concentrated to leave

the residue (4-triazolide). The residue was dissolved in a mixture of dioxane (80 ml) and 40% aqueous methylamine (12 ml), and the solution was kept at room temperature for 15 min. The solvent was removed in vacuo and the residue was dissolved in conc.$NH_4OH$ (50 ml), and then kept at 50°C for 3 hr. The solvent was removed in vacuo and the residue was dissolved in hot EtOH. The separated crystals were collected to give **8** (2.6 g, 10.8 mmol, 62%)[17], mp 181-184°C. An aliquot was recrystallized from EtOH for analysis. Anal. Calcd for $C_{10}H_{15}N_3O_4$ (241.25): C, 49.78; H, 6.27; N, 17.42. Found: C, 49.64; H, 6.37; N, 17.33.

Compound **8** (100mg, 0.41mmol) was dehydraterd by distillation twice with anhydrous pyridine and the residue was dissolved in 20ml of pyridine. DMTrCl (154mg, 1.1eq) was added and the whole was kept at room temperature for 5hr. The solvent was removed in vacuo and the residue was taken up in $CHCl_3$, washed with 5% $NaHCO_3$. The organic layer was separated through a Whatman 1PS filter paper, then the solvent was removed in vacuo. The residue was triturated in ether-n-hexane to give 180mg (0.33mmol, 81%) of 5'-O-dimethoxytrityl-2'-deoxy-$N^4$-methylcytidine (**9**). FD-MS m/z: 543($M^+$). $^1$H-NMR($CDCl_3$): 7.42-6.81(15H, m, H-6, H-$N^4$ and dimethoxytrityl group), 6.34(1H, br, H-1'), 5.35(1H, d, H-5), 4.51(1H, m, H-3'), 4.05(1H, m, H-4'), 3.79(6H, s, methoxy groups of dimethoxytrityl) 3.43(2H, m, H-5'), 2.97(3H, br, $N^4$-Me), 2.63-2.55(1H, m, H-2'a), 2.25-2.15(1H, m, H-2'b).

Compound **9** (50mg, 0.09mmol) was converted to **7** (64mg, 0.08 mmol, 83%, Fig.1) by the procedure described previously. In this case the N-4 benzoylation of 2'-deoxy-$N^4$-methylcytidine was omitted in the step of phosphorylation. UV in MeOH max 274nm. FD-MS m/z: 835($M^+$), 846($M^+$+11), 937($M^+$+102), 948($M^+$+113). $^1$H-NMR($CDCl_3$): 12.00(1H, br, triethylammonium), 7.59-6.77(19H, H-6, H-$N^4$, dimethoxytrityl group and o-chlorophenyl group), 6.40(1H, br, H-1'), 5.28(1H, d, H-5), 5.12(1H, m, H-3'), 4.32(1H, m, H-4'), 3.77(6H, s, methoxy groups of dimethoxytrityl), 3.42-3.37(2H, m, H-5'), 3.05-2.94(9H, m, $N^4$-Me and methylene groups of triethylamine), 2.40-2.15(2H, m, H-2'), 1.29(9H, t, methyl groups of triethylamine).

Synthesis of decadeoxyribonucleotides

The decanucleotides listed in Table 1 were prepared by the

solid phase phosphotriester method.[18] The protected oligomers on the polymer support were released from the resin and deprotected with tetramethylguanidinium pyridine-2-aldoximate[19] followed by treatment with ammonia, then with 80% acetic acid. All the decamers were purified by anion exchange column chromatography followed by reversed phase HPLC to a purity of over 99%. Although the yields of decamers containing $N^6$-methyladenine or $N^4$-methylcytosine were rather low due to the formation of unknown side products, the sequence of the purified materials was confirmed by the 5'-phosphate labeling and successive partial digestion with snake venom phosphodiesterase[15] and the mobility shift analysis of the respective partial hydrolyzates. Some typical examples of the autoradiograms are shown in Fig 2.

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 spectropolarimeter.

5'-Labeling of decanucleotides

The oligonucleotide (1 nmol) to be labeled was incubated at 37°C for 1 hr with $\gamma$-$^{32}$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 15 ul. The product was adsorbed on Whatman DE 50 (bicarbonate form) and washed with 0.4 M triethylammonium bicarbonate, then eluted with 2M triethylammonium bicarbonate. The eluate was concentrated *in vacuo* to leave the product.

Digestion of decanucleotides

Method A) $^{32}$P-labeled oligonucleotide (5000 to 10000 cpm) was incubated with Bgl II (24 units) in 10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 100 mM NaCl, and 7 mM 2-mercaptoethanol at 20°C for 12 hr. In the case of other restriction enzymes, the following buffers were used: Sau 3AI (30 units); 10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$ and 100 mM NaCl: Mbo I (120 units); 100 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, and 100 mM NaCl: Mfl I (12 units); 10 mM Tris-

HCl (pH 8.0), 7 mM $MgCl_2$, and 7 mM mercaptoethanol.

Method B) $^{32}P$-labeled oligonucleotide (5000 to 10000 cpm) and related carrier oligonucleotide duplex were incubated at 20°C for 12 hr with Bgl II (24 units) in 10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 100 mM NaCl and 7 mM 2-mercaptoethanol, or with Sau 3AI (30 units) in 10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$ and 100 mM NaCl, or with Mbo I (120 units) in 100 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$ and 100 mM NaCl, or with Mfl I (12 units) in 10 mM Tris-HCl (pH 8.0), 7 mM $MgCl_2$, and 7 mM 2-mercaptoethanol.

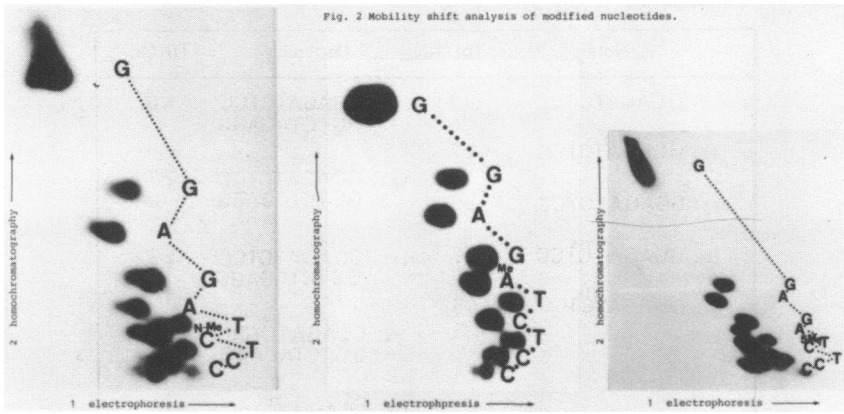
Method C) $^{32}P$-labeled GGAGATCTCC (5000 to 10000 cpm) and cold GGAGATCTCC (final concentration of $1.2 \times 10^{-6}$M) were incubated with Bgl II (12 units), in the presence of a modified decamer in appropriate concentrations, in 10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 100 mM NaCl and 7 mM 2-mercaptoethanol at 20°C. An aliquot of the reaction mixture was taken out and adsorbed on a POLYGRAM CEL 300 DEAE-cellulose plate (Macherey-Nagel) at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 minutes after incubation to determine the initial velocities.

All digests were developed by homochromatography and the radioactive spots were excised and counted using a toluene based scintillator on a Beckman LS-230 liquid scintillation counter.

## Results and Discussion

In the synthesis of decanucleotides containing N-methylated bases by the solid-phase phosphotriester method, the prior protection of the exocyclic methylamino function by acylation was thought to be necessary. However, the benzoylation of the N-methyl group of 2'-deoxy-$N^6$-methyladenosine and $N^4$-methylcytidine proceeded sluggishly. Therefore, we used the nucleotides with an unprotected N-methylbase for the present purpose, although the yields of coupling of the nucleotide fragments containing unprotected N-methylated base are generally lower than compared to those of the natural N-protected nucleotides. The structures of the decanucleotides containing N-methylbases were confirmed by mobility shift analyses of the partial hydrolyzates (Fig. 2).

Melting temperatures (Tm) of the decanucleotide duplexes are shown in Table 1. The decamers containing $N^6$-methyladenine and




$N^4$-methylcytosine have lower Tm's than compared with the usual decamers. It is expected that, in the case of N-methyl-cytosine or -adenine[21], the usual base-pairing is possible only when the N-methyl groups occupy the anti-orientation to the $N^3$-(or $N^1$)-atom in the pyrimidine (or purine) moiety, which should exert at the same time some steric hindrance between the N-methyl groups and the C-5 in the cytosine or N-7 in the adenine, respectively.[22] The Tm of the duplex containing 5-methylcytosine in both strands, on the other hand, was higher than that of the usual duplex as can be expected. The circular dichroism (CD) spectra of the decamer duplexes showed that these duplexes have the right-handed regular duplex conformations (Fig. 3). The relatively weak molar ellipticities of the heteroduplexes are due to the nature of the heteroduplex in general as discussed in our previous study[11,20], and not due to incomplete base-pair formation.

The rates of hydrolysis by restriction endonucleases of the duplexes containing modified bases in both strands are summarized in Table 2. Bgl II and Sau 3AI hydrolyzed the duplex containing $N^6$-methyladenine in position 5 (from the 5'-end, decamer II) which was consistent with the reported observations[23]. On the other hand, Bgl II did not cleave the duplex having the $N^6$-methyladenine in position 3 from the 5'-end (decamer III).

No enzymes tested here cleaved the duplexes having $N^4$-methyl-cytosine (decamer IV) or 5-methylcytosine[24] (decamer V) in place of the cytosines in both strands of the recognition sequences.

Table 1 Melting temperatures of decamer duplexes.

| Nucleotide | Tm (°c) | Nucleotide | Tm (°c) |
|---|---|---|---|
| I. GGAGATCTCC | 39 | VI. CCAGATCTCC<br>GGTCTAGAGG | 40 |
| II. GGAG$\overset{Me}{A}$TCTCC | 27 | | |
| III. GG$\overset{Me}{A}$GATCTCC | 33 | VII. CCAG$\overset{Me}{A}$TCTCC<br>GGTCTAGAGG | 27 |
| IV. GGAGAT$\overset{NHe}{C}$TCC | 27 | VIII. CC$\overset{Me}{A}$GATCTCC<br>GGTCTAGAGG | 22 |
| V. GGAGAT$\overset{5Me}{C}$TCC | 45 | | |
| | | IX. CCAGAT$\overset{NMe}{C}$TCC<br>GGTCTAGAGG | 27 |
| | | X. CCAGAT$\overset{5Me}{C}$TCC<br>GGTCTAGAGG | 35 |

Tm's of 1,2,3,4,5,6,10, were measured in a buffer of 0.1 M NaCl, 0.01 M sodium cacodylate, pH 7.5 and Tm's of 7,8,9, were measured in a buffer of 0.1 M NaCl, 0.01 M $MgCl_2$, 0.01 M sodium cacodylate, pH 7.5.

The rates of enzymatic cleavge of the duplexes containing modified bases in one strand are summarized in Table 3. The numbers on the upper side of the oblique lines in the Table express the cleavage rates (%) of the upper strands, and the numbers on the lower side for the lower strands. While the duplexes containing one $N^4$-methylcytosine (duplex IX) or 5-methylcytosine (duplex X) were resistant to hydrolysis by Bgl II and

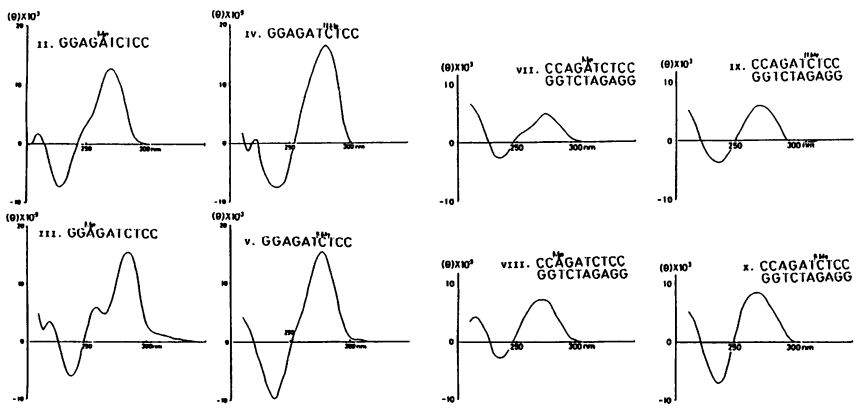



Fig. 3 The CD spectra of decamer duplexes in 0.1 M NaCl, 0.01 M $MgCl_2$, 0.01 M sodium cacodylate pH 7.5 at 17°C.

Table 2 The rates of hydrolysis of the duplexes containing modified bases by four enzymes.

| | ▼AGATCT | ▼GATC | ▼GATC | Pu▼GATCPy |
|---|---|---|---|---|
| | BglII | Sau3AI | MboI | MflI |
| I. GGAGATCTCC | 100 | 61 | 100 | 97 |
| II. GGAG$^{Me}$ATCTCC | 77 | 28 | 0 | 0 |
| III. GG$^{Me}$AGATCTCC | 0 | 44 | 100 | 3 |
| IV. GGAGATC$^{NH_2}$TCC | 0 | 0 | 0 | 0 |
| V. GGAGATC$^{5NH_2}$TCC | 0 | 0 | 0 | 0 |

The conditions are described in Method A.

Sau 3AI[25], both chains of these were cleaved by Mbo I[24] and Mfl I. In addition, the rates of cleavage with these two enzymes is reversed, the natural strand being cleaved faster by Mbo I, while the modified strand is cleaved faster by Mfl I. A similar phenomenon was observed in the case of a heteroduplex containing $N^6$-methyladenine at position 5 of the upper strand (duplex VII), except for cleavage by Sau 3AI. It may be concluded that the restriction endonuclease recognizing a hexanucleotide sequence (Bgl II and Mfl I) cleaves the modified strand faster in the heteroduplexes, which again confirmed the mode of cleavage we proposed previously.[4] Furthermore, a single alteration of the structure of the recognition site at the 5'-terminus of the AGATCT sequence to $N^6$-methyladenine (the decamer VIII in row 3 of

Table 3 The rates of hydrolysis of the duplexes containing modified bases in one strand by four enzymes.

| | ▼AGATCT | ▼GATC | ▼GATC | Pu▼GATCPy |
|---|---|---|---|---|
| | BglII | Sau3AI | MboI | MflI |
| VI. CCAGATCTCC / GGTCTAGAGG | 97 / 78 | 47 / 66 | 97 / 90 | 95 / 72 |
| VII. CCAG$^{Me}$ATCTCC / GGTCTAGAGG | 99 / 13 | 75 / 0 | 3 / 50 | 7 / 0 |
| VIII. CC$^{Me}$AGATCTCC / GGTCTAGAGG | 0 / 0 | 0 / 3 | 0 / 84 | 0 / 0 |
| IX. CCAGATC$^{NH_2}$TCC / GGTCTAGAGG | 0 / 1 | 0 / 3 | 49 / 83 | 53 / 3 |
| X. CCAGATC$^{5NH_2}$TCC / GGTCTAGAGG | 0 / 0 | 0 / 0 | 16 / 49 | 89 / 22 |

The conditions are described in Method B.

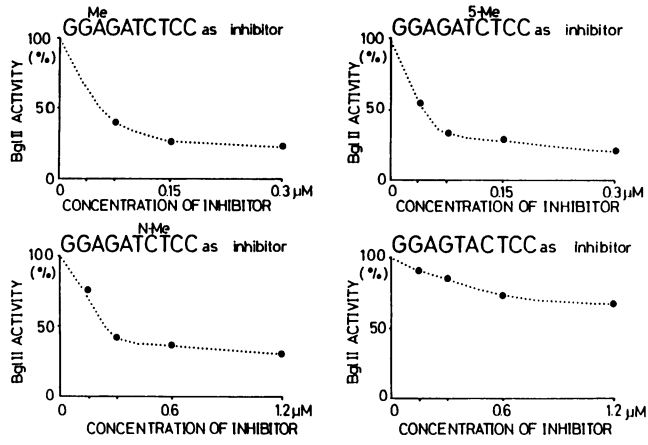



Fig.4 The inhibition experiment in the Bgl II digestion of GGAGATCTCC.
The conditions are described in Method C.

Table 3) is enough to result complete resistance to the enzymes. It is to be noted that Sau 3AI and Mbo I have different recognition modes, although they cleave the same GATC sequence.

It is conceivable that alteration of the base moiety in the recognition sequence may cause either a loss (or decrease) of binding to the enzymes, or inability of phosphoester cleavage by the altered mode of binding. To clarify this point we carried out the inhibition experiment of the cleavage of GGAGATCTCC by Bgl II in the presence of modified decamers. The results are summarized in Fig. 4. It is evident that methylation of the one base in the recognition sequence showed concentration-dependent inhibition of the cleavage of the parent decamer. The biological DNA methylation which inhibits the cleavage by enzymes has two possible modes; namely, methylation of the amino residue blocks the proton-donor ability of the amino group thus inhibiting hydrogen bond formation, or the methyl residue inhibits the association with the enzyme protein by steric hindrance. Now it can be speculated from the present results that the steric effect of the methyl group introduced in the adenine or cytosine moiety is not operative. Thus some different mode of binding due to the loss of proton-donor ability of the amino group must have occurred, and in that form the cleavage of the phosphodiester linkage is impos-

sible. The well known biological importance of the 5-methylation of cytosine may be also a consequence of the essential change of the binding mode of nucleic acids and proteins.

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References and Notes


1. Part 73 see: Suzuki,Y., Matsuda,A., Ueda,T., Chem. Pharm. Bull., submitted.
2. Wells,R.D., Klein,R.D., Singleton,C.K. (1981) The Enzymes, Vol.XIV, 157-191, Academic Press,Inc.
3. a) Connolly,B.A., Potter,B.V., Eckstein,F., Pigoud,A., Grotjahn,L. (1984) Biochemistry,**23**, 3443-3453
   b) Alves,J., Pingoud,A., Haupt,W., Langowski,J., Peters,F., Maass,G., Wolf,C. (1984) Eur. J. Biochem.,140, 83-92
   c) Ohtsuka,E., Ishino,Y., Ibaraki,K., Ikehara. (1984) Eur. J. Biochem.,139, 447-450
   d) Wolfes,H., Fliess,A., Pingoud, A. (1985) Eur. J. Biochem., 150, 105-110
   e) Kita,K., Hiraoka,N., Kimizuka,F., Obayasi,A., Kojima,H., Takahashi,H., Saito,H. (1985) Nucleic Acids Res.,13, 7015-7024
   f) Berkner,K.L., Folk,W.R. (1977) J. Biol. Chem.,252, 3185-3193
   g) Gruenbaum,Y., Cedar,H., Rajin,A. (1981) Nucleic Acids Res., 9, 2509-2515
   h) Bodnar,J.W., Zempsky,W., Wander,D., Bergson,C., Ward,D.C. (1983) J. Biol. Chem.,258, 15206-15213
   i) Jiricny,J., Wood,S.G., Martin,D., Ubasawa,A. (1986) Nucleic Acids Res.,14, 6579-6590
   j) Brennan,C.A., Van Cleve,M.D., Gumport,R.I. (1986) J. Biol. Chem.,261, 7279-7286
4. Ono,A., Sato,M., Ohtani,Y., Ueda,T. (1984) Nucleic Acids Res., 12, 8939-8949
5. Ehrlich,M., Y.-H.Wang,R. (1981) Science, 212, 1350-1357
6. a) Ehrlich,M., Gama-Sosa,M.A., Caweira,L.H., Ljungdahl,L.G., Kuo,K.C., Gehrke,C.W. (1985) Nucleic Acids Res., 13, 1399-1412
   b) Butkus,V., Klimasauskas,S., Kersulyte,D., Vaitkevicius,D., Lebionka,A., Janulaitis,A. (1985) Nucleic Acids Res.,13, 5727-5746
7. a) Pirrotta,V. (1976) Nucleic Acids Res.,3, 1747-1760
   b) Duncan,C.H., Wilson,G.A., Young,F.E. (1978) J. Bacteriol., 134, 338-344
8. a) Sussenbach,J.S., Monfoort,C.H., Schiphof,R., Stobberingh,E. E. (1976) Nucleic Acids Res.,3, 3193-3202
   b) Streeck,R.E. (1980) Gene, 12, 267-275
9. Gelinas,R.E., Myers,P.A., Roberts,R.J. (1977) J. Mol. Biol., 114, 169-179
10. Hiraoka,N., Kita,K., Nakajima,H., Obayashi,A. (1984) J. Ferment. Technol., 62, 583-588
11. Ueda,T., Sato,M., Ono,A. (1986) Chemica Scripta, 26, 199-203
12. Coddington,A. (1962) Biochim. Biophys. Acta, 59, 472-474
13. Broka,C., Hozumi,T., Arentzen,R., Itakura,K. (1980) Nucleic Acids Res., 8, 5461-5471

14. Sung,W.L. (1981) J. Chem. Soc. Chem. Commun., 1089
15. Divakar,K.J., Reese,C.B. (1982) J. Chem. Soc. Perkin I, 1171-1176
16. Sung,W.L. (1982) J. Org. Chem.,47, 3623-3630
17. Wempen,I., Duschinsky,R., Kaplan,L., Fox,J.J. (1961) J. Am. Chem. Soc.,83, 4755-4766
18. Miyoshi,K., Arentzen,R., Huang,T., Itakura,K. (1980) Nucleic Acids Res.,8, 5507-5517
19. Reese,C.B., Yan,L. (1978) Tetrahedron Letters, 4443-4446
20. Silberklang,M., Gillum,A.M., RajBhandary,U.L. (1977) Nucleic Acids Res., 4, 4091-4108
21. Fazakerley,G.V., Teoul,R., Guy,A., Fritzsche,H., Guschlbauer, W. (1985) Biochemistry, 24, 4540-4548
22. Sato,M., Ono,A., Higuchi,H., Ueda,T. (1986) Nucleic Acids Res., 14, 1405-1416
23. McClelland,M. (1983) Nucleic Acids Res., 11, r169-173
24. Dybving et al reported that Mbo I cleaved the recognition sequences methylated by Acholeplasma laidlawii's methylase which introduces 5-methylcytosine, but it was not clear that these recognition sequences were completely (both strands) methylated or half (one strand) methylated. A combination of our results and that of Dybving show that the methylase of Acholeplasma laidlawii introduces a 5-methylcytosine into one strand of the recognition sequence: Dybving,K., Swinton,D., Maniloff,J., Hattman,S. (1980) J. Bacteriol., 151, 1420-1424.
25. Streeck reported[8b] that Sau 3AI cleaved the recognition sequence containing 5-methylcytosine in one strand, but an excess of enzyme and a long incubation time was needed.