Synthesis and Characterization of Oligodeoxynucleotides Containing 4-*O*-Methylthymine†

Benjamin F. L. Li,*,‡ Colin B. Reese,§ and Peter F. Swann‡

Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London W1P 7PN, England, and Department of Chemistry, King's College London, Strand, London WC2R 2LS, England

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ABSTRACT: The carcinogenicity of *N*-nitroso compounds is believed to result from the alkylation of DNA, particularly on O-6 of the guanine and O-4 of the thymine residues. In order to study the base-pairing properties of 4-*O*-methylthymidine (T*) residues and the structural changes produced in DNA by the presence of this alkylated nucleoside, the oligodeoxyribonucleotides T*GCG, CGCAAGCTT*GCG, CGCGAGCTT*GCG, and CGCAAGCTTGCG were synthesized by the phosphotriester approach in solution. The 4-*O*-methylthymidine required for oligonucleotide synthesis was prepared by treating the 4-(3-nitro-1,2,4-triazolo) derivative of 3′,5′-bis-*O*-(methoxyacetyl)thymidine with 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) in methanol solution. The susceptibility of the 4-*O*-methyl group of T* toward nucleophiles enables this group of 4-*O*-methylthymidine-containing oligomers to be labeled by a direct exchange reaction with [^{13}C]- or [^{14}C]methanol in the presence of DBU. Although it has been previously suggested that 4-*O*-methylthymine forms stable base pairs with guanine, the thermal melting profiles of the double helices formed by these dodecamers suggest that the presence of 4-*O*-methylthymine paired to either adenine or guanine destablizes the helix. The melting curve of the sequence containing a 4-*O*-methylthymine residue base paired to guanine was biphasic and similar to that of an analogous sequence containing 6-*O*-methylguanine paired to thymine.

The alkylation of guanine on O-6 and thymine on O-4 in DNA is believed to play a crucial role in the carcinogenic and mutagenic action of *N*-nitroso compounds [reviewed by Pegg (1977)]. O-6-Alkylation of guanine residues is more important for the nitrosamines donating methyl groups (Loveless, 1969; Goth & Rajewsky, 1974; Pegg, 1984), but O-4-alkylation of thymine residues becomes increasingly important for nitrosamines donating larger alkyl groups (Singer, 1984; Swenberg et al., 1984). We have begun to undertake the synthesis of oligodeoxynucleotides containing these alkylated bases in order (a) to study the changes carcinogens produce in the structure of DNA, (b) to assay and study the mechanism of action of the DNA repair enzymes that play a crucial role in the body's defence against carcinogenic nitrosamin s, and (c) to study the biological effect of introduction of these alkylated bases into specific sites in the genome. This paper reports the synthesis by the phosphotriester approach in solution of self-complementary dodecadeoxyribonucleotides containing 4-*O*-methylthymine residues in the quantity (ca. 400 A_{260} units) and purity needed for structural studies by two-dimensional (2D) NMR and crystallography.

Two aspects of structure that are of particular interest to biologists could be studied if oligodeoxynucleotides containing 4-*O*-alkylthymine were available. The first is the base-pairing properties of the alkylated base, which is important because of the postulated relationship between mutagenesis and carcinogenesis. The second is the conformation and stability of the region of DNA containing the alkylated base, which is of interest in the context of both the speculation that sequence-specific aspects of DNA structure may play an important role in gene regulation (Rich et al., 1984) and that the conformational changes in DNA may be important in the recognition of damaged DNA by the repair enzymes that defend the cell against carcinogens. These have not been satisfactorily studied before because 4-*O*-methylthymine represents only a low proportion (0.06%) of the methylated bases produced in DNA by a carcinogen such as *N*-methyl-*N*-nitrosourea (McCarthy et al., 1984) and after a carcinogenic dose only about one thymine in 10^7 bases is methylated.

The synthesis of the desired oligodeoxynucleotides required the synthesis of 4-*O*-methylthymidine (T*). 4-*O*- and 2-*O*-alkylthymidines have previously been synthesized by the alkylation of thymidine with 2-bromopropane, followed by displacement with the appropriate alkoxide ions (Singer et al., 1983). However, this procedure gives a mixture of 4-*O*- and 2-*O*-alkylthymidines, and careful purification is required to obtain the individual isomers. We report here a facile and high-yielding synthesis of pure 4-*O*-methylthymidine (Scheme I) using the 3-nitro-(1,2,4-triazolo) derivative of 3′,5′-bis-*O*-(methoxyacetyl)thymidine (compound **2** in Scheme I). The latter compound **2** is also a key intermediate in the synthesis of 4-*O*-phenylthymidine (**3**) (Reese & Skone, 1984) and 5-methyl-2′-deoxycytidine (**5**).

The strategy used for oligonucleotide synthesis is illustrated in Scheme II. Each cycle of the synthesis comprised two stages. First, a nucleotide, dimer, or tetramer with a free 5′-hydroxy function but otherwise fully protected was condensed in the presence of an excess of 1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MSNT) with the 3′-phosphodiester of a nucleotide, dimer, or tetramer with protecting groups on the 5′-OH, amino functions, and internucleotide linkages. The product of this condensation was fully protected. It was then partially unblocked by removal of either the 5′-OH protecting group or the 2-cyanoethyl group protecting the 3′-phosphate residue, to give material that could be used in another condensation reaction.

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* Address correspondence to this author.
‡ Middlesex Hospital Medical School.
§ King's College London.

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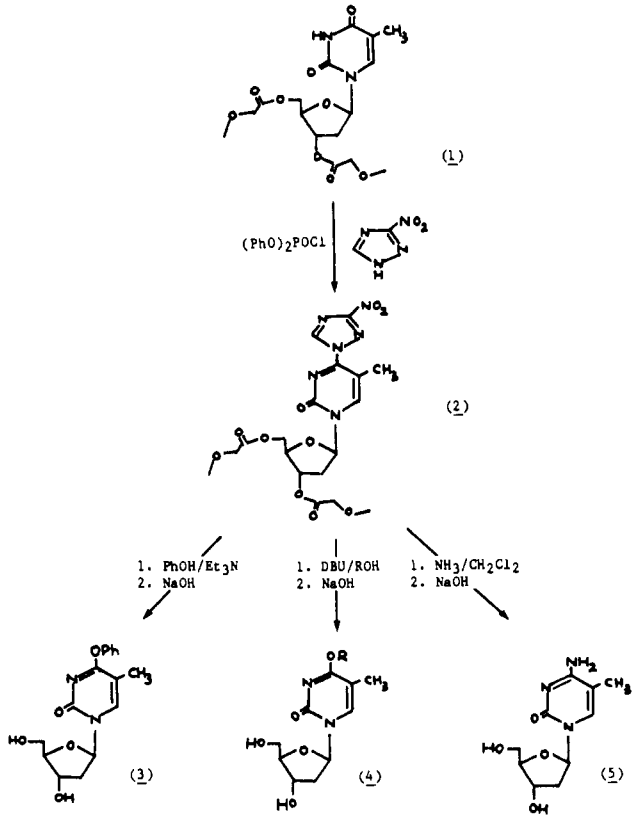
Scheme I: Synthesis of 4-*O*-Phenylthymidine (**3**), 4-*O*-Alkylthymidine (**4**), and 5-Methyldeoxycytidine (**5**) from the 4-(3-Nitro-1,2,4-triazolo) Derivative (**2**) of 3′,5′-Bis-*O*-(methoxyacetyl)thymidine (**1**)



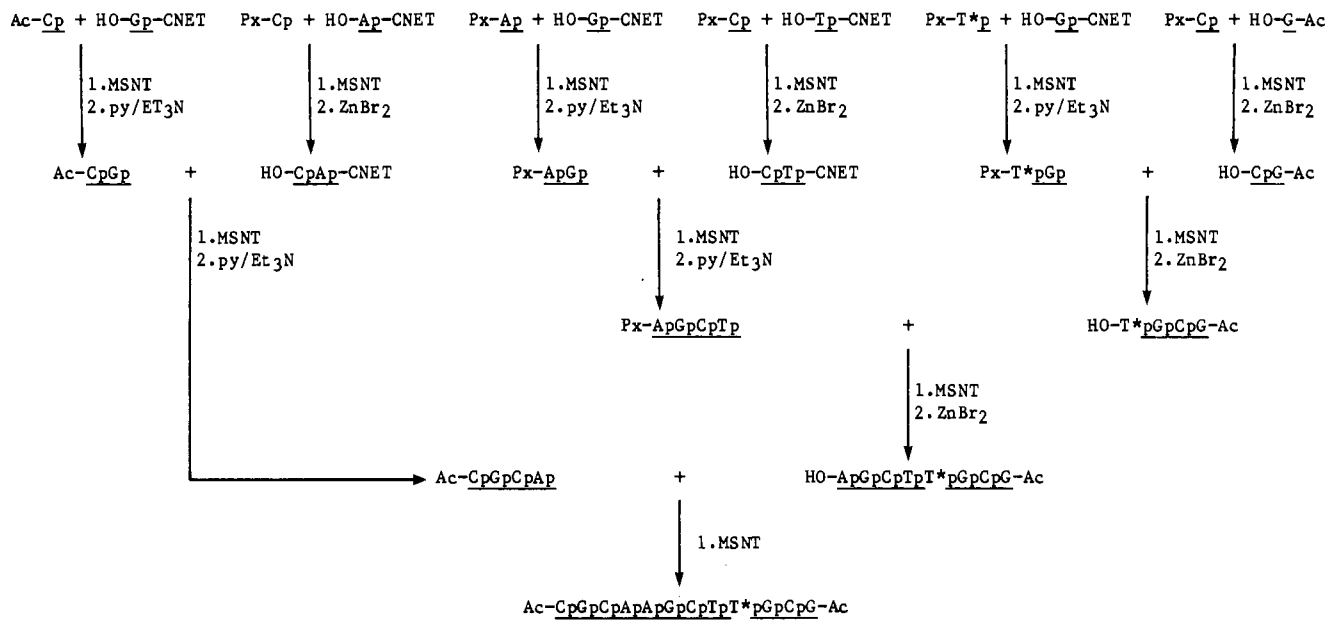
In the synthesis of the oligonucleotides, 2′-deoxyguanosine was protected as its 2-*N*-acetyl-6-*O*-(3-chlorophenyl) derivative and thymidine as its 4-*O*-phenyl derivative. The synthesis of oligomers containing 4-*O*-methylthymidine proved to be more difficult than the synthesis of oligomers containing 6-*O*-methyldeoxyguanosine because 4-*O*-methylthymidine is susceptible to both acidic hydrolysis (Singer et al., 1978) and nucleophilic attack. The normal unblocking procedure in which *N*-acyl protecting groups are removed by ammonolysis had to be avoided because ammonia attacks 4-*O*-methylthymidine (Figure 4). Similarly, a protic acid could not be used for removal of the 5′-*O*-(9-phenylxanthen-9-yl) group because of the susceptibility of 4-*O*-alkylthymidine residues to dealkylation (Singer et al., 1978). Therefore zinc bromide was used to remove the 5′-*O*-(9-phenylxanthen-9-yl) group, acetyl groups were used to protect the 3′- and 5′-hydroxy functions of the 3′- and 5′-terminal nucleotides in the dodecamer, the 2-cyanoethyl group was used for temporary phosphate protection, and DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) in methanol solution was used to remove the *N*-acyl protecting groups. Although it presented problems in oligonucleotide synthesis, the susceptibility of 4-*O*-methylthymidine toward nucleophilic substitution has the advantage that radioactive or heavy atoms can be introduced on the *O*-methyl group of 4-*O*-methylthymidine-containing oligomers by use of labeled methanol with DBU for the final deblocking step.

Experimental Procedures

Materials. 3-Nitro-1,2,4-triazole, 1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MSNT), 9-chloro-9-phenylxanthene, and 2-chlorophenyl phosphorodichloridate were prepared following the published procedures (Chattopadhyaya & Reese, 1980). Pyridine and triethylamine were purified by distillation, first over 2-mesitylenesulfonyl chloride and then over KOH. When used in the synthesis of oligomers containing 4-*O*-methylthymine, these two solvents were not distilled over calcium hydride; they were stored over KOH, and under nitrogen. Tetrahydrofuran (THF) was distilled over calcium hydride and acetonitrile over phosphorus pentoxide.

Purification Methods and Analytical Methods. Protected nucleosides and their phosphotriesters were purified by column

Scheme II: Strategy for Synthesis of Oligodeoxynucleotides Containing 4-*O*-Methylthymidine (T*)[a]



[a] When after deprotection two molecules of this dodecanucleotide anneal to form a double helix, it will contain two T*·A base pairs. The unmodified oligonucleotide containing *T·A* base pairs and the oligonucleotide containing T*·*G* base pairs were obtained by substituting appropriate monomers in the scheme. Abbreviations: py, pyridine; MSNT, 1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazole; Px, 9-phenylxanthen-9-yl; Ac, acetyl; T*, 4-*O*-methylthymidine. Protected residues are underlined: A, 6-*N*-benzoyl-2′-deoxyadenosine; C, 4-*N*-benzoyl-2′-deoxycytidine; G, 2-*N*-acetyl-6-*O*-(3-chlorophenyl)-2′-deoxyguanosine; T, 4-*O*-phenylthymidine. Phosphate residues protected by 2-chlorophenyl groups are represented by p, and p-CNET represents phosphate protected by both 2-chlorophenyl and 2-cyanoethyl groups.

chromatography on Kieselgel 60H and protected oligonucleotides on Kieselgel 60 (Merck) under gradient elution with an increasing concentration of ethanol in chloroform. The products were precipitated with petroleum ether (bp 40–60 °C) and stored at −20 °C. After deprotection, oligonucleotides were desalted on a Sephadex G-25 or G-50 (DNA grade, Pharmacia) column (15 × 750 mm) that was eluted with ethanol water (1:4 v/v). The desalted products were purified by liquid chromatography (LC) on reverse-phase (Nova-Pak C18; Waters Inc.) or ion-exchange (Partisil 10 SAX; Whatman) columns. The precise elution conditions used for LC are shown in the figures. After chromatography the oligonucleotides were desalted with either Waters Sep-Pak C18 cartridges (Atkinson & Smith, 1984) or dialysis (2000 molecular weight cut-off, Spectrum Medical Industry). Thin-layer chromatography (TLC) was carried out on Merck HPTLC plates that were eluted with chloroform–methanol mixtures as described below.

DNA Melting. Melting curves were obtained by measuring the change in absorption at 260 nm in a Unicam SP500 spectrophotometer (Pye Unicam, Cambridge, U.K.) fitted with a Gilford 222 photometer and a Gilford 2527 thermoprogrammer (Gilford Instruments, Oberlin, OH). All melting curves were measured on solutions with an initial absorbance at 260 nm of 1.25 at 12 °C. The temperature was increased 1 deg/min.

NMR Spectroscopy. ^{1}H NMR spectra of the dodecadeoxynucleotides were measured with a Bruker 500-MHz spectrometer in the laboratory of Professor D. J. Patel, Columbia University, New York.

Synthetic Procedures. (1) Protected Naturally Occurring 2′-Deoxynucleosides. 4-*N*-Benzoyl-2′-deoxycytidine and 6-*N*-benzoyl-2′-deoxyadenosine were synthesized with the transient protection procedure (Gaffney et al., 1982), and 2-*N*-acetyl-6-*O*-(3-chlorophenyl)-2′-deoxyguanosine and 4-*O*-phenylthymidine were synthesized by the procedure of Reese and Skone (1984).

(2) 4-O-Methylthymidine. 3′,5′-Bis-*O*-(methoxyacetyl)-thymidine (0.39 g, 1.0 mmol) was added to a stirred solution of diphenyl phosphorochloridate (0.67 mL, 2.5 mmol), 3-nitro-1,2,4-triazole (0.29 g, 2.5 mmol), and triethylamine (0.5 mL, 5 mmol) in acetonitrile (5 mL). After 90 min, the solution was filtered into water (0.5 mL) and evaporated to a small volume. The residue was dissolved in dichloromethane (10 mL) and washed first with an equal volume of saturated aqueous $NaHCO_3$ and then with water. The organic layer was dried ($MgSO_4$), and the solvent was then removed under reduced pressure. The residue was dissolved in dry dichloromethane (4 mL) and cooled to −5 °C. Dry methanol (0.28 mL, 7 mmol) and DBU (0.22 mL, 1.5 mmol) were then added. After 10 min, the reaction mixture was poured into cold 0.5 M aqueous KH_2PO_4 buffer (pH 6.5, 10 mL) and extracted with dichloromethane (8 mL). The dichloromethane layer was washed with an equal volume of saturated aqueous $NaHCO_3$ and then dried and evaporated under reduced pressure. The resultant gum was dissolved in pyridine (5 mL) and water (1 mL) and cooled to −5 °C. Precooled 2.5 M aqueous NaOH (1 mL) was added, and the solution was kept at −5 °C for 2 min. The reaction was quenched with Dowex 50 (pyridinium form, 1.5 mL). The resin was removed by filtration, and the filtrate was evaporated to a gum. This material was purified by chromatography on a silica gel column and was then recrystallized from chloroform–methanol (98:2 v/v) to give 4-*O*-methylthymidine (0.18 g, 70%). The product had mp 170–172 °C [Anal. Calcd for $C_{11}H_{16}N_2O_5$: C, 51.6; H, 6.3; N, 10.9. Found: C, 51.3; H, 6.3; N, 10.8.]: UV λ_{max} 282 nm, λ_{min} 242 nm, ξ_{max} 6.4 × 10^3 [Singer et al. (1978) report λ_{max} 281 nm, ξ_{max} 6.7 × 10^3]; ^{1}H NMR ([$^2H_6$]Me_2SO, 250 MHz) δ 2.58 (3 H, s, 5-CH_3), 2.00, 2.16 (2 H, m, $H_{2',2''}$), 2.58 (2 H, m, $H_{5'5''}$), 3.80 (1 H, m, $H_{4'}$), 3.82 (3 H, s, OCH_3), 4.22 (1 H, br m, $H_{3'}$), 5.02 (1 H, t, OH exchangeable), 5.19 (1 H, d, OH exchangeable), 6.12 (1 H, t, H_1), 7.98 (1 H, s, H_6). The LC elution profile of the product is illustrated in Figure 1.

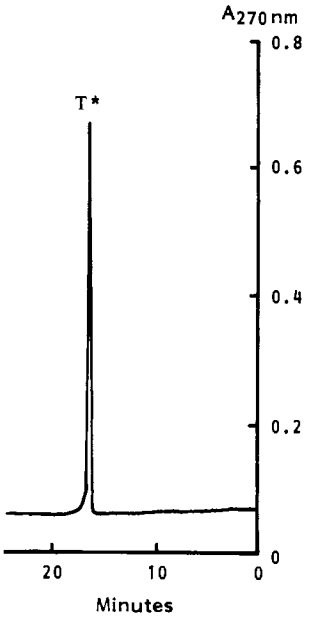


FIGURE 1: LC analysis of 4-*O*-methylthymidine synthesized from the 4-*O*-(3-nitro-1,2,4-triazolo) derivative of 3′,5′-bis-*O*-(methoxyacetyl)thymidine (**2**). The chromatography was carried out at 25 °C on a Waters Z module Nova Pak C18 cartridge eluted at a flow of 3 mL/min with a gradient from 0 to 7.5% acetonitrile in 50 mM aqueous KH_2PO_4 (pH 4.5) over 30 min.

(3) 5′-O-(9-Phenylxanthen-9-yl)-N-acyl Derivatives of 2′-Deoxynucleosides. The synthesis of the 5′-*O*-(9-phenylxanthen-9-yl) derivatives followed the published procedure (Chattopadhyaya & Reese, 1978). In general, the starting *N*-acyl derivatives were dried by evaporation of their pyridine (2 mL/mmol) solutions; they were then dissolved in pyridine (3 mL/mmol), and a solution of 9-chloro-9-phenylxanthene (PxCl, 1.1 molar equiv) in pyridine was added dropwise. The reactions were followed by TLC with chloroform–methanol (95:5 v/v) as eluant. The reactions were quenched by the addition of saturated aqueous $NaHCO_3$ (1 mL/mmol of nucleoside building block). After 10 min, the solutions were poured into saturated aqueous $NaHCO_3$ (15 mL/mmol of nucleoside building block), and the products were extracted with chloroform (15 mL/mmol of nucleoside building block). The organic layer was then dried ($MgSO_4$) and concentrated under reduced pressure, and the products were purified by column chromatography on silica gel. Except in the case of the 4-*N*-benzoyl-2′-deoxycytidine derivative, which had to be loaded in chloroform–pyridine (98:2 v/v), the products were loaded on the silica gel column in chloroform–ether (1:1 v/v). The columns were washed with 3 bed volumes of ether and were then eluted with a gradient of increasing concentrations of ethanol in chloroform. The desired products usually eluted between 98:2 (v/v) and 96:4 (v/v) chloroform–ethanol. The solvent was removed under reduced pressure, and the residues were dissolved in dichloromethane or, in the case of the 4-*N*-benzoyl-2′-deoxycytidine derivative, in chloroform–pyridine (95:5 v/v) and precipitated by addition dropwise into petroleum ether (bp 40–60 °C). The products were stored at −20

°C. Yields ranged from 75 to 85%.

(*4*) *Triethylammonium Salts of 5′-O-(9-Phenylxanthen-9-yl)-N-acyl-2′-deoxynucleoside 3′-(2-Chlorophenyl phosphates*). Phosphorylation followed the published procedure (Chattopadhyaya & Reese, 1980). In general, triethylamine (0.84 mL, 6 mmol), 1,2,4-triazole (0.46 g, 6.6 mmol), 2-chlorophenyl phosphorodichloridate (0.49 mL, 3 mmol) and *N*-methylimidazole (0.32 mL, 4 mmol) were stirred together in dry THF (20 mL) for 15 min. The 5′-*O*-(9-phenylxanthen-9-yl)-*N*-acyldeoxynucleoside (1 mmol) was then added, and the solution was stirred for 15 min (the reaction was followed by TLC). The solution was filtered into a 50-mL flask containing triethylamine (1.64 mL, 12 mmol) and water (1 mL). The filtrate was evaporated to a small volume and dissolved in dichloromethane (30 mL). The organic solution was washed twice with an equal volume of saturated aqueous $NaHCO_3$ and then twice with water. However, in the case of the 2′-deoxycytidine derivatives, the dichloromethane solution was washed 4 times with 0.1 M aqueous triethylammonium bicarbonate (pH 7). Emulsions, when formed, were cleared by the addition of saturated aqueous NaCl followed by centrifugation. The dichloromethane solution was dried ($MgSO_4$) and evaporated to a white foam. This material was dissolved in small volume of dichloromethane and precipitated by adding the solution dropwise into petroleum ether (bp 40–60 °C). The yields usually ranged from 90 to 95%.

(*5*) *N-Acyl-2′-deoxynucleoside 3′-(2-Chlorophenyl 2-cyanoethyl phosphates*). These compounds were synthesized by a slight modification of the published procedure (Wreesmann et al., 1983). 5′-(9-Phenylxanthen-9-yl)-*N*-acyl-2′-deoxynucleosides were dried by evaporation from pyridine solution (3 mL/mmol). The phosphorylating agent was prepared by stirring 2-chlorophenyl phosphorodichloridate (0.22 mL, 1.3 mmol) and 1-hydroxybenzotriazole (0.35 g, 2.6 mmol) together in dry THF (3 mL) and pyridine (1 mL) for 15 min. A solution of 5′-*O*-(9-phenyl-xanthen-yl)-*N*-acyl-2′-deoxynucleoside in pyridine (2 mL) was added to the stirred phosphorylating agent, and the reaction was allowed to proceed for 15 min [reactions were followed by TLC with chloroform–methanol (95:5 v/v); the product had R_f * 0]. 2-Cyanoethanol (0.21 mL, 1.5 mmol) was then added, and the solution allowed to stand for 90 min. Water (0.5 mL) was then added. After 5 min the solution was evaporated to a small volume and redissolved in dichloromethane (20 mL), and the solution was then washed with an equal volume of saturated aqueous $NaHCO_3$ and then water. The organic layer was evaporated under reduced pressure and the product dried by repeated evaporation of a toluene solution. The product was dissolved in dichloromethane–propan-2-ol (85:15 v/v, 25 mL), and zinc bromide (5.63 g, 25 mmol) was added. The resulting solution was stirred for 20 min and was then poured into a separating funnel containing 1 M aqueous ammonium acetate (75 mL). After vigorous shaking, the yellow organic solution became colorless. The dichloromethane layer was separated and evaporated to give a white foam, which was redissolved in dichloromethane (25 mL), and the solution was washed with 1 M aqueous ammonium acetate (75 mL). The product was purified by column chromatography on silica gel. The pure product was precipitated from the eluate with petroleum ether (bp 40–60 °C) and stored at −20 °C. The yields ranged from 70 to 80%.

Triethylammonium 5′-*O*-acetyl-4-*N*-benzoyl-2′-deoxycytidine 3′-(2-chlorophenyl phosphate) was prepared by treating 4-*N*-benzoyl-2′-deoxycytidine 3′-(2-chlorophenyl 2-cyanoethyl phosphate) with acetic anhydride (2.0 molar equiv) in pyridine solution and then removing the 2-cyanoethyl group by the procedure below.

(*6*) *Oligodeoxynucleotide Synthesis*. The synthetic strategy is illustrated in Scheme II. Condensations involving more than 0.15 mmol of the component with a free 5′-hydroxy function was carried out with MSNT (3.5 molar equiv of 5′-OH component) and 3′-phosphodiester component (1.2 molar equiv) in pyridine (6 mL/mmol of 5′-OH component), and condensations involving less than 0.15 mmol of the 5′-OH component were carried out with MSNT (0.34 mmol) in pyridine (1 mL). The phosphodiester and the 5′-hydroxy components were dried together by evaporation of a pyridine solution (3 mL/mmol; 1 mL for less than 0.15 mmol) before the addition of the MSNT. The coupling reactions were followed by TLC (which is satisfactory up to the octamer level). Reaction times ranged from 20 to 70 min (see Table I). The reactions were then quenched with saturated aqueous $NaHCO_3$ (1 mL/mmol of phosphodiester). After 10 min, the solution was poured into saturated aqueous $NaHCO_3$ (20 mL/mmol of the 5′-OH component) and extracted with CH_2Cl_2 (20 mL/mmol of the 5′-OH component). After being washed with saturated aqueous $NaHCO_3$, the dichloromethane layer was dried ($MgSO_4$) and coevaporated with toluene to give a foam or powder. At the dimer level, the fully protected dimer was first purified by silica gel chromatography before the 5′-*O*-(9-phenylxanthen-9-yl) or the 2-cyanoethyl group was removed, but the tetramers and octamers were not purified before the unblocking reactions.

The 5′-(9-phenylxanthen-9-yl) protecting group was removed according to a published procedure (Ohtsuka et al., 1983). The dimers or tetramers were treated with $ZnBr_2$ [5.6 g, 25 mmol for each mmol of 5′-*O*-(9-phenylxanthen-9-yl)-protected 3′-phosphodiester used in the condensation] in dichloromethane–propan-2-ol (85:5 v/v; 25 mL). The time required for 5′ unblocking varied from 10 to 70 min and increased with increasing length of the oligomers. The octamers were unblocked with twice the amount of solvent and $ZnBr_2$ used for dimers and tetramers. The reactions were quenched with 1 M aqueous ammonium acetate (75 mL). After removal of 5′ protection the oligomers were purified by silica gel chromatography using a gradient of ethanol in chloroform. The 5′-unprotected oligonucleotides were usually eluted with chloroform–ethanol (93:7 v/v).

The 2-cyanoethyl group was removed from the 3′-phosphotriester with triethylamine–pyridine (1:1 v/v). 2-Cyanoethyl derivatives were first dried by evaporation of their pyridine (3 mL/mmol) solutions, and triethylamine (3 mL/mmol) and pyridine (3 mL/mmol) were added to the reaction. The reaction times ranged from 20 (monomers) to 90 min (longer oligomers). When TLC indicated that the reactions were complete, the solvents were removed by evaporation under reduced pressure, and the residue was dissolved in a small volume of dichloromethane. The solution was added dropwise to petroleum ether (bp 40–60 °C) and the precipitate collected by centrifugation. The conditions of condensation reactions and the yield of products are summarized in Table I.

Deprotection and Purification. The deprotection procedures consisted of two stages.

(1) The first stage was treatment with a solution of (*E*)-2-nitrobenzaldoxime (0.3 M) and N^1,N^1,N^3,N^3-tetramethylguanidine (TMG) (0.27 M) in acetonitrile–water (4:1 v/v) at room temperature for 20 h. This removes the 2-chlorophenyl protecting groups from the internucleotide linkage, the phenyl group from 4-*O*-phenylthymine residues, and the 3-chlorophenyl group from 2-*N*-acetyl-6-*O*-(3-chlorophenyl)-

Table I: Condensation Reactions[a]

3'-Phosphodiesters (mmol)		5'OH-components	(mmol)	MSNT (mmol)	Pyridine (mL)	Reaction time (min)	Product	Isolated yield (%)
Px-Cp	1.76	HO-G-Ac	1.47	4.39	8.0	20	HO-CpG-Ac	82
Px-Cp	0.274	HO-Tp-CNET	0.25	0.82	2.5	20	HO-CpTp-CNET	80
Px-Cp	2.20	HO-Gp-CNET	2.00	5.0	10.5	20	HO-CpGp-CNET	76
Px-Cp	1.00	HO-Ap-CNET	0.90	2.06	3.5	20	HO-CpAp-CNET	74
Ac-Cp	1.78	HO-Gp-CNET	1.55	4.66	9.0	20	Ac-CpGp	89
Px-Tp	0.396	HO-Gp-CNET	0.360	1.18	2.5	20	Px-TpGp	89
Px-Ap	0.630	HO-Gp-CNET	0.550	1.69	4.0	20	Px-ApGp	85
Px-T*p	0.310	HO-Gp-CNET	0.281	0.93	2.0	20	Px-T*pGp	75
Px-T*pGp	0.240	HO-CpG-Ac	0.210	0.71	2.0	35	HO-T*pGpCpG-Ac	73
Px-TpGp	0.104	HO-CpG-Ac	0.093	0.42	1.0	40	HO-TpGpCpG-Ac	64
Px-ApGp	0.119	HO-CpTp-CNET	0.108	0.94	2.0	35	Px-ApGpCpTp	70
Ac-CpGp	0.163	HO-CpAp-CNET	0.148	0.65	1.5	35	Ac-CpGpCpAp	77
Ac-CpGp	0.396	HO-CpGp-CNET	0.360	1.18	3.0	35	Ac-CpGpCpGp	83
Px-ApGpCpTp	0.112	HO-T*pGpCpG-Ac	0.102	0.34	1.0	50	HO-ApGpCpTpT*pGpCpG-Ac	60
Px-ApGpCpTp	0.068	HO-TpGpCpG-Ac	0.062	0.34	1.0	50	HO-ApGpCpTpTpGpCpG-Ac	61
Ac-CpGpCpAp	0.052	HO-ApGpCpTpT*pGpCpG-Ac	0.047	0.34	1.0	80	Ac-CpGpCpApApGpCpTpT*pGpCpG-Ac	75
Ac-CpGpCpGp	0.048	HO-ApGpCpTpT*pGpCpG-Ac	0.042	0.34	1.0	80	Ac-CpGpCpGpApGpCpTpT*pGpCpG-Ac	71
Ac-CpGpCpAp	0.016	HO-ApGpCpTpTpGpCpG-Ac	0.016	0.34	1.0	80	Ac-CpGpCpApApGpCpTpTpGpCpG-Ac	N.D.

[a] Abbreviations: MSNT, 1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazole; Px, 9-phenylxanthen-9-yl; T*, 4-*O*-methylthymidine. Protected residues are underlined: A, 6-*N*-benzoyl-2'-deoxyadenosine; C, 4-*N*-benzoyl-2'-deoxycytidine; G, 2-*N*-acetyl-6-*O*-(3-chlorophenyl)-2'-deoxyguanosine; T, 4-*O*-phenylthymidine. Phosphate residues protected by 2-chlorophenyl groups are represented by p, and p-CNET represents phosphate residues protected by both 2-chlorophenyl and 2-cyanoethyl groups. ND, not determined.

guanine residues. Ten molecular equivalents of (*E*)-2-nitrobenzaldoxime and 9 molecular equivalents of TMG were used for each protecting group.

(2) In the second stage, the *N*-acyl protecting groups were removed from standard oligonucleotides containing only naturally occurring unmodified bases with concentrated aqueous ammonia (d = 0.88), but DBU–tetrahydrofuran–methanol (1:3:3 v/v/v) was used for oligomers containing 4-*O*-methylthymine residues. For the standard oligonucleotides, the products of treatment with (*E*)-2-nitrobenzaldoxime and TMG were concentrated under reduced pressure to a small volume and were then treated with excess concentrated aqueous ammonia (d = 0.88) for 30 h. For the sequences containing 4-*O*-methylthymine residues, the products of treatment with (*E*)-2-nitrobenzaldoxime and TMG were evaporated to a small volume and then dried by repeated evaporation of a pyridine solution (3 × 5 mL) before addition of DBU–tetrahydrofuran–methanol (1:3:3 v/v//v) (7.5 mL for each 0.1 g of dodecamer). After 60 h at room temperature, the solution was neutralized with glacial acetic acid (1.5 molecular equivalents with respect to DBU), and water (10 mL) was added. The pH of the aqueous solution was then adjusted to 6.5 with 5% aqueous acetic acid, and the solution was then extracted with ether (2 × 20 mL), dichloromethane (3 × 20 mL), and then again with ether (20 mL). It was then desalted [Sephadex G-25 (tetramer) and G-50 (dodecamers)] with ethanol–water (1:4 v/v) as eluting solvent. The oligomers were purified by reverse-phase LC (Nova Pak C18). The conditions for purification and base analysis are indicated in the legend of Figure 2.

Stability of 4-O-Methylthymidine and ^{14}C and ^{13}C Labeling of the 4-O-Methyl Group. (1) Stability of 4-O-Methylthymidine in Concentrated Aqueous Ammonia (d = 0.88). 4-*O*-Methylthymidine (ca. 1.0 mg) was suspended in concentrated aqueous ammonia (d = 0.88 mL). After 45 min at room temperature, the ammonia was evaporated under reduced pressure, and the residue was redissolved in water (1 mL). The solution was then analyzed by LC (Figure 4).

*(2) Replacement of the 4-O-Methyl Group of Protected T*GCG by $^{14}CH_3$ during Deblocking.* Fully protected T*GCG (5.04 mg, 2.1 μmol) was dissolved in a solution of (*E*)-2-nitrobenzaldoxime (20 mg; 0.12 mmol) and TMG (15 μL; 0.11 mmol) in dry acetonitrile (0.4 mL). After 40 h, the reaction mixture was evaporated to a small volume, and dry THF (0.25 mL), dry methanol (0.2 mL), [^{14}C]methanol (0.05 mL, 3321 dpm/μmol), and DBU (25 μL, 0.17 mmol) were added. The solution was stirred at room temperature for 4 days. Aqueous acetic acid (10%, 1.5 mL) was added at 0 °C, and the products were immediately chromatographed on DNA-grade Sephadex G-25. The resultant tetramer had specific radioactivity of 740 dpm/μmol (i.e., 21% replacement).

*(3) Incorporation of ^{13}C into the 4-O-Methyl Group of T*GCG by Exchange with [^{13}C]Methanol.* Fully deprotected T*GCG (10 μmol) was stirred in dry THF (0.125 mL), [^{13}C]methanol (3.1 mmol), and [^{14}C]methanol (4.67 μCi, to act as tracer) for 3 days at room temperature. The solution was evaporated to a small volume and was immediately chromatographed on Sephadex G-25. The extent of exchange was assessed by measurement of the radioactivity of the T*GCG. A total of 10% of the 4-*O*-methyl groups had been replaced.

Results and Discussion

The synthesis of oligodeoxyribonucleotides containing a 4-*O*-methylthymine residue is described in these experiments. The 4-*O*-methylthymidine (**4**; R = Me) used as starting material was prepared from the 4-(3-nitro-1,2,4-triazolo) derivative of 3',5'-bis-*O*-(methoxacetyl)thymidine (**2**). This approach leads to a high yield of pure 4-*O*-methylthymidine (Figure 1). The susceptibilities of 4-*O*-methylthymine toward nucleophilic attack (see Figure 4) and to dealkylation by acid (Singer et al., 1978) are the major problems encountered in

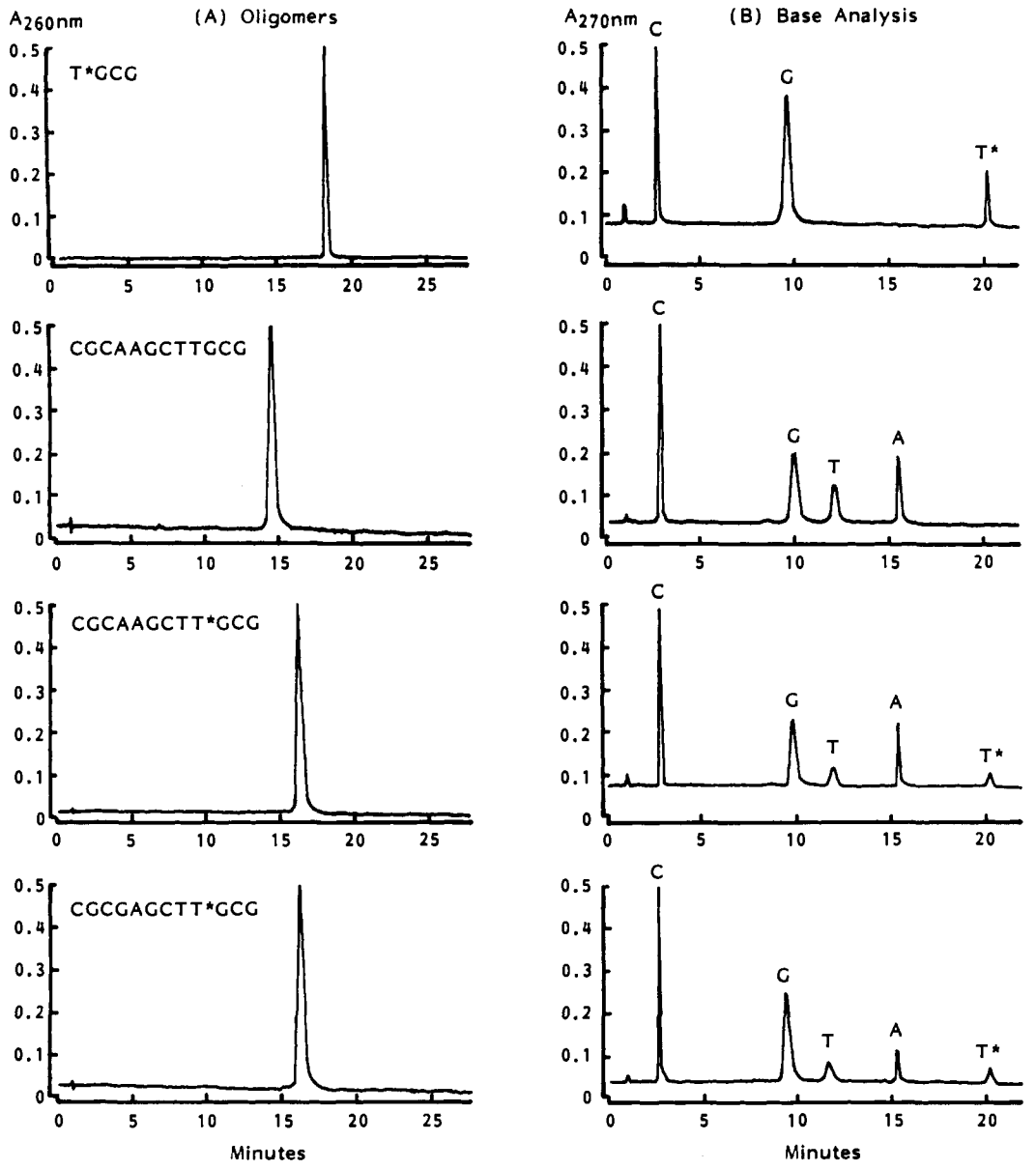


FIGURE 2: Reverse-phase LC analysis of oligodeoxynucleotides and of their constituent nucleosides. Oligonucleotides were degraded to nucleosides by treatment with snake venom diesterase and alkaline phosphatase. The chromatography was carried out on a Waters Nova Pak C18 cartridge with a flow of 3 mL/min. Oligonucleotides were chromatographed at 45 °C with a gradient of 0–11% acetonitrile in 50 mM aqueous KH_2PO_4 (pH 6.3) over 30 min. Nucleosides were chromatographed at 25 °C. The column was eluted for 8 min with 1.25% acetonitrile in 50 mM aqueous KH_2PO_4 (pH 4.5), and then the concentration of acetonitrile was increased to 12.5% over the following 14 min.

the synthesis of oligodeoxynucleotides containing 4-*O*-methylthymine residues. These problems were overcome by the use of methoxide ions (DBU–methanol) rather than ammonia to remove the *N*-acyl protecting groups and zinc bromide rather a protic acid to remove the 9-phenylxanthen-9-yl group from the protected oligonucleotide intermediates in the synthesis. The terminal 5′-OH and 3′-OH functions of the dodecamer were protected by the base-sensitive acetyl group so that these functions could be unblocked without the use of acid. Coupling yields were high (Table I), and the resulting oligonucleotides were eluted as single components by both reverse-phase and ion-exchange LC. Enzymic hydrolysis and chromatography of the resultant nucleosides gave no evidence of impurities arising from side reactions during synthesis (Figure 2).

The 500-MHz ^1H NMR spectra of T*GCG, CGCAAGCTT*GCG, and CGCGAGCTT*GCG are illustrated in Figure 3. The resonance signal assigned to the 4-*O*-methyl protons of 4-*O*-methylthymine is well resolved for T*GCG and CGCGAGCTT*GCG but is broad for CGCAAGCTT*GCG. The *O*-methyl protons resonate at δ 3.75, 3.57, and 3.62, respectively, in these three oligomers. The chemical shifts of protons in oligonucleotides are influenced by the extent of the stacking interaction. Since the 4-*O*-methylthymine residue for the tetramer is terminal, there is probably little stacking interaction, and as expected, the chemical shift (δ 3.75) is close to that of the nucleoside (δ 3.82). Thus, the appreciably lower chemical shift of the 4-*O*-methyl proton resonances in the dodecamers suggested that T* is stacked into the helix in both cases: the slight difference between the sequence with the T*·A (δ 3.62) and the T*·G (δ 3.57) base pairs suggests that the duplex with 4-*O*-methylthymidine base paired to adenine may be less stable than the duplex with 4-*O*-methylthymine base paired to guanine. It is noteworthy that 4-*O*-methylation of a thymine residue causes the 5-CH_3 and 6-H resonance signals to shift downfield. The 5-CH_3 of thymidine resonates at δ 1.75 while 5-CH_3 of 4-*O*-methylthymidine resonates at δ 1.87. Similarly, the 6-H protons resonate at δ 7.68 and 7.99, respectively.

Figure 5 shows the thermal melting curves of the parent

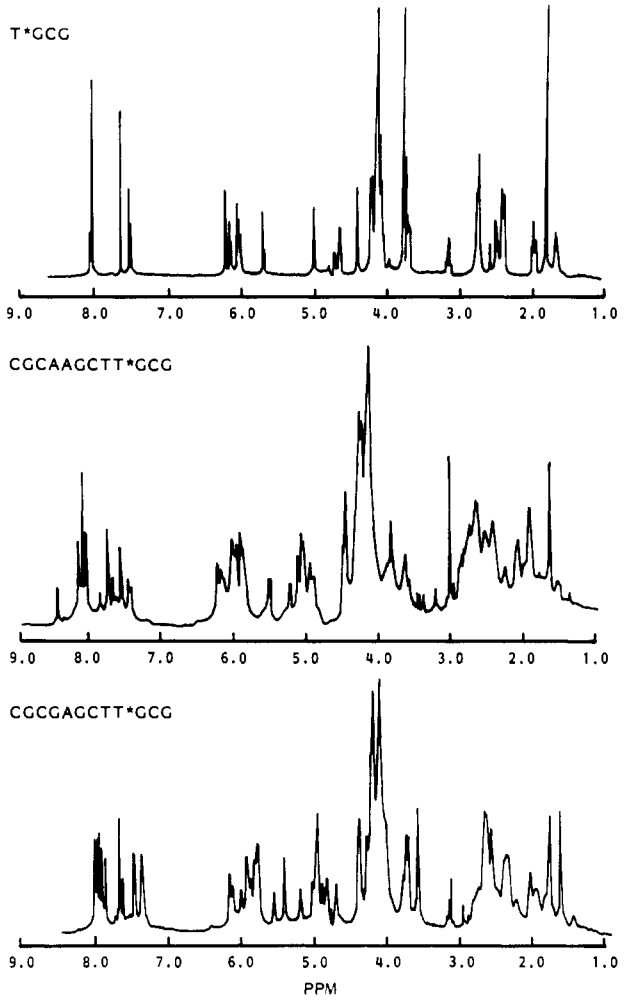


FIGURE 3: The 500-MHz ^1H NMR spectra of oligodeoxynucleotides. Oligonucleotides (400 units at 260 nm) were dissolved in 0.4 mL of D_2O containing 0.1 M NaCl, 1.0 mM EDTA, and 10 mM KH_2PO_4 (pH 7).

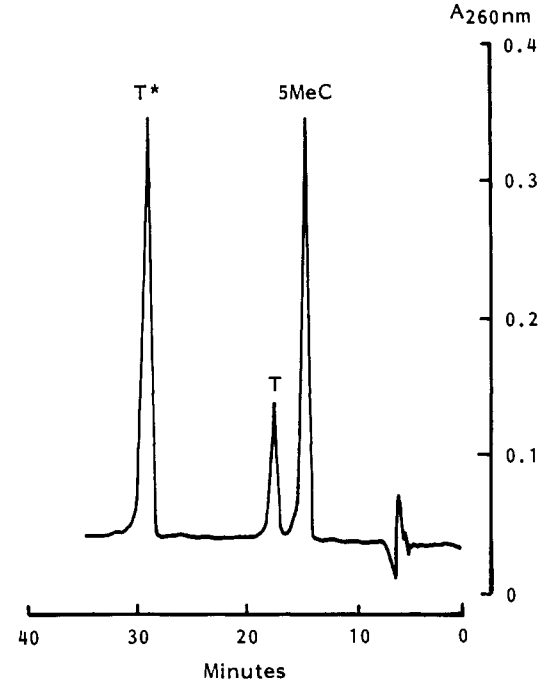


FIGURE 4: LC analysis of products after 45 min of the reaction of 4-*O*-methylthymidine in concentrated aqueous ammonia (d = 0.88) at 25 °C. The products were chromatographed on a Waters Nova Pak C18 steel column (3.9 × 150 mm) at 25 °C with a flow of 1.5 mL/min of a gradient of 5–30% acetonitrile in 0.1 M aqueous triethylammonium acetate (pH 7). Abbreviations: T*, 4-*O*-methylthymidine; 5MeC, 5-methyl-2′-deoxycytidine; T, thymidine.

oligonucleotide duplexes and those containing 4-*O*-methylthymine (T*) paired with adenine, T* paired with guanine, and 6-*O*-methylguanine (G*) paired with thymine. The solution had similar absorbances at 12 °C ($A_{1cm,260nm}$ = 1.25), and the chart axes are identical so that a direct visual comparison is possible. Melting profiles of short oligomers are very sensitive to small structural changes. For example, the two parent oligomers CGC*A*AGCT*T*GCG and CGC*G*AGCT*C*GCG shown in Figure 5 differ by only two bases (i.e., two A·T base pairs in the first sequence are substituted by two G·C base pairs in the second), yet they have appreciably different melting profiles, and the T_m's differ by about 8 °C. Melting of the oligomers containing T* or 6-*O*-methylguanine (G*) extends over a wider temperature range than that of the respective parents. In the case where T* is paired with guanine and G* paired with thymine, the melting is biphasic. In addition, the hypochromicity of the sequences containing T* and G* was less than that of the unalkylated sequences.

The lower and extended melting temperature of the oligonucleotides containing 4-*O*-methylthymine paired with either adenine or guanine suggests that the presence of this alkylated base destabilizes the duplex, and the lower total hypochromicity suggests that the presence of the methylated base decrease the overall extent of base stacking. We do not know why the melting curves were biphasic. NMR studies show clearly that the greater part, and perhaps all, of the oligomer is present in solution as a DNA–like duplex, and therefore we tentatively suggest that the biphasic curve may be caused by preferential melting of the base pairs around the methylated base. Gaffney et al. (1984) have reported that a dodecanucleotide containing G* base paired with T had a lower T_m than the reference oligomer with G base paired with C, but unfortunately, they did not illustrate, or comment upon, the shape of the melting curves or the relative hypochromicity of the sequences. These changes in melting profile and hypochromicity (Figure 5) conflict with the report by Singer et al. (1983) that the presence of 4-*O*-methylthymine in poly(dA·dT) had no effect on melting or hypochromicity; they are also apparently inconsistent with the proposal that 4-*O*-methylthymine forms a stable base pair with guanine (Abbott & Saffhill, 1977; Singer et al., 1983, 1984). However, they are consistent with deductions from the crystal structure of 4-*O*-methylthymidine (Brennan et al., 1986). In the crystal the 4-*O*-methyl group laid in the same plane as the pyrimidine ring and the methyl group adopted a syn conformation relative to N-3. In addition, there was no base–base stacking in the crystal. The proposed T*·G base pair involves N-3 as an acceptor for a hydrogen bond with the N-1 H of guanine (Abbott & Saffhill, 1977). The syn conformation would not allow the proposed T*·G base pairing because of steric interaction between the 4-*O*-methyl group and *O*-6 of the guanine residue and because of the reduced accessiblity of N-3 of 4-*O*-methylthymine residues for hydrogen bonding. The proposed T*·G mispairing requires the 4-*O*-methyl group to be in the anti conformation. This is possible but is energetically unfavorable (Brennan et al., 1986). The view that 4-*O*-methylthymine mispairs with guanine was gained indirectly from experiments in which guanine was misincorporated when DNA or RNA synthesis was carried out on substrates of poly(dA·dT) or oligo(dA), each containing a small percentage of 4-*O*-methylthymine residues. Both poly(dA·dT) and poly(dA)·poly(dT) form double helices of unusual structure

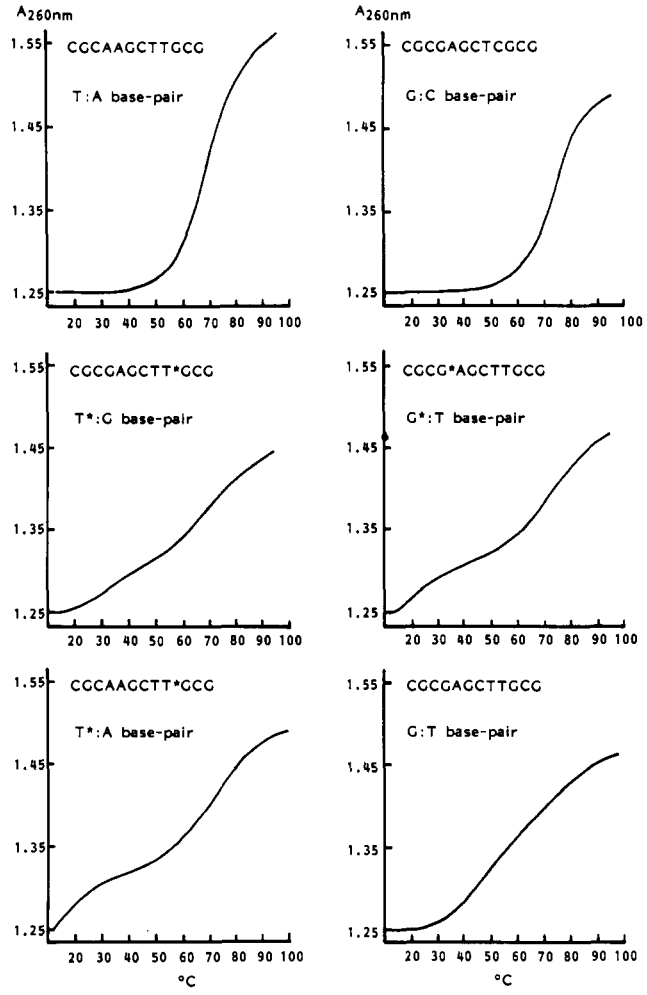


FIGURE 5: Thermal melting curves of oligonucleotides containing 4-*O*-methylthymidine (T*) base paired to A and to G, compared to the melting curved of the parent oligomer and analogous oligomers with 6-*O*-methylguanine (G*) base paired to T. Oligonucleotides containing G* were synthesized by solution-phase methods (Li et al., 1987). All oligonucleotide solutions had an initial absorbance of 1.25 at 260 nm at 12 °C in buffer: 1 M NaCl, 10 mM KH_2PO_4, and 1 mM EDTA (pH 7). The cuvette was heated at 1 deg/min.

[see Saenger (1984)], and therefore, these experiments cannot be regarded as conclusive evidence for the formation of the stable 4-*O*-methylthymine–guanine base pairs. Base pairing can be investigated by NMR spectroscopy (Patel et al., 1985), and therefore, NMR spectroscopic measurements are being carried out on these dodecamers in order to get direct and conclusive information on the base-pairing properties of 4-*O*-methylthymine.

Registry No. Px-*Cp*, 79527-01-8; Ac-*Cp*, 106251-60-9; Px-*Tp*, 106251-61-0; Px-*Ap*, 106251-62-1; Px-T**p*, 106251-63-2; HO-*G*-Ac, 106251-64-3; HO-*Tp*-CNET, 106251-65-4; HO-*Gp*-CNET, 106251-66-5; HO-*Ap*-CNET, 69434-23-7; HO-*CpG*-Ac, 106266-51-7; HO-*CpTp*-CNET, 106251-67-6; HO-*CpGp*-CNET, 106251-68-7; HO-*CpAp*-CNET, 106251-69-8; Ac-*CpGp*, 106251-70-1; Px-*TpGp*, 106266-52-8; Px-*ApGp*, 106266-53-9; Px-T**pGp*, 106251-71-2; HO-T**pGpCpG*-Ac, 106266-54-0; HO-*TpGpCpG*-Ac, 106251-72-3; Px-*ApGpCpTp*, 106251-73-4; Ac-*CpGpCpAp*, 106251-74-5; Ac-*CpGpCpGp*, 106251-75-6; HO-*ApGpCpTp*T**pGpCpG*-Ac, 106266-24-4; HO-*ApGpCpTpTpGpCpG*-Ac, 106266-25-5; Ac-*CpGpCpApApGpCpTp*T**pGpCpG*-Ac, 106295-46-9; Ac-*CpGpCpGpApGpCpTp*T**pGpCpG*-Ac, 106266-26-6; Ac-*CpGpCpApApApGpCpTpTpGpCpG*-Ac, 106318-36-9; T*GCG, 106266-55-1; CGCAAGCTTGCG, 106295-47-0; CGCAAGCTT*GCG, 106251-76-7; CGCGAGCTT*GCG, 106266-56-2; T*, 50591-13-4; PxCl, 42506-03-6; *C*, 4836-13-9; *T*, 92447-15-9; *A*, 4546-72-9; Px-*C*, 69075-27-0; Px-*T*, 92447-16-0; Px-*A*, 78699-78-2; Px-T*, 106251-77-8; 3′,5′-bis-*O*-(methoxyacetyl)thymidine, 92447-12-6.

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