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Oligonucleotide *N*-Alkylphosphoramidates: Synthesis and Binding to Polynucleotides†

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ABSTRACT: A few different methods for the preparation of oligonucleotide *N*-alkylphosphoramidates were compared directly. One of these, involving the use of protected nucleoside phosphites as building blocks, provided the requisite *N*-alkylphosphoramidates via oxidation of the intermediate dinucleoside methyl phosphites with iodine in the presence of the appropriate alkylamine. This method was found to have several attractive features, including the use of building blocks identical with those employed for the synthesis of DNA and compatibility with procedures and instruments employed for the stepwise synthesis of oligonucleotides by solution and solid-phase methods. This procedure was used to make several di-, tri-, and tetranucleotide *N*-alkylphosphoramidates derived from deoxyadenosine and thymidine; alkyl substituents included *N*,*N*-dimethyl, *N*-butyl, *N*-octyl, *N*-dodecyl, and *N*-(5-aminopentyl). The aminoalkyl derivative of d(TpT) (**24**) was used to demonstrate the feasibility of introducing an intercalative agent to the alkylphosphoramidate moiety of such derivatives. The oligonucleotide *N*-alkylphosphoramidates were separated into their component diastereomers and characterized structurally by a number of techniques including circular dichroism, high-field ^{1}H NMR spectroscopy, FAB mass spectrometry, and enzymatic digestion to authentic nucleosides and nucleotides. Physicochemical characterization of several di- and trinucleotide alkylphosphoramidates revealed that the adenine nucleotide analogues formed stable complexes with poly(thymidylic acid). The stabilities of these complexes were found to increase with increasing chain length of the *N*-alkylphosphoramidate substituents. The finding that *N*-alkylphosphoramidate substituents can enhance the binding of certain oligonucleotides to their complementary polynucleotides suggests the existence of a novel source of polynucleotide affinity.

The preparation of oligonucleotides containing modified phosphodiester linkages is of current interest as a source of sequence-specific nucleic acid probes (Miller et al., 1981; Letsinger & Schott, 1981; Asseline et al., 1984; Chu & Orgel, 1985; Dreyer & Dervan, 1985; Thuong et al., 1987). Nucleoside phosphoramidates have been prepared previously by several procedures involving both tri- and pentavalent phosphorous intermediates. These have included the condensation of nucleoside phosphate diesters with amines in the presence of triphenylphosphine–CCl_4 (Appel 1975; Stec, 1983), nucleophilic substitution of nucleoside phosphate triesters with alkylamines (Meyer et al., 1973; Juodka & Smrt, 1974; Letsinger et al., 1986), addition of alkyl and aryl azides to phosphites (Cramer et al., 1972; Letsinger & Schott, 1981), as well as the oxidation of intermediate nucleoside phosphites (Nemer & Ogilvie, 1980a,b) or nucleoside hydrogen phosphonate diesters (Froehler, 1986) with iodine in the presence of alkylamines.

Presently, we describe experiments that define the scope and utility of such transformations for the preparation of oligonucleotide phosphoramidates, by both solution and solid-phase techniques. Also described is chromatographic resolution and analysis of the formed diastereomers and their analysis by spectral and degradative techniques.

The association of a series of diadenosine *N*-alkylphosphoramidates with poly(thymidylic acid) was characterized by measurement of T_m and hypochromicity values, as well as by determination of the stoichiometry of association. These measurements indicated that the *N*-alkyl groups promoted the binding of the diadenosine *N*-alkylphosphoramidate derivatives to poly(T) and that affinity increased with increasing alkyl

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chain length. The implications of this novel source of affinity for the design of sequence-specific nucleic acid probes is discussed.

Experimental Procedures

Thymidine and tetrazole were obtained from Aldrich Chemicals. 2′-Deoxyadenosine and 3′-*O*-benzoylthymidine were purchased from Sigma Chemicals as were poly(dA) and poly(T). 5′-(Dimethoxytrityl)-2′-deoxyadenosine was obtained from Bachem. Anhydrous acetonitrile was distilled from calcium hydride and stored over 4A molecular sieves. Anhydrous tetrahydrofuran was heated at reflux over lithium aluminum hydride for 1 h prior to use. Oligonucleotide synthesis was carried out on a manual polynucleotide synthesizer (Matteucci & Caruthers, 1981) using Vydac TP-20 spherical silica gel (The Separations Group) as a solid support. Nuclease P1 (*Penicillium citrinum*; 1 unit catalyzes the hydrolysis of 1 μmol of phosphodiester linkages in yeast RNA in 1 min at 37 °C) and alkaline phosphatase [calf intestine; 1 unit catalyzes the hydrolysis of 1 μmol of *p*-nitrophenyl phosphate in 1 min at pH 10.4 (glycine buffer) and 37 °C] were obtained from Boehringer-Mannheim. Chromatographic separations were carried out on silica gel columns [Merck silica gel 60, 70–230 mesh (230–400 mesh for flash chromatography)] or TLC[1] plates (Merck silica gel 60, F-254, 0.25- or 2-mm thickness). HPLC analysis was carried out on an Alltech 10-μm C_{18} column (250 × 4.6 mm) using CH_3CN–H_2O mixtures (either 40 mM triethylammonium acetate, pH 6.0, or 0.1 M ammonium formate buffers). For preparative isolations, the appropriate fractions were collected and concentrated under diminished pressure and then diluted with water and desalted on a Bond Elut C_{18} cartridge (Analytichem International).

NMR spectra were obtained on Varian EM-390 or Nicolet NT-360 spectrometers. Ultraviolet spectra were obtained on Cary 15 or 17 spectrophotometers. Circular dichroism spectra were obtained on a Jasco J-500C spectrometer. Melting profiles were obtained on a Perkin-Elmer Lambda 5 spectrophotometer.

5′-O-(Methoxytrityl)thymidine 3′-(O-Methyl N,N-diisopropylphosphoramidite) (**15**, *R* = *MTr*). A solution containing 772 mg (1.50 mmol) of 5′-*O*-(*p*-methoxytrityl)thymidine (Schaller et al., 1963) in 8 mL of CH_2Cl_2 was treated with 1.75 mL (10.0 mmol) of *N,N*-diisopropylethylamine. Methyl *N,N*-diisopropylphosphoramidochloridite (400 μL; 2.25 mmol) was added slowly, and the combined solution was maintained at 25 °C for 20 min prior to addition of 100 mL of CH_2Cl_2. The reaction mixture was extracted with 150 mL of saturated aqueous $NaHCO_3$, and the organic phase was dried (Na_2SO_4) and concentrated. The resulting foam was purified by silica gel flash column chromatography (Still et al., 1978); elution was with 6:4 CH_2Cl_2–hexane. The appropriate fractions were combined and concentrated under diminished pressure to afford the desired product as a gum. The product was dissolved in 6 mL of toluene and added dropwise to 200 mL of cold (−78 °C) hexane, which effected precipitation of the product as a white powder. Filtration afforded 979 mg (97%) of the activated nucleoside phosphoramidite, silica gel TLC R_f 0.85 (ethyl acetate).

5′-O-(Methoxytrityl)thymidylyl(3′→5′)-3′-O-benzoylthymidine (**2**). A solution containing 338 mg (0.5 mmol) of 5′-*O*-(*p*-methoxytrityl)thymidine 3′-(O-methyl-*N,N*-diisopropylphosphoramidite) in 2 mL of dry CH_3CN was treated with 140 mg (2.0 mmol) of tetrazole and 115 mg (0.33 mmol) of 3′-*O*-benzoylthymidine (de Rooji et al., 1979) under N_2 at 25 °C for 15 min. The phosphite intermediate was oxidized by addition of an aqueous pyridine solution containing 0.1 M iodine, and the reaction mixture was partitioned between CH_2Cl_2 and H_2O. The dried (Na_2SO_4) organic phase was concentrated, and the product was purified by flash chromatography on silica gel; elution was with mixtures of CH_2Cl_2 and ethyl acetate. Dinucleoside monophosphate **1** was isolated as a colorless foam (~310 mg) and was employed directly for the preparation of dinucleoside monophosphate **2**.

Demethylation of **1** was carried out by treatment with 4 mL of dioxane, 2 mL of triethylamine, and 2 mL of thiophenol at 25 °C for 3 h. The reaction mixture was concentrated under diminished pressure, and the residue was triturated with hexane. The residue was purified by flash chromatography on silica gel; elution was with mixtures of CH_2Cl_2 and CH_3OH containing 1% triethylamine. The appropriate fractions were combined and concentrated and then applied to a 10-mL Amberlite IR-120 column (pyridinium form) as a methanolic solution. Elution with methanol afforded dinucleoside monophosphate **2** in quantitative yield as the pyridinium salt. The product was isolated as a powder following precipitation from a large volume of *n*-hexane–ether, silica gel TLC R_f 0.48 (100:10:2 CH_2Cl_2–CH_3OH–triethylamine).

P-(n-Butylamino)-P-deoxy-5′-O-(methoxytrityl)thymidylyl(3′→5′)-3′-O-benzoylthymidine (**3**). A mixture of 68 mg (~70 μmol) of pyridinium dinucleoside monophosphate **2** and 52 mg (0.20 mmol) of triphenylphosphine was rendered anhydrous by repeated evaporations of portions of dry acetonitrile. The mixture was then dissolved in 1.5 mL of acetonitrile and 0.2 mL of dry pyridine and treated with 17 μL (27 mg; 0.18 mmol) of CCl_4. The reaction mixture was stirred at 25 °C for 4 h and then treated with 150 μL (110 mg; 1.5 mmol) of *n*-butylamine and maintained at 25 °C for an additional 30 min. The reaction mixture was concentrated under diminished pressure, and the residue was purified by preparative silica gel TLC; development was with 10:1 CH_2Cl_2–CH_3OH. The product was isolated as a yellow powder, yield 16 mg (24%): ^1H NMR [Table I of the supplementary material (see paragraph at end of paper regarding supplementary material)].

P-(n-Butylamino)-P-deoxy-5′-O-(methoxytrityl)thymidylyl(3′→5′)thymidine (**4**). Dinucleoside monophosphate **3** (20 mg; 20 μmol) was dissolved in 2 mL of 1:1 CH_3OH–*tert*-butylamine. The reaction mixture was maintained at 40–45 °C for 2 days and then concentrated under diminished pressure. The residue was purified by preparative silica gel TLC; development was with 9:1 CH_2Cl_2–CH_3OH. Dinucleoside monophosphate **4** was isolated as an off-white gummy solid following lyophilization, yield 15 mg (84%): silica gel TLC R_f 0.50 (10:1 CH_2Cl_2–CH_3OH); ^1H NMR [$CDCl_3$, $(CH_3)_4Si$] δ 0.8–0.9 (m), 1.2–1.35 (m), 1.32 (s), 1.81 (s), 2.1–2.9 (m), 3.00 (m), 3.4–4.3 (m), 3.71 (s), 4.4 (m), 4.6 (m), 5.04 (m), 6.07 (t), 6.35 (m), 6.78 (d), 7.2–7.4 (m), 7.50 (s), and 8.46 (br s).

Synthesis of P-(n-Butylamino)-P-deoxy-5′-O-(methoxytrityl)thymidylyl(3′→5′)thymidine (**4**) *on a Solid Support.* A 100-mg sample of Vydac TP-20 silica gel was derivatized with ~3 μmol of fully protected dinucleoside monophosphate **5** by the method of Matteucci and Caruthers (1981). Demethylation of the phosphate ester was accomplished by treatment of the derivatized support with 2 mL of 1:2:1 tri-

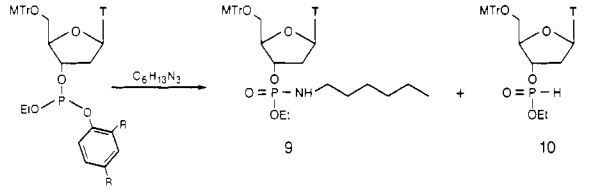
[1] Abbreviations: TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography, DMTr, dimethoxytrityl; MTr, methoxytrityl; FAB mass spectrometry, fast atom bombardment mass spectrometry; CD, circular dichroism; T_m, melting temperature.

ethylamine–dioxane–thiophenol. The mixture was shaken at 25 °C for 3 h and then filtered and washed successively with dioxane, pyridine, methanol, and ether prior to overnight drying.

The silica gel containing putative **6** was then treated with 52 mg (0.20 mmol) of triphenylphosphine, 17 μL (27 mg; 0.18 mmol) of CCl_4, 8 μL (8 mg; 0.1 mmol) of dry pyridine, and enough dry acetonitrile to permit the mixture to be shaken at 25 °C for 3 h. The mixture was then treated with 100 μL (73 mg; 1.0 mmol) of *n*-butylamine and maintained at 25 °C for an additional 2 h. Putative dinucleoside monophosphate **7** was treated with 3 mL of 1:1 CH_3OH–*tert*-butylamine at 40 °C for 16 h. The silica gel was filtered and washed extensively with methanol. The filtrate was concentrated, and the residue was purified by preparative silica gel TLC. The product (2.1 μmol; ~70% yield) was isolated as a solid, λ_{max} (pH 7) 268 nm. This material was shown to be identical with dinucleoside monophosphate **4** prepared via solution-phase synthesis (vide supra).

5′-O-(Methoxytrityl)thymidine 3′-(O-Ethyl N-n-hexylphosphoramidate) (**9**). A solution containing 619 mg (1.20 mmol) of 5′-*O*-(methoxytrityl)thymidine (Schaller et al., 1963) in 3 mL of dry tetrahydrofuran was added dropwise to a reaction vessel (N_2, −78 °C) containing 220 μL (343 mg; 1.30 mmol) of 2,4-dichlorophenyl phosphorodichloridite (Tolkmith, 1958), 280 μL (274 mg; 3.5 mmol) of dry pyridine, and 5 mL of tetrahydrofuran. The reaction mixture was stirred at −78 °C for 10 min and then treated with 100 μL (79 mg; 1.70 mmol) of absolute ethanol and allowed to warm to room temperature. The reaction mixture was partitioned between CH_2Cl_2 and water, and the organic extract was dried (Na_2SO_4) and concentrated under diminished pressure.

Putative nucleoside **8c** was dissolved in 15 mL of dry tetrahydrofuran and treated with 1.5 mL of *n*-hexyl azide (Grundman, 1965) at 25 °C for 4 days. The reaction mixture

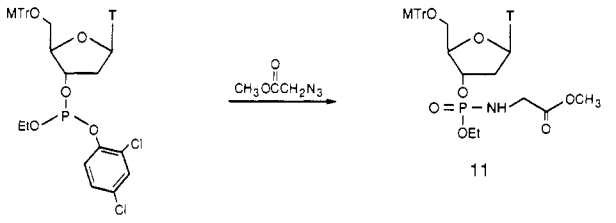


8 a R, R′ = H
b R = H, R′ = Cl
c R, R′ = Cl

was concentrated under diminished pressure, and the residue was triturated with 30 mL of *n*-hexane. Purification of the crude product was effected by flash chromatography on silica gel (Still et al., 1978); elution was with increasing amounts of ethyl acetate in CH_2Cl_2 and then with 10:1 CH_2Cl_2–CH_3OH. 5′-*O*-(Methoxytrityl)thymidine 3′-(*O*-ethyl *N*-*n*-hexylphosphoramidate) (**9**) was isolated as a white powder, yield 37? mg (54%): silica gel TLC R_f 0.47 (ethyl acetate), 0.53 (10:1 CH_2Cl_2–CH_3OH); ^1H NMR [$CDCl_3$, $(CH_3)_4Si$] δ 0.79 (m, 3), 1.1–1.3 (m, 11), 1.33 (s, 3), 2.2–2.9 (m, 4), 3.33 (br s, 2), 3.66 (s, 3), 3.94 (q, 2), 4.18 (m, 1), 5.02 (m, 1), 6.35 (dd, 1), 6.7–7.4 (m, 14), 7.45 (s, 1), and 9.35 (br s, 1).

5′-O-(Methoxytrityl)thymidine 3′-[O-Ethyl N-(carbomethoxymethyl)phosphoramidate] (**11**). A solution containing 129 mg (0.25 mmol) of 5′-*O*-(methoxytrityl)thymidine (Schaller et al., 1963) in 1.5 mL of dry tetrahydrofuran was added dropwise over a period of 5 min to a reaction vessel (N_2, −78 °C) containing 47 μL (73 mg; 0.28 mmol) of 2,4-dichlorophenyl phosphorodichloridite (Tolkmith, 1958), 64 μL (63 mg; 0.80 mmol) of dry pyridine, and 2 mL of dry tetrahydrofuran. The reaction mixture was stirred at −78 °C for 10 min and then treated with 24 μL (19 mg; 0.40 mmol) of absolute ethanol and allowed to warm to room temperature. The reaction mixture was partitioned between CH_2Cl_2 and water, and the organic phase was dried (Na_2SO_4) and concentrated.

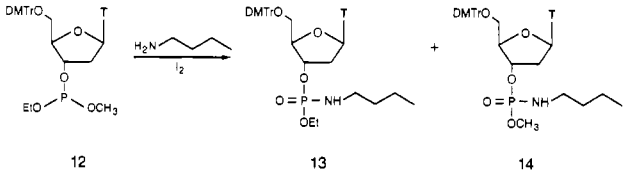
Putative **8c** was dissolved in 3 mL of dry tetrahydrofuran and treated with 375 μL of methyl azidoacetate (Grundman, 1965) at 25 °C for 2 days. The mixture was concentrated



8c

under diminished pressure, and the residue was purified by silica gel flash column chromatography; elution was with CH_2Cl_2 containing increasing amounts of CH_3OH (up to 10%). 5′-*O*-(Methoxytrityl)thymidine 3′-[*O*-ethyl *N*-(carbomethoxymethyl)phosphoramidate] (**11**) was isolated as a white powder, yield 118 mg (68%): silica gel TLC R_f 0.36 (ethyl acetate), 0.50 (10:1 CH_2Cl_2–CH_3OH); ^1H NMR [$CDCl_3$, $(CH_3)_4Si$] δ 1.15 (t, 3), 1.28 (s, 3), 2.0–2.6 (m, 2), 3.3–3.7 (m, 10), 3.96 (q, 2), 4.17 (m, 1), 5.09 (m, 1), 6.36 (dd, 1), 6.7–7.4 (m, 14), 7.47 (s, 1), and 9.39 (br s, 1).

5′-O-(Dimethoxytrityl)thymidine 3′-(O-Ethyl N-n-butylphosphoramidate) (**13**). A solution containing 338 mg (0.62 mmol) of 5′-*O*-(dimethoxytrityl)thymidine (Schaller et al., 1963) in 1.8 mL of dry tetrahydrofuran was added dropwise to a reaction vessel (N_2, −30 °C) containing 57 μL (80 mg; 0.60 mmol) of methyl phosphorodichloridite (Martin & Pizzolato, 1950), 160 μL (158 mg; 2.0 mmol) of dry pyridine, and 4 mL of tetrahydrofuran. The reaction mixture was stirred at −30 °C for 5 min and then treated with 100 μL (79 mg; 1.70 mmol) of absolute ethanol. The reaction mixture was allowed to warm to 0 °C and then added to ice water and extracted with portions of $CHCl_3$. The combined $CHCl_3$ extract was dried (Na_2SO_4) and concentrated. Putative nucleoside phosphite derivative **12** was dried carefully by coe-



12 13 14

vaporation of portions of dry toluene and tetrahydrofuran and then treated with a solution containing 200 mg (0.8 mmol) of iodine in 2 mL of dry tetrahydrofuran and 1 mL of *n*-butylamine. The reaction mixture was stirred at 25 °C for 5 min and then partitioned between $CHCl_3$ and aqueous $NaHSO_3$. The organic extract was dried (Na_2SO_4) and concentrated under diminished pressure. The residue was purified by silica gel flash chromatography (20-g column); elution was with 0–2% CH_3OH in ethyl acetate. 5′-*O*-(Dimethoxytrityl)thymidine 3′-(*O*-ethyl *N*-*n*-butylphosphoramidate) (**13**) was obtained as a white solid by precipitation from a large volume of hexane, yield 196 mg (45%): silica gel TLC R_f 0.26 (ethyl acetate), 0.50 (20:1 $CHCl_3$–CH_3OH); ^1H NMR [$CDCl_3$, $(CH_3)_4Si$] δ 0.8 (t, 3), 1.05–1.2 (m, 10), 2.15–2.9 (m, 4), 3.32 (br s, 2), 3.67 (s, 6), 3.88 (q, 2), 4.15 (m, 1), 5.00 (m, 1), 6.35 (dd, 1), 6.65–7.3 (m, 13), 7.49 (s, 1), and 9.03 (s, 1).

Nucleoside phosphoramidate **13** was characterized further by ^1H NMR following deprotection (81% yield) with 80%

Table I: Preparation of Dinucleoside *N*-Alkylphosphoramidates **17** and **19**[a]

compd	base	R	R′	yield (%)	R_f value[b]
3	T	MTr	n-C_4H_9	48	
17a	T	MTr	n-C_8H_{17}	76	
17b	T	MTr	n-$C_{12}H_{25}$	56	
17c	A^{Bz}	DMTr	n-C_4H_9	54	
17d	A^{Bz}	DMTr	n-C_8H_{17}	83	
17e	A^{Bz}	DMTr	n-$C_{12}H_{25}$	79	
17f	A^{Bz}	DMTr	$(CH_3)_2$[c]	87	
19g	T	H	n-C_4H_9	58	0.45
19a	T	H	n-C_8H_{17}	77	0.60
19b	T	H	n-$C_{12}H_{25}$	100	0.66
19c	A	H	n-C_4H_9	81	0.26
19d	A	H	n-C_8H_{17}	59	0.38
19e	A	H	n-$C_{12}H_{25}$	77	0.50
19f	A	H	$(CH_3)_2$[c]	94	0.26

[a] See Experimental Procedures for methods of preparation. [b] Silica gel TLC, development with 5:1 CH_2Cl_2–CH_3OH. [c] *N*,*N*-Dimethylphosphoramidate derivative.

aqueous HOAc (data not shown).

General Procedure for the Preparation of Dinucleoside Phosphoramidates **17**. In a typical experiment, 0.3 mmol of 5′-hydroxy-3′-O-benzoylated nucleoside **16** (Eckstein, 1967; Ogilvie, 1973) was treated with 0.40–0.45 mmol of nucleoside 3′-(*O*-methyl *N*,*N*-diisopropylphosphoramidite) **15** in 1.5 mL of dry acetonitrile containing 1.6–2.0 mmol of tetrazole. The reaction mixture was maintained at 25 °C for 20–30 min and then partitioned between CH_2Cl_2 and water. The CH_2Cl_2 layer was dried (Na_2SO_4) and concentrated under diminished pressure. After codistillation of several portions of dry tetrahydrofuran from the residue, the resulting foam was dissolved in 2 mL of dry tetrahydrofuran and treated with 0.40–0.45 mmol of iodine in 2 mL of tetrahydrofuran and 1 mL of alkylamine. After 5 min, the reaction mixture was poured into aqueous sodium bisulfite and extracted with portions of CH_2Cl_2. The CH_2Cl_2 extract was dried (Na_2SO_4) and concentrated. The residue was purified by silica gel flash chromatography (Still et al., 1978) or by preparative silica gel TLC using CH_2Cl_2–CH_3OH mixtures.

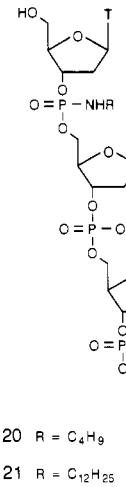
In this fashion, 0.45 mmol (304 mg) of 5′-*O*-(methoxytrityl)thymidine 3′-(*O*-methyl *N*,*N*-diisopropylphosphoramidite) and 0.30 mmol (114 mg) of 3′-*O*-benzoylthymidine afforded P-(*n*-octylamino)-*P*-deoxy-5′-(*O*-methoxytrityl)thymidylyl(3′→5′)-3′-*O*-benzoylthymidine (**17a**) as a white solid following chromatographic purification and precipitation from a large volume of 2:1 hexane–ether, yield 237 mg (76%): silica gel TLC R_f 0.36 and 0.43 (ethyl acetate), 0.32 and 0.36 (10:1 CH_2Cl_2–CH_3OH); ^1H NMR [$CDCl_3$, $(CH_3)_4Si$] δ 0.87 (t), 1.2–1.3 (m), 1.37 (s), 1.5 (m), 1.92 (s), 1.94 (s), 2.2–3.0 (m), 3.3–3.6 (m), 3.77 (s), 3.79 (s), 4.2–4.4 (m), 5.15 (m), 5.47 (d), 5.56 (d), 6.3–6.5 (m), 6.8–6.9 (2 d), 7.2–7.6 (m), 7.53 (s), 8.0 (2 d), and 8.4 (br s). The yields of the other dinucleoside phosphoramidates prepared are given in Table I; ^1H NMR values for these compounds are also provided (Table I of the supplementary material).

Virtually identical yields of these products were obtained in one-pot procedures that omitted isolation of the intermediate dinucleoside phosphites.

General Procedure for Deprotection of Dinucleoside Phosphoramidates **17**. In a typical experiment, 0.1 mmol of the fully protected dinucleoside phosphoramidate was debenzoylated to afford **18** by stirring overnight with 10 mL of 1:1 *tert*-butylamine–methanol at 45–50 °C. The solvent was removed under diminished pressure, and the residue was purified by silica gel flash chromatography or by preparative silica gel TLC using CH_2Cl_2–CH_3OH mixtures. Detritylation was effected by treatment with 2 mL of 80% aqueous acetic acid at 25 °C for 1 h (dimethoxytrityl derivatives) or 16 h (monomethoxytrityl derivatives), affording deprotected dinucleoside phosphoramidate **19**. Following removal of acetic acid under diminished pressure, the residue was coevaporated with portions of toluene and then purified by preparative silica gel TLC (development with CH_2Cl_2–CH_3OH mixtures).

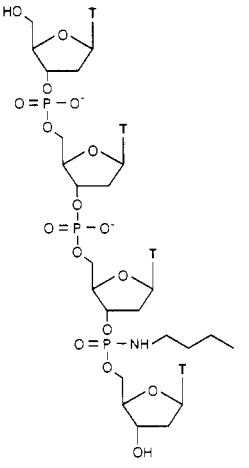
In this fashion, 100 μmol (104 mg) of *P*-(*n*-octylamino)-*P*-deoxy-5′-*O*-(methoxytrityl)thymidylyl(3′→5′)-3′-*O*-benzoylthymidine (**17a**) was deprotected to provide *P*-(*n*-octylamino)-*P*-deoxythymidylyl(3′→5′)thymidine (**19a**) as a white solid following preparative silica gel TLC (10:1 CH_2Cl_2–CH_3OH) and lyophilization from dioxane, yield 53 mg (77%): silica gel TLC R_f 0.07 (10:1 CH_2Cl_2–CH_3OH), 0.60 (5:1 CH_2Cl_2–CH_3OH); ^1H NMR [$CDCl_3$, $(CH_3)_4Si$] δ 0.87 (t), 1.3 (m), 1.5 (m), 1.90 (s), 1.92 (s), 2.1–2.9 (m), 3.6 (m), 3.8 (m), 4.0–4.2 (m), 4.45 (m), 5.08 (m), 6.30 (dd), 7.32 (s), 7.41 (s), and 7.68 (s). The yields of the other dinucleoside phosphoramidates prepared are given in Table I; ^1H NMR values for these compounds are also summarized (Table II of the supplementary material).

Synthesis of d(TpTpTpT) Phosphoramidates **20–22**. The requisite tetrathymidylate derivatives were prepared on a manual polynucleotide synthesizer as described by Matteucci and Caruthers (1981). Derivatized silica gel (71 μmol of



20 R = C_4H_9

21 R = $C_{12}H_{25}$



22

DMTr groups/g of silica gel) was used in 400-mg columns. Individual couplings were carried out as described (Matteucci & Caruthers, 1981) by using 5′-*O*-(dimethoxytrityl)thymidine 3′-(*O*-methyl *N*,*N*-diisopropylphosphoramidite) and tetrazole in tetrahydrofuran. Oxidation of individual phosphite bonds was also carried out as described by using 0.1 M I_2 in 2:1:1 tetrahydrofuran–H_2O–lutidine, except that introduction of the *N*-alkylphosphoramidate linkage was accomplished by substitution of 0.1 M I_2 in 2:1 tetrahydrofuran–alkylamine for introduction of the appropriate internucleotide bond.

After completion of each tetranucleotide synthesis the silica gel containing the fully protected tetranucleotide was treated with 2 mL of 2:1:1 dioxane–thiophenol–triethylamine at 25 °C for 90 min. The silica gel was washed successively with dioxane, CH_3OH, and CH_2Cl_2, after which the tetranucleotide was hydrolyzed from the support by treatment overnight with 2 mL of 1:1 *tert*-butylamine–CH_3OH at 45 °C, and the tritylated oligomers were purified by preparative HPLC on a 10-μm C_{18} column; elution was effected with 0.04 M triethylammonium acetate, pH 6.9, containing 35% (for DMTr-**22**), 38% (for DMTr-**20**), or 51% (for DMTr-**21**) acetonitrile. Purification by HPLC also effected separation of the diastereomers (retention times: 10.9 and 15.8 min for

DMTr-**20**; 9.7 and 12.5 min for DMTr-**21**; 10.3 and 14.0 min for DMTr-**22**). Approximately 20% of each crude tetranucleotide preparation was purified by HPLC, yielding in each case ~8 A_{260} units of the individual diastereomers.

Deprotection of individual diastereomers of DMTr-**20–22** was carried out with 1 mL of 80% aqueous HOAc at 25 °C overnight. The solvent was concentrated under diminished pressure, and the residue was coevaporated with portions of toluene and triturated with ether. Purification was effected by C_{18} reverse-phase HPLC using CH_3CN in 0.04 M triethylammonium acetate, pH 6.9. Approximately 5 A_{260} units of each tetranucleotide was obtained.

Enzymatic Digestion of d(TpTpTpT) Phosphoramidates **20–22**. Digestion of tetranucleotides **20–22** (0.1–0.2 A_{260} unit scale) was carried out in 100 μL of 0.25 M Tris·HCl, pH 7.0, containing 6–9 units of *P. citrinum* nuclease P1. The digestions were carried out at 37 °C for 4 h, and half of the reaction mixtures were analyzed by HPLC. The remaining half of each reaction mixture was combined with 2 units of calf intestine alkaline phosphatase and incubated for an additional 30 min prior to HPLC analysis.

Also analyzed in the same fashion was a diastereomeric mixture of *P*-(*n*-butylamino)-*P*-deoxythymidylyl(3′→5′)thymidine.

6-Chloro-9-(p-chlorophenoxy)-2-methoxyacridine. A reaction mixture consisting of 1.39 g (5.0 mmol) of 6,9-dichloro-2-methoxyacridine and 3.0 g (23 mmol) of *p*-chlorophenol was heated at 80 °C for 4 h. The hot reaction mixture was poured into hot 5% aqueous NaOH. The combined solution was stirred until a fine yellow crystalline precipitate appeared. The crystals were filtered, washed to neutrality with water, and dried at 70 °C in vacuo. Recrystallization from pyridine afforded 6-chloro-9-(*p*-chlorophenoxy)-2-methoxyacridine as yellow microcrystals, yield 1.06 g (57%), mp 151.5–153 °C: λ_{max} (CH_3OH) 400, 381, 351, 333, 318 (sh), and 262 nm; ^1H NMR [$CDCl_3$, $(CH_3)_4Si$] δ 3.76 (s, 3), 6.7–6.9 (m, 2), 7.1–7.6 (m, 5), and 7.8–8.2 (m, 3). Anal. Calcd for $C_{20}H_{13}NO_2Cl_2$: C, 64.88; H, 3.54; N, 3.78. Found: C, 65.09; H, 3.68; N, 3.83.

P-[(5-Aminopentyl)amino]-P-deoxy-5′-O-(methoxytrityl)thymidylyl(3′→5′)-3′-O-(methoxytrityl)thymidine (**24**). A solution containing 370 mg (0.55 mmol) of 5′-*O*-(methoxytrityl)thymidine 3′-(*O*-methyl *N*,*N*-diisopropylphosphoramidite) (**15**, R = MTr) in 2 mL of dry acetonitrile was treated with 178 mg (2.5 mmol) of tetrazole and 232 mg (0.45 mmol) of 3′-(methoxytrityl)thymidine (Ogilvie & Letsinger, 1967; Matteucci & Caruthers, 1980). The reaction mixture was maintained at 25 °C for 20 min and then treated with 125 mg (0.49 mmol) of iodine in 3 mL of 2:1 tetrahydrofuran–1,5-diaminopentane. After 5 min at 25 °C, the reaction mixture was concentrated under diminished pressure and the residue was partitioned between CH_2Cl_2 and 50% aqueous methanol. The organic phase was dried (Na_2SO_4) and concentrated. The residue was purified by silica gel flash chromatography (30-g column); elution was carried out by using CH_2Cl_2 containing increasing amounts of methanol. In this fashion each of the diastereomers of *P*-[(5-aminopentyl)amino]-*P*-deoxy-5′-*O*-(methoxytrityl)thymidylyl(3′→5′)-3′-*O*-(methoxytrityl)thymidine (**24**) was obtained as an off-white powder in chromatographically homogeneous form, yield 125 mg (24%) of the less polar isomer [silica gel TLC R_f 0.48 (5:1 CH_2Cl_2–CH_3OH)] and 166 mg (31%) of the more polar isomer (R_f 0.43); ^1H NMR (less polar isomer) [$CDCl_3$, $(CH_3)_4Si$] δ 1.38 (s), 1.78 (s), 1.2–2.0 (m), 2.2–2.8 (m), 3.2–3.7 (m), 3.75 (s), 3.78 (s), 3.9–4.3 (m), 5.03 (br t), 6.17 (t), 6.33 (dd), 6.83 (dd), 7.2–7.5 (m), 7.50 (s), and 8.38 (s); ^1H NMR (more polar isomer) δ 1.34 (s), 1.77 (s), 1.2–1.8 (m), 2.2-2.9 (m), 3.3–3.7 (m), 3.75 (s), 3.77 (s), 3.9–4.2 (m), 5.08 (br t), 6.22 (t), 6.33 (dd), 6.8–6.9 (m), 7.2–7.4 (m), 7.54 (s), and 8.42 (s).

P-[[5-[(6-Chloro-2-methoxyacridin-9-yl)amino]pentyl]-amino]-P-deoxythymidylyl(3′→5′)thymidine (**26**). To solutions of 118 mg (0.10 mmol) of each of the diastereomers of **24** in 400 μL of pyridine was added 167 mg (0.45 mmol) of 6-chloro-9-(*p*-chlorophenoxy)-2-methoxyacridine, and the reaction mixtures were heated overnight at 60 °C. Each reaction mixture was diluted with CH_3OH, filtered to remove insoluble material, and concentrated under diminished pressure. The yellow solid product obtained from each was purified by preparative silica gel TLC, development with 9:1 CH_2Cl_2–CH_3OH. The individual diastereomers (putative **25**) were treated separately with 6 mL of 2% trifluoroacetic acid in CH_2Cl_2 (30 min, 25 °C) to effect detritylation. The individual diastereomers were isolated as yellow powders following precipitation of each from a large volume of ether as the presumed trifluoroacetate salt, yield 63 mg (64%) of the less polar isomer, 53 mg (54%) of the more polar isomer: ^1H NMR (less polar isomer) ($CDCl_3$, DMSO-d_6) δ 1.2–1.4 (m), 1.78 (s), 2.0–2.8 (m), 3.65 (s), 3.84 (t), 3.92 (s), 4.0–4.1 (m), 4.28 (m), 4.5 (br s), 4.95 (t), 6.22 (q), 7.6–7.7 (m), 7.79 (d), 7.84 (s), and 8.23 (d); ^1H NMR (more polar isomer) δ 1.2–1.6 (m), 1.90 (s), 2.0–2.6 (m), 3.8–4.2 (m), 3.92 (s), 4.4 (br s), 4.6 (br s), 5.05 (m), 6.3 (m), 7.6–7.7 (m), 7.79 (d), 7.84 (s), and 8.23 (d).

Compound **26** was also prepared on a solid support in analogy with the synthesis of *n*-alkylphosphoramidates **20–22**.

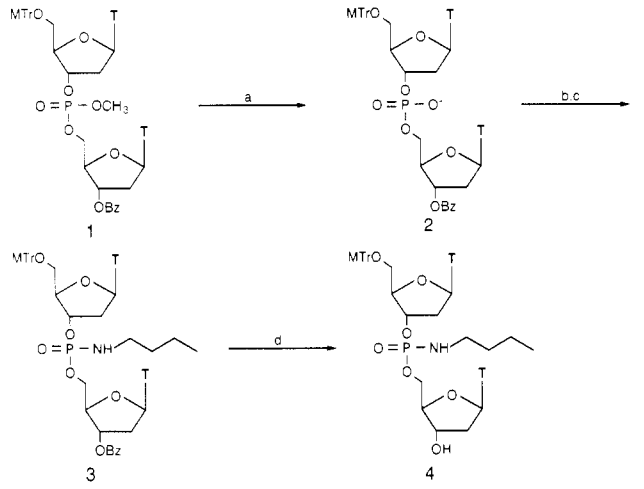
Determination of Melting Temperatures for Complexes of Oligoadenylate Analogues and Poly(dT). Solutions of both the oligonucleotides and poly(thymidylic acid) were prepared at nucleotide concentrations of 6×10^{-5} M, and mixed in a nucleotide ratio of 1:2 (dA:dT) in 10 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$. In some cases, 15% CH_3OH was added to facilitate dissolution of oligonucleotide *N*-alkylphosphoramidates. The spectroscopic behavior upon cooling or heating was monitored by UV at 260 nm. Hypochromicities of individual oligonucleotide analogues were determined as described (Tazawa et al., 1970; Miller et al., 1981). Molar extinction coefficients of 8.52×10^3 (264 nm) for poly(dT) and 9.39×10^3 (257 nm) for poly(dA) were used.

Results and Discussion

Preparation and Analysis of Oligodeoxynucleoside N-Alkylphosphoramidates. Because there has been no direct comparison of the several methods available for the preparation of oligodeoxynucleoside *N*-alkylphosphoramidates, we initiated our studies by comparison of a few promising methods.

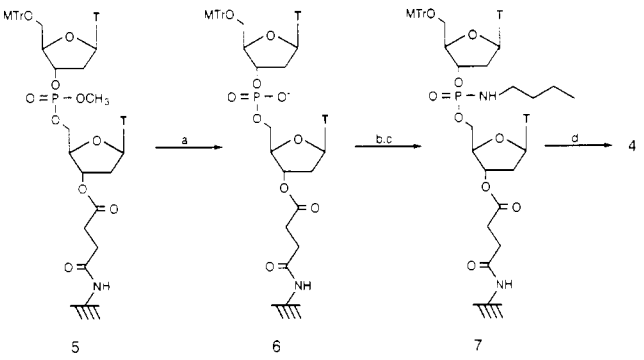
The condensation of nucleoside phosphate diesters with amines in the presence of triphenylphosphine–CCl_4 (Appel, 1975) has been used previously for the preparation of nucleoside phosphoranilidate derivatives (Stec, 1983); the desired products were obtained in moderate yield. Application of this method for the synthesis of deoxynucleoside *N*-alkylphosphoramidates was attempted by using fully protected thymidylyl(3′→5′)thymidine derivative **2** (Scheme I). Successive treatments of **2** with ~3 equiv of triphenylphosphine–CCl_4 in CH_3CN–pyridine, and then with an excess of *n*-butylamine, afforded dinucleoside *n*-butylphosphoramidate **3** in 24% isolated yield. The structures of **3**, and its debenzoylated derivative **4**, were consistent with the behavior of each on silica gel TLC and with their measured UV and high-field ^1H NMR spectra (Table I of the supplementary material). Dinucleoside *n*-butylphosphoramidate **4** was also synthesized

Scheme I[a]



[a] Reagents: (a) C_6H_5SH, Et_3N, dioxane; (b) $(C_6H_5)_3P$, CCl_4; (c) n-$C_4H_9NH_2$; (d) t-$C_4H_9NH_2$, CH_3OH.

Scheme II[a]



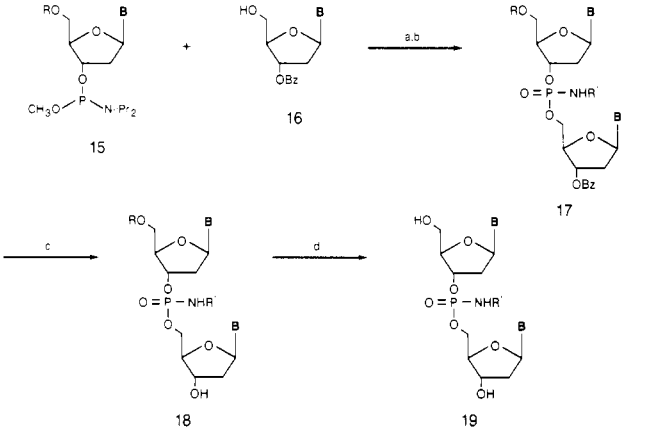
[a] Reagents: (a) C_6H_5SH, Et_3N, dioxane; (b) $(C_6H_5)_3P$, CCl_4; (c) n-$C_4H_9NH_2$; (d) t-$C_4H_9NH_2$, CH_3OH.

on a polymeric support by modification of the method of Matteucci and Caruthers (1981) (Scheme II). Compound **4** was obtained in ~70% overall yield from the resin-bound precursor **5**; it was shown to be identical in all respects with the product obtained by solution-phase synthesis.

Also studied was the preparation of nucleoside N-alkylphosphoramidates via treatment of nucleoside phenyl phosphite derivatives **8** with n-hexyl azide (25 °C, 4 days). The use of **8a** and **8b**, containing phenyl and 4-chlorophenyl phosphites, respectively, led only to solvolysis and other decomposition products. On the other hand, nucleoside **8c** provided the desired nucleoside n-hexylphosphoramidate derivative **9** in 54% yield, along with quantities of nucleoside hydrogen phosphonate **10** and 5′-O-(methoxytrityl)thymidine. Also studied for comparative purposes was the reaction of **8c** with methyl azidoacetate, a transformation more closely analogous to that reported by Letsinger and Schott (1981). Not surprisingly, this reaction proceeded with greater facility, providing compound **11** in 68% isolated yield after 2 days at 25 °C.

The procedure reported by Nemer and Ogilvie (1980), involving oxidation of an intermediate dinucleoside trichloroethyl phosphite with iodine in the presence of an alkylamine, appeared particularly attractive as the oxidation was rapid and essentially quantitative, and the reported conditions appeared amenable for adaptation to solid-phase synthesis. Because the formation of an N-alkylphosphoramidate by this procedure must involve the loss of one of the original phosphite substituents, we first sought to determine the extent to which this step might proceed selectively. Accordingly, we prepared

Scheme III[a]



[a] Reagents: (a) tetrazole, CH_3CN; (b) I_2, $R'NH_2$, THF; (c) t-$C_4H_9NH_2$, CH_3OH, 45 °C; (d) aqueous HOAc.

deoxynucleoside 3′-phosphite **12** and treated this intermediate with a slight excess of I_2 in dry tetrahydrofuran–n-butylamine. Workup of the reaction mixture provided 5′-O-(dimethoxytrityl)thymidine 3′-(O-ethyl N-n-butylphosphoramidate) (**13**) in 45% isolated yield; very little (<10%) of the putative O-methyl analogue **14** could be detected. The selective loss of the methyl substituent suggested that this approach might well afford the requisite oligonucleotide N-alkylphosphoramidates. Further, as O-methyl protection is frequently employed for phosphate esters during solid-phase oligonucleotide synthesis (Matteucci & Caruthers, 1981), the protected nucleoside 3′-(O-methyl N,N-diisopropylphosphoramidites) employed as building blocks in such schemes could potentially be employed as precursors both for phosphate ester and phosphoramidate linkages.

As outlined in Scheme III, two different dinucleoside O-methyl phosphites were prepared, and each was treated with a few different alkylamines in the presence of I_2. The resulting fully protected dinucleoside N-alkylphosphoramidates (**3**, **17a–f**) were purified by chromatography on silica gel; the yields of each (48–87%) are given in Table I. Successive debenzoylation (t-$BuNH_2$, CH_3OH) and detritylation (80% aqueous CH_3COOH) afforded the respective dinucleoside N-alkylphosphoramidates **19a–g** in isolated yields of 58–100%, as shown in the table. The final products were characterized by silica gel TLC and reverse-phase HPLC, as well as by UV and 360-MHz ^1H NMR spectroscopy (Table II of the supplementary material). Dinucleoside N-alkylphosphoramidates **19a**, **19b**, and **19g** were also characterized by the appearance of the respective molecular ions in their FAB mass spectra (positive and negative ion; glycerol matrix). Each of the tritylated and deprotected dinucleoside N-alkylphosphoramidates (**18** and **19**, respectively) could be separated by reverse-phase HPLC (CH_3CN–H_2O mixtures) into two components present in approximately equal amounts (see, e.g., Figure 1). In each case, the high-field ^1H NMR spectra of the constituents were consistent with their formulation as diastereomers. In some cases the two diastereomers were evaluated separately for their ability to associate with the complementary single-stranded nucleic acid (vide infra).

To evaluate the applicability of this approach for the elaboration of oligodeoxynucleotide N-alkylphosphoramidates, we prepared a few different tetranucleotides by solid-phase synthesis. Each of these contained a single N-alkylphosphoramidate linkage at one of the two terminal positions. Synthesis of the oligonucleotides was carried out by the method of Matteucci and Caruthers (1981), with the exception that

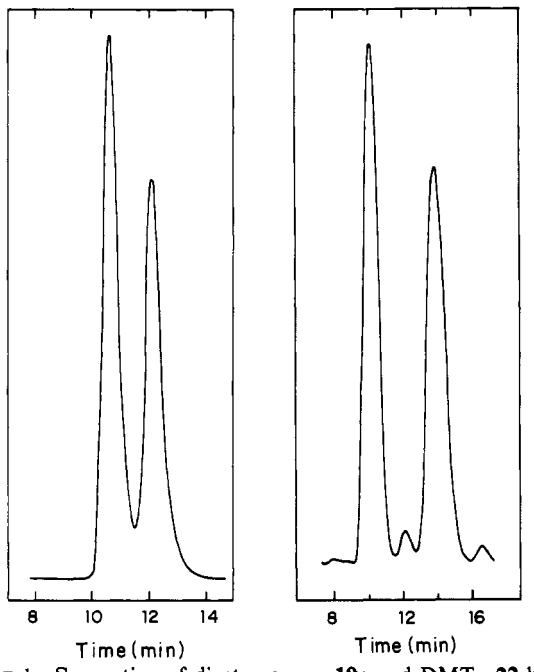


FIGURE 1: Separation of diastereomers **19a** and DMTr–**22** by reverse-phase HPLC. Left panel: Diastereomeric dinucleoside *n*-octylphosphoramidates were applied to a 25 × 0.46 cm Alltech C_{18} column (10 μm) and washed with 35% CH_3CN in 0.1 N ammonium formate at a flow rate of 1.0 mL/min. The column was monitored at 254 nm. Right panel: Diastereomeric tetranucleotides DMTr–**22** were separated preparatively on a 25 × 1.0 cm C_{18} column (10 μm) which was washed with 35% CH_3CN in 0.02 N triethylammonium acetate, pH 6.9, at a flow rate of 6 mL/min.

introduction of the alkylphosphoramidate linkages was accomplished by substitution of I_2–alkylamine treatment for I_2–H_2O in the oxidative step of the coupling cycle. Following partial deblocking and removal from the column (*tert*-butylamine–CH_3OH), the tritylated tetranucleotides were purified by preparative HPLC on a C_{18} reverse-phase column. Elution with 0.02–0.04 M tetraethylammonium acetate, pH 6.9, containing an appropriate amount of acetonitrile effected purification of the oligonucleotides and separation of the diastereomers, as illustrated in Figure 1 for tetranucleotide DMTr–**22**.

Because the method of solid-phase synthesis employed here proceeds from the 3′-end of the nascent oligonucleotide, the successful synthesis of tetranucleotide analogue **22** demonstrated the stability of the *N*-alkylphosphoramidate bond during the subsequent condensation, oxidation, and detritylation procedures. In addition, during the synthesis of **22** a small amount of the solid support was removed after the first condensation–oxidation cycle and subjected to complete deprotection. This procedure yielded a diastereomeric mixture identical in all respects (^1H NMR, silica gel TLC, reverse-phase HPLC) with **19g** prepared by solution-phase synthesis.

Characterization of tetrathymidylate analogues **20**–**22** included enzymatic digestion of each with nuclease P1 and alkaline phosphatase (Connolly et al., 1984); dinucleoside *N*-alkylphosphoramidate **19a** was shown to be resistant to nuclease P1 under conditions that led to complete hydrolysis of d(TpT). Treatment of a single diastereomer of **22** with nuclease P1 gave three products, including equal amounts of pdT and dT. Further treatment with calf intestine alkaline phosphatase converted the product mixture to a mixture of thymidine and a single diastereomer of **19g**. Analogous digestion of **20** and **21** with nuclease P1 resulted in the formation of **19g** and **19b**, respectively, plus pdT. In each case (**20**–**22**), digestion of the diastereomeric mixture of tetranucleotides was found to produce both diastereomers of its respective dinucleoside *N*-alkylphosphoramide **19**; each of the diastereomers of the tetranucleotide was shown to lead exclusively to one of the two diastereomers of **19**.

Table II: Binding of Adenine Oligonucleotides to Poly(thymidylic acid)[a]

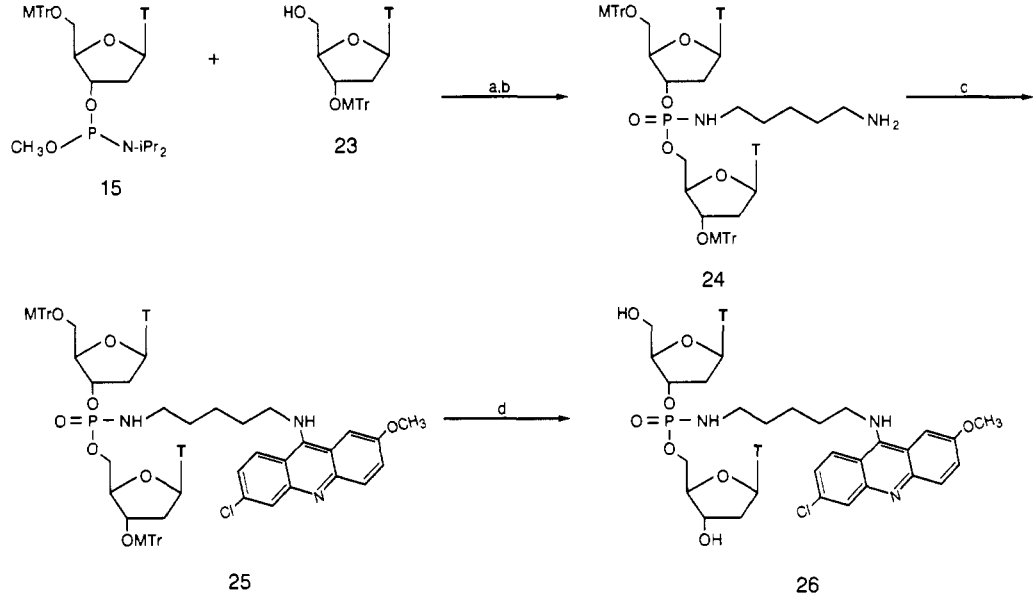
compd	hypochromicity (%) single strand	hypochromicity (%) annealing[b]	T_m (°C) determined by heating[b,c]	T_m (°C) determined by cooling[b,c]
d(ApA)	17[g]	36	9	8
		30[d]	7[d]	5[d]
d[Ap[N(CH_3)$_2$]A] (**19f**)	7	–	–	–
d[Ap(NHC$_4$H$_9$)A] (**19c**)	7	48	17	17
		42[d]	12[d]	10[d]
d[Ap(NHC$_8$H$_{17}$)A] (**19d**)	7	–	–	–
d[Ap(NHC$_{12}$H$_{25}$)A] (**19e**)	7	30[d]	20[d]	16[d]
19e, more polar diastereomer[e]	–	31[d]	20[d]	16[d]
19e, less polar diastereomer[e]	–	26[d]	19[d]	15[d]
d(ApApA)	27[h]	36	29 (0.1)	28
			29 (1.0)	
d[Ap(NHC$_{12}$H$_{25}$)-ApA][f]	10	32	40 (1)	31 (0.5)
			40 (2)	31 (1)

[a] Carried out as described under Experimental Procedures. [b] All determinations were made in 10 mM Tris·HCl, pH 7.5, containing 10 mM $MgCl_2$, except where CH_3OH was added in addition. [c] Heating and cooling were carried out at rates of 1.0 and 0.5 deg/min, respectively, except where noted otherwise in parentheses. [d] Contained 15% CH_3OH. [e] As judged by relative retention on reverse-phase (C_{18}) HPLC; elution was with 40% CH_3CN in 0.1 N ammonium formate. [f] Prepared by solid-phase synthesis in analogy with the preparation of **21**. [g] From Miller et al. (1971). [h] From Cassani and Bollum (1969).

In recent years, numerous reports have described the synthesis of oligonucleotides modified to contain adjuvants useful in DNA binding (Letsinger & Schott, 1981; Asseline et al., 1984; Hélène et al., 1985; Thuong et al., 1987; Letsinger et al., 1988), site-selective alkylation (Vlassov et al., 1985; Knorre et al., 1985; Knorre & Vlassov, 1985; Zarytova et al., 1986; Iverson & Dervan, 1987), or cleavage (Boutorin et al., 1984; Chu & Orgel, 1985; Dreyer & Dervan, 1985; Le Doan et al., 1986; Boidot-Forget et al., 1986, 1987). It seemed likely that the chemistry described here should be applicable for the synthesis of such modified oligonucleotides, and we attempted to prepare a representative dinucleoside phosphoramidate (**26**). As outlined in Scheme IV, key intermediate **24** was prepared by I_2–diaminopentane oxidation of the dinucleoside *O*-methyl phosphite derived from **15** and **23**. Individual diastereomers of **24** were obtained by silica gel flash chromatography; each was treated separately with 6-chloro-9-(*p*-chlorophenoxy)-2-methoxyacridine at 60 °C overnight to produce **25**. Following detritylation (2% CF_3COOH in CH_2Cl_2, 30 min) each of the diastereomers of **26** was isolated by precipitation of its trifluoroacetate salt from a large volume of ether. The less and more polar isomers were obtained in overall yields from **24** of 64% and 54%, respectively. Compound **26** was also prepared by solid-phase synthesis in analogy with the synthesis of **20**–**22**.

The ultraviolet hypochromicities and circular dichroism (CD) spectra of the individual oligoadenylate *N*-alkylphosphoramidates indicated a diminution of base stacking relative to d(ApA) and d(ApApA). The magnitude of the hypochromic effect was not affected by the nature of the *N*-alkyl group (Table II), in contrast to earlier reports concerning the hypochromicities of alkyl phosphotriesters (Miller et al., 1971; Letsinger et al., 1986). The lack of dependence

Scheme IV[a]



[a] Reagents: (a) tetrazole, CH_3CN; (b) I_2, $NH_2(CH_2)_5NH_2$; THF; (c) 6-chloro-9-(*p*-chlorophenoxy)-2-methoxyacridine; (d) 2% CF_3COOH in CH_2Cl_2.

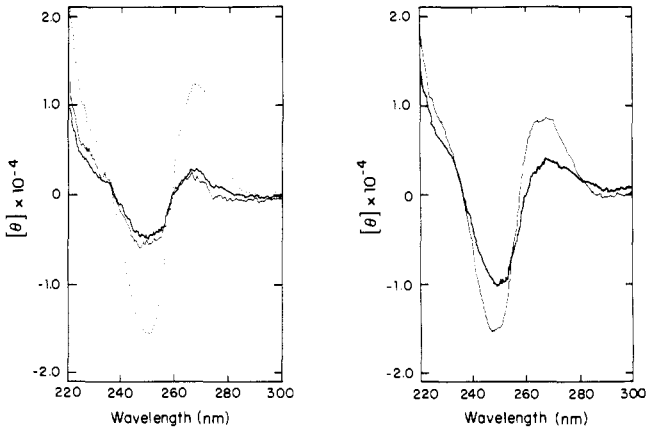


FIGURE 2: Circular dichroic spectra of representative oligonucleotide *N*-alkylphosphoramidates. Left panel: Circular dichroic spectra of d(ApA) (···), d[Ap(NMe_2)dA] (**19f**) (—), and d[Ap(NHC_4H_9)dA] (**19c**) (boldface —) in 0.01 M Tris·HCl (pH 7.5)–0.01 M $MgCl_2$ at 25 °C. The nucleotide concentration was 6×10^{-5} M. Right panel: Circular dichroic spectra of d(ApApA) (—) and d[Ap($NHC_{12}H_{25}$)ApA] (boldface —) in 0.01 M Tris·HCl (pH 7.5)–0.01 M $MgCl_2$ at 25 °C. The nucleotide concentration was 6×10^{-5} M.

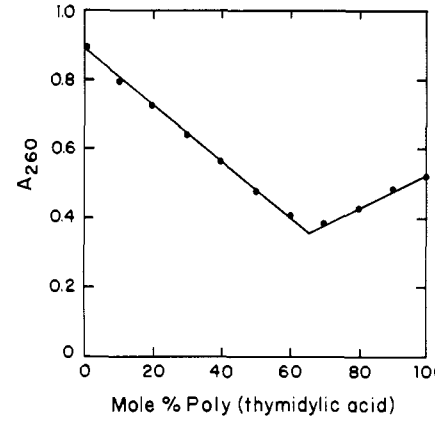


FIGURE 3: Mixing profile of dinucleoside *n*-butylphosphoramidate **19c** and poly(thymidylic acid). The complex was formed in 10 mM Tris·HCl, pH 7.5, containing 10 mM $MgCl_2$. Equimolar stock solutions of **19c** and poly(dT) (6×10^{-5} M nucleotide concentration) were mixed in different ratios and allowed to reach equilibrium at 0 °C, after which A_{260} was recorded.

on alkyl chain length was also apparent in the CD spectra. As can be seen in Figure 2, for example, the spectra of **19c** and **19f** were very similar; this was also true for the CD spectrum of **19d** (data not shown). The wavelengths of their maximum and minimum ellipticity in these spectra matched those of d(ApA), but their amplitudes were greatly reduced, as was seen with alkyl phosphotriesters (Miller et al., 1971). Also carried out was a comparison of the CD spectrum of d(ApApA) with that of d[Ap($NHC_{12}H_{25}$)ApA]. As anticipated, both spectra exhibited maximum and minimum ellipticity at the same wavelengths, but the spectrum of d[Ap($NHC_{12}H_{25}$)ApA] was diminished in amplitude (Figure 2). Both the CD and hypochromicity results are consistent with decreased interaction between adjacent bases (Kondo et al., 1970; Miller et al. 1971) in the *N*-alkylphosphoramidates.

Oligonucleotide Binding of Adenine Nucleoside N-Alkylphosphoramidates. The interaction of individual oligonucleotide *N*-alkylphosphoramidates was studied initially by the use of absorbance mixing curves. These experiments readily demonstrated the formation of complexes between oligoadenylate *N*-alkylphosphoramidates and poly(thymidylic acid), but not between oligothymidylate **21** and poly(dA); accordingly, the former were studied further. As shown in Figure 3, when measurement was done in 10 mM Tris·HCl, pH 7.5, containing 10 mM $MgCl_2$, dinucleotide analogue **19c** gave an absorption minimum at 66 mol % of poly(dT), corresponding to a dA:dT nucleotide stoichiometry of 1:2. Since triple helix formation by polymers of adenine and thymine (uracil) nucleotides is well documented (Stevens & Felsenfeld, 1964; Davies, 1967; Tazawa et al., 1970; Miller et al., 1971, 1981; Arnott et al., 1976; Letsinger et al., 1986), this result was not unexpected. Essentially the same result was obtained with the other tested oligoadenylate-derived phosphoramidates.

A number of oligoadenylate *N*-alkylphosphoramidate analogues were employed for measurements of hypochromicity and melting temperature (Table II). The T_m values and hypochromicities in the presence of complementary oligonucleotides were determined simultaneously; in the case of **19e**, 15% methanol was included to effect dissolution of the nucleotide analogue. The T_m values were determined both by

heating of the formed oligonucleotide–poly(dT) complexes and by slow cooling of solutions initially maintained above the T_m. As shown in the table, the introduction of an *n*-butylphosphoramidate moiety in place of the phosphate ester [i.e., **19c** vs d(ApA)] resulted in a species whose binding to poly(thymidylic acid) was altered, as judged by a substantial increase in percent hypochromicity and T_m. Precisely the same effect was obtained in the presence of 15% CH_3OH, although the absolute values for percent hypochromicity and T_m were slightly lower. The enhanced binding of **19c** to poly(dT) was consistent with the observations of Letsinger et al. (1986), who found that the unsubstituted phosphoramidate of d(ApA) also exhibited an enhanced affinity for poly(dT). These authors attributed the increased binding to the absence of charge repulsion by the uncharged phosphoramidate $-NH_2$ moiety and its ability to form H bonds in aqueous media.

Also apparent in Table II is the effect of increasing alkyl chain length on the affinity of the oligoadenylate *N*-alkylphosphoramidates for poly(dT). Direct comparison of d(ApA), **19c**, and **19e** (in 15% CH_3OH) revealed an increase in T_m with increasing chain length. This was true for both methods of T_m determination. A significant increase in T_m was also observed when one of the phosphate esters in d(ApApA) was replaced by an *N*-dodecylphosphoramidate. This increase in affinity is entirely consistent with the results of Letsinger et al. (1986), who found that trichloroethyl and 1,1-dimethyltrichloroethyl esters of d(ApA) and d(ApApA) bound to poly(dT) with significantly enhanced affinity. The present results suggest that lipophilic substituents may act more generally to stabilize DNA helix structure. It is worthy of note that both diastereomers of **19e** had essentially the same T_m, whether measured by heating or cooling.

One interesting facet of the measurements made in the presence of poly(thymidylic acid) involved the change in percent hypochromicity observed with increasing alkyl chain length (Table II). Following an initial increase in percent hyopchromicity as the *N*-alkylphosphoramidate substituent was introduced, further increases in alkyl chain length actually resulted in a *decrease* of the measured hypochromicity for both the di- and trinucleotide analogues studied. While the magnitude of the hypochromicity obviously reflects a complex variety of factors, the observed pattern is intriguing. Another effect was observed in parallel with this decrease in percent hypochromicity. As illustrated in Table II, for most of the nucleotide analogues studied the T_m values were essentially identical whether determined by heating or cooling. However, for the most lipophilic di- and trinucleotide derivatives studied, the T_m's measured by heating were consistently several degrees higher than those measured by cooling. This was true for both diastereomers of **19e** and for d[Ap($NHC_{12}H_{25}$)ApA]; the effect was unaltered by reasonable changes in the rate of heating or cooling. These observations suggest that the larger lipophilic substituents may cause disruption of the base stacking normally associated with polynucleotides and form duplexes that are structurally altered to permit accommodation of the lipophilic substituents within the least polar regions of the formed complexes, thereby avoiding the interaction of the lipophilic groups with the polar aqueous medium. A relatively slow rate of formation and dissociation of such "disordered" oligonucleotide complexes could well be consistent with the observed differences in T_m according to the method of measurement employed.

The observed increase in affinity for a polynucleotide by its complementary oligonucleotide bearing a lipophilic alkyl substituent suggests the existence of a novel source of polynucleotide affinity, consistent with the behavior of the natural product model (Scannell et al., 1988) that provided the conceptual basis for the design of the oligonucleotide *N*-alkylphosphoramidates. It also suggests that the alkyl groups sometimes used to covalently tether more classical DNA binding agents to polynucleotides could well be participants in the overall process by which polynucleotide binding is enhanced. It is important to note that the present measurements were carried out with oligonucleotides of a type known to form nucleic acid triple helices readily (Stevens & Felsenfeld, 1964; Davies, 1967; Tazawa, 1970; Miller et al., 1971, 1981; Arnott et al., 1976; Letsinger et al., 1986); indeed, the mixing profile obtained for **19c** and poly(dT) is suggestive of a triple-strand structure. It may be the case that the novel source of affinity noted here is most readily apparent where the potential for formation of a triple-strand structure exists. Indeed, this might offer some interesting opportunities for manipulation of nucleic acid structure (Moser & Dervan, 1987).

In the context of the design of sequence-specific nucleic acid probes, the oligonucleotide *N*-alkylphosphoramidates described here are of interest for a few different reasons. These include probable resistance to nuclease degradation, as observed upon incubation with *P. citrinum* nuclease P1, and the possibility that the presence of a lipophilic alkylphosphoramidate moiety may render mammalian cells permeable to the oligonucleotide [see, e.g., Miller et al. (1985)]. Further, it seems reasonable to anticipate that oligonucleotide *N*-alkylphosphoramidate derivatives may exhibit unusual properties in systems that model DNA assembly (Behr, 1986) and encapsulation within lipid membranes (Jay & Gilbert, 1987).

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SUPPLEMENTARY MATERIAL AVAILABLE

Table I, giving 360-MHz ^1H NMR spectra of dinucleoside alkylphosphoramidate derivatives **3** and **17a–f**, and Table II, giving 360-MHz ^1H NMR spectra of dinucleoside alkylphosphoramidate derivatives **19a–g** (3 pages). Ordering information is given on any current masthead page.

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