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Synthesis and Properties of Phosphoramidite Derivatives of Modified Nucleosides

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Protected N^6-methyl-2′-deoxyadenosine (d-m^{6}A), 2-amino-2′-deoxyadenosine (d-a^{2}A), 2′-deoxyinosine (dI), 5-methyl-2′-deoxycytidine (d-m^{5}C) and deoxyuridine (dU) were reacted with bis(diisopropylamino)methoxyphosphine in the presence of diisopropylammonium tetrazolide as the activating reagent to give the corresponding phosphoramidite derivatives in yields of 100, 65, 90, 78 and 65%, respectively. The ^{31}P-nuclear magnetic resonance spectra of the products were measured. Using these compounds, dinucleotides and trinucleotides were synthesized on a long-chain alkylamine controlled pore glass (LCA-CPG) in quantitative yields.

The stability of 6-methyldeoxyadenosine and *N,N*-diisobutyryl-2-aminodeoxyadenosine to acid was examined. When protected di- and trinucleotides (m^{6}A-T, a^{2}A-T, T-m^{6}A-T, T-a^{2}A-T) bound to the support (LCA-CPG) were treated with 3% trichloroacetic acid in dichloromethane, depurination was negligible within 10 min (dinucleotide) or 60 min (trinucleotide).

Keywords——phosphoramidite; solid-phase synthesis; phosphite method; acid treatment; enzyme degradation

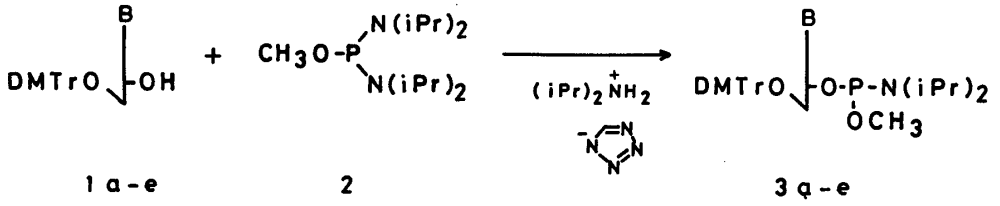
Chemical oligodeoxyribonucleotide synthesis can be performed very rapidly by the phosphotriester or phosphitetriester method, especially on a polymer support. To date, chemically synthesized oligodeoxyribonucleotides have proven to be very useful tools for biological studies[1] or conformational studies.[2] For example, chemically synthesized oligomers were used as probe in cloning complementary deoxyribonucleic acids (cDNAs).[3] Since amino acid codons are degenerate, a probes for a specific amino acid sequence should contain all possible sets of degenerate codons. Recently 2′-deoxyinosine has been successfully used instead of a mixed oligonucleotide probe.[4]

5-Methyl-2′-deoxycytidine and 6-methyl-2′-deoxyadenosine (found in natural DNA) and 2-amino-2′-deoxyadenosine are of interest in connection with conformational studies, especially because 5-methyl-2′-deoxycytidines stabilize the left-handed *Z*-conformation of double-stranded DNA, if they are located in alternating purine–pyrimidine sequences such as m^{5}C-G-m^{5}C-G-m^{5}C-G.[5] We are also interested in studying the effect of the methyl group on the conformation of DNA. For this study, oligonucleotides containing deoxyuridine are necessary.

However, the phosphoramidite derivatives of such modified 2′-deoxynucleosides have not been reported so far. We report here the synthesis and characterization by ^{31}P-nuclear magnetic resonance (^{31}P-NMR) of the phosphoramidite derivatives of dI, d-m^{5}C, d-m^{6}A, dU and d-a^{2}A.

Results

The syntheses of phosphoramidite derivatives of d-m^{6}A, d-a^{2}A, dI, d-m^{5}C and dU are illustrated in Fig. 1. d-m^{6}A was synthesized by the reported procedure.[6] The 5′-hydroxyl



	B	
a	m^6A	DMTr, 4,4′-dimethoxytrityl
b	N^2,N^6-$(ibu)_2a^2A$	iPr, isopropyl
c	I	
d	N^4-bzm^5C	
e	U	

Fig. 1

TABLE I. Yields and ^{31}P-NMR Spectra Data of Compounds **3a—e**

Compound	Yield (%)	^{31}P-NMR chemical shift (ppm)
3a	100	−146.3, −146.2
3b	65	−146.4, −146.2
3c	90	−146.5, −146.4
3d	78	−146.5, −146.0
3e	65	−146.7, −146.2

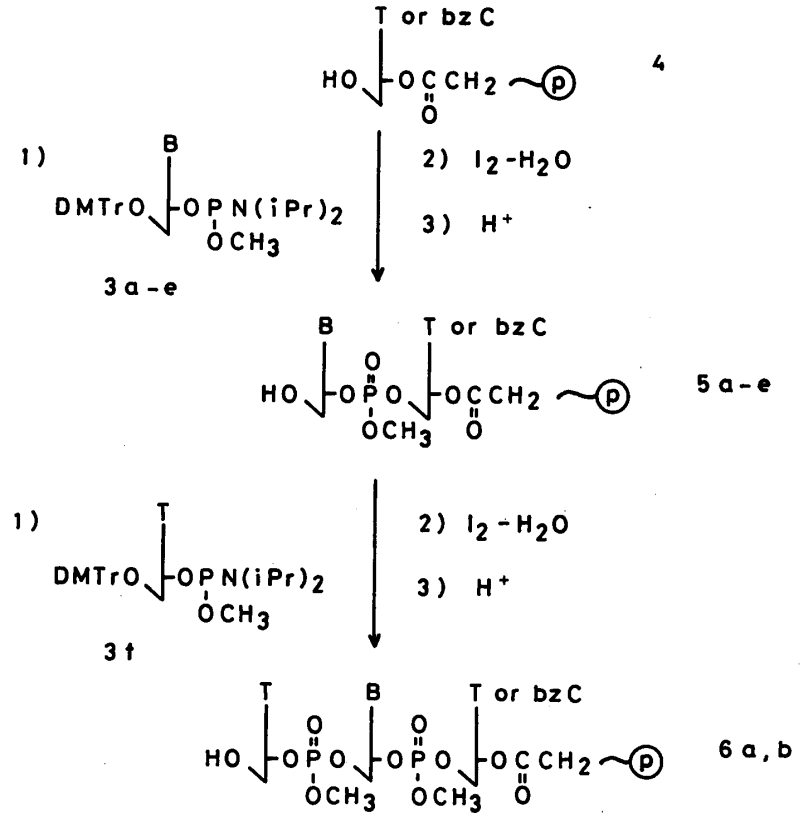
The chemical shifts are down-field with respect to trimethyl phosphate in $CDCl_3$ as an external standard.

TABLE II. Hydrolysis of Di- and Trinucleotides by Snake Venom Phosphodiesterase

Compound	Enzyme degradation					
d (m^6A-T)	d-m^6A	(0.98)	pT	(1)		
d (a^2A-T)	d-a^2A	(1.00)	pT	(1)		
d (m^5C-C)	d-m^5C	(1.04)	dpC	(1)		
d (I-C)	dI	(1.01)	dpC	(1)		
d (U-C)	dU	(1.02)	dpC	(1)		
d (T-m^6A-T)	T	(0.99)	dpm^{6}A	(1.03)	pT	(1)
d (T-a^2A-T)	T	(1.01)	dpa^{2}A	(0.98)	pT	(1)

group of dI, dU, and d-m^6A was protected with dimethoxytrityl chloride in dimethylformamide–pyridine or pyridine solution. 5′-*O*-Dimethoxytrityl-d-m^5C was prepared from 5′-*O*-dimethoxytritylthymidine by Sung's procedure.[7] 5′-*O*-Dimethoxytrityl-N^2,N^6-diisobutyryl-2-amino-2′-deoxyadenosine (**1b**) was prepared according to Gaffney *et al.* with some modification.[8] Compounds **1a—e** thus prepared were phosphitylated with bis(diisopropylamino)methoxyphosphine in the presence of diisopropylammonium tetrazolide according to Barone *et al.*[9] Compounds **3a—e** were isolated by silica gel short column chromatography. The yields and ^{31}P-NMR data are summarized in Table I.

In order to investigate the reaction conditions and the stability, we synthesized dimers (**5**) and trimers (**6**). Compounds **3a—e** (20 eq) were coupled with thymidine or N^4-benzoylcytidine bound to long-chain alkylamine controlled-pore glass beads (LCA-CPG)[10] in acetonitrile for 5 min at room temperature. Then after oxidation of phosphite to phosphate with iodine–water, the 5′-*O*-dimethoxytrityl group was removed with 3% trichloroacetic acid (TCA) in dichloromethane for 2 min.[11] The coupling yields were quantitatively estimated by



B = **a**, m^6A; **b**, N^2N^6-$(ibu)_2a^2A$; **c**, I; **d**, N^4-bzm^5C; **e**, U; **f**, T
DMTr = 4,4′-dimethoxytrityl; iPr, isopropyl
Ⓟ = long chain alkylamine controlled-pore glass

Fig. 2

measuring released dimethoxytrityl cation. For the preparation of trimer bound to the resin (**6**), the resin (**5**) was condensed with **3f** and 1*H*-tetrazole followed by oxidation and detritylation as above. The resin was treated with thiophenol–triethylamine, then concentrated ammonia to remove the phosphate and base-protecting groups and to release the product from the resin, and all dimers and trimers were analyzed by reversed-phase (C_{18} silica gel) high-pressure liquid chromatography (HPLC). They were completely hydrolyzed to nucleoside and nucleoside-5′-phosphate components by snake venom phosphodiesterase (Table II).

We next examined the stability of d-m^6A and d-a^2A to acid. In the case of N^6-benzoyldeoxyadenosine (d-bzA), it was reported that 5′-terminal d-bzA (bzAp) is more sensitive to acid than internal d-bzA (pbzAp).[12] Accordingly, we prepared the resins **5a**, **b** and **6a**, **b**. The resins **5a**, **b** were treated with 3% trichloroacetic acid in dichloromethane for various periods (1, 3, 5 and 10 min), although acid treatment to remove dimethoxytrityl groups usually ends within 2 min. Each support was treated with ammonia to release the nucleotidic materials from the resin and the supernatant was analyzed by reversed-phase HPLC (Fig. 3A, C). However, no depurination was detected even after acid treatment for 10 min. In the case of **5b**, we obtained the same result as with **5a** (data not shown). Then, the trinucleotide resins (**6a**, **b**) were treated with 3% trichloroacetic acid in dichloromethane for prolonged periods (5, 10, 30 and 60 min), because internal d-m^6A or d-a^2A are exposed to multiple acid treatments during the course of synthesis. After deblocking and releasing the nucleotidic materials as usual from the resin, the supernatant was analyzed by reversed-phase HPLC (Fig. 3B, D). However, it did not show any change even after acid treatment for

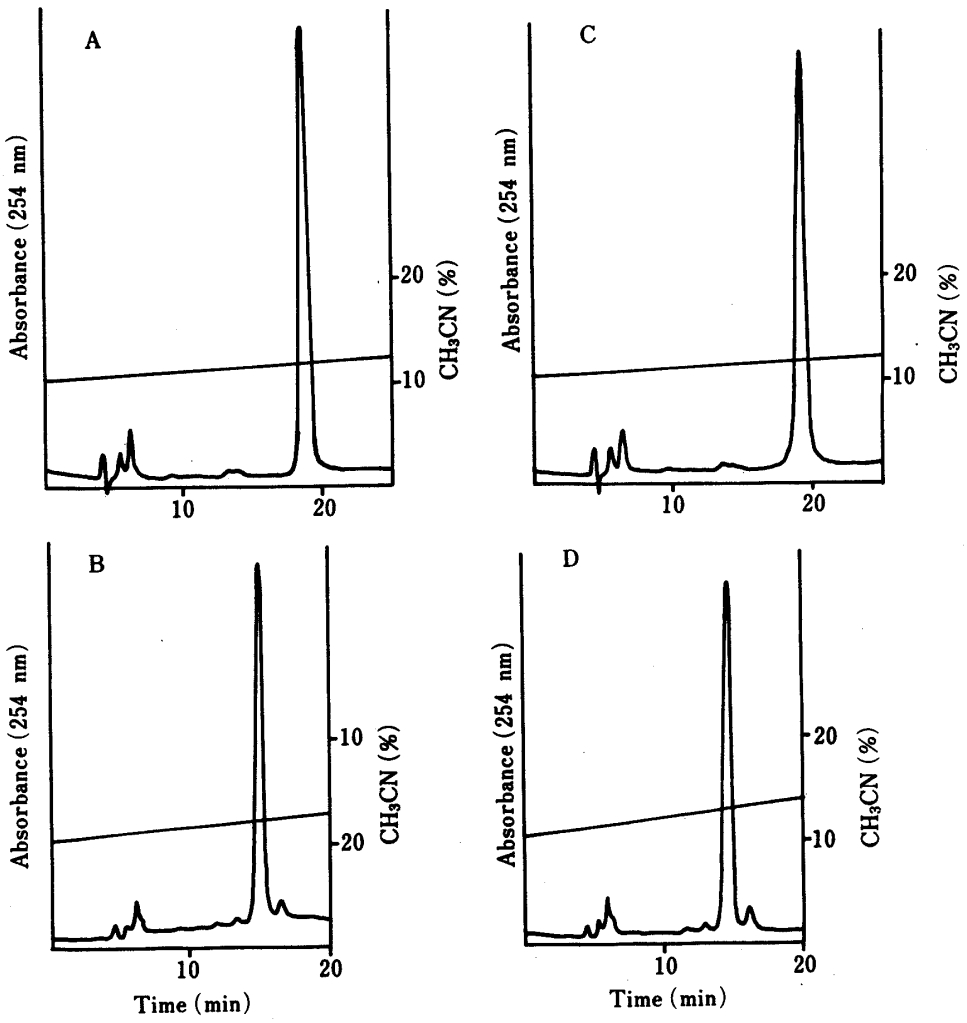


Fig. 3. A (**5a**) and B (**6a**) were Deblocked as Described in the Experimental Section. C (**5a**) and D (**6a**) were Treated for Another 10 and 60 min, Respectively, with 3% Trichloroacetic Acid in Dichloromethane then Deblocked

The nucleotide products were analyzed by HPLC without further purification, using a column of silica gel (C-18) with a gradient of acetonitrile in 0.1 M triethylammonium acetate (pH 7). The flow rate was 1 ml/min.

60 min. In the case of **6b**, we obtained the same result as with **6a** (data not shown). That means oligonucleotides containing d-m^{6}A or d-a^{2}A could be synthesized by the same procedures as used in standard DNA synthesis.

Materials and Methods

Thin-layer chromatography (TLC) was performed on plates of Kieselgel 60 F_{254} (Merck). For column chromatography, Kieselgel 60 (Merck) was used. Paper partition chromatography was performed on Toyo filter paper No. 51-A in isopropanol–concentrated NH_4OH–water (7 : 1 : 2, v/v). Long chain alkylamine controlled-pore glass bead (LCA-CPG) (pore size 500A, particle size 125—177 μm) were purchased from Pierce Chemical Company.

HPLC was performed on an Altex 322 MP chromatography system. The ^{31}P-NMR spectra were recorded with a JEOL GX500 spectrometer operating at 202.42 MHz using trimethyl phosphate as an external standard. Ultraviolet (UV) spectra were measured on a Hitachi model 200-10 spectrophotometer. 2′-Deoxyuridine was purchased from Sigma Chemical Company, 2′-deoxyinosine from Yamasa Biochemicals, and venom phosphodiesterase from Boehringer Mannheim.

The Synthesis of Phosphoramidite Derivatives (3a—e)——Bis(diisopropylamino)methoxyphosphine (280 μl, 1.5 mmol) was added to the 5′-dimethoxytrityl-*N*-protected nucleoside (1 mmol) and diisopropylamine hydrotetrazolide (0.5 mmol) in dry dichloromethane (5 ml) with stirring. Completion of the reaction was checked by TLC (AcOEt), then the mixture was evaporated to a small volume. The residue was dissolved in ethyl acetate (30 ml) and washed twice with sat. $NaHCO_3$, then with water. The organic solvent was evaporated off, and the product was applied to a column (ϕ 2.5 × 5 cm) of silica gel and eluted with a mixture of dichloromethane, ethyl acetate and triethylamine (48 : 48 : 4, v/v).[13]

The fraction which contained the desired product was pooled and evaporated to provide a foam. The yields and the ^{31}P-NMR data are shown in Table I. These amidite derivatives were stable for at least two months if they were stored at −20 °C under nitrogen.

The Synthesis of Dimers and Trimers on a Polymer Support——5′-*O*-Dimethoxytritylthymidine or 5′-*O*-dimethoxytrityl-*N*-benzoylcytidine bound to the resin (**4**) (1 μmol) was placed in a glass tube (ϕ 1 × 8 cm) with a sintered glass filter and treated with 3% trichloroacetic acid in dichloromethane (2 ml) for 2 min. The resin was washed with dichloromethane (2 ml), pyridine (2 ml), and then dichloromethane (2 ml × 2). The phosphoroamidite derivative (**3**) (20 eq) and 1*H*-tetrazole (60 eq) in dry acetonitrile (0.2 ml) were added to the resin. After being shaken for 5 min, the resin was washed twice with dichloromethane (2 ml) and treated with 0.1 M iodine in tetrahydrofuran–pyridine–H_2O (8 : 1 : 1, v/v) (2 ml) for 3 min. The resin was washed with acetonitrile (2 ml × 2), and dichloromethane (2 ml × 2), then treated with 3% trichloroacetic acid in dichloromethane for 2 min. The released dimethoxytrityl cation was collected and measured to calculate the condensation yield. For the synthesis of trimer, the resin (**5a, b**) was successively condensed with **3f** and 1*H*-tetrazole, then subjected to oxidation and detritylation. The operations were the same as described above.

The resin was treated with a mixture of thiophenol–triethylamine–dioxane (1 : 1 : 2, v/v, 1 ml) for 30 min then with concentrated ammonia (1 ml) at 55 °C overnight. The NH_4OH solution was collected and lyophilized. The residue was dissolved in H_2O and separated by reversed phase HPLC.

The Stability to Acid——The nucleotide resins (**5a, b** and **6a, b**) were treated with 3% trichloroacetic acid in dichloromethane (2 ml) for 10 min and 60 min at room temperature, respectively. After removal of the acidic solvent, the resins were washed successively with dichloromethane (2 ml), acetonitrile (2 ml × 2) and ether (2 ml × 2), and then dried. The resins were treated with thiophenol–triethylamine–dioxane (1 : 1 : 2, v/v) (1 ml) for 30 min then with concentrated ammonia (1 ml) at 55 °C overnight. The supernatant was lyophilized and analyzed by reversed phase HPLC.

Enzyme Digestion——d-m^{6}AT, d-a^{2}AC, d-m^{5}C, dIC, dUC, d-Tm^{6}AT and d-Ta^{2}AC 1.5A_{260} each isolated by reversed-phase HPLC were each hydrolyzed by snake venom phosphodiesterase. The mixture comprised 1 M triethylammonium bicarbonate (pH 7.5) (20 μl), H_2O (70 μl) and enzyme (mg/ml, 10 μl), and was incubated at 37 °C for 4.5 h. The mixture was analyzed by paper chromatography. The nucleoside and nucleoside-5′-phosphate components were eluted with 50 mM triethylammonium acetate (pH 7) and the UV spectra were measured. The results are shown in Table II.

References

1) *a*) K. Itakura, F. Bolivar and H. W. Boyer, *Science*, **198**, 1056 (1977); *b*) F. Sanger, S. Nicklen and A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 5463 (1977); *c*) M. J. Zoller and M. Smith, *Methods Enzymol.*, **100**, 468 (1983).
2) *a*) S. B. Zimmerman, *Annu. Rev. Biochem.*, **51**, 395 (1982); *b*) A. Rich, A. Nordheim and A. H. Wang, *Annu. Rev. Biochem.*, **53**, 791 (1984).
3) *a*) D. V. Goeddel, H. M. Shepard, E. Yelverton, D. Leung, R. Crea, A. Sloma and S. Pestka, *Nucleic Acids Res.*, **8**, 4057 (1980); *b*) S. H. Chang, A. Majumdar, R. Dunn, O. Makabe, U. L. RajBhandary, H. G. Khorana, E. Ohtsuka, T. Tanaka, Y. Taniyama and M. Ikehara, *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 3398 (1981).
4) *a*) E. Ohtsuka, S. Matsuki, M. Ikehara, Y. Takahashi and K. Matsubara, *J. Biol. Chem.*, **260**, 2605 (1985); *b*) Y. Takahashi, K. Kato, Y. Hayashizaki, T. Wakabayashi, E. Ohtsuka, S. Matsuki, M. Ikehara and K. Matsubara, *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 1931 (1985).
5) *a*) D. Fujii, A. H. J. Wang, G. van der Marel, J. H. van Boom and A. Rich, *Nucleic Acids Res.*, **10**, 7879 (1982); *b*) W. F. Bubsy, Jr., V. N. Reinhold, G. Büchi and G. N. Wagan, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 1870 (1977).
6) O. Dimroth, *Justus Liebigs Ann. Chem.*, **373**, 336 (1910).
7) W. L. Sung, *Nucleic Acids Res.*, **9**, 6139 (1981).
8) B. L. Gaffney, L. A. Marky and R. A. Jones, *Tetrahedron*, **40**, 3 (1984).
9) A. D. Barone, J.-Y. Tang and M. H. Caruthers, *Nucleic Acids Res.*, **12**, 4051 (1984).
10) S. P. Adams, K. S. Kavka, E. J. Wykes, S. B. Holder and G. R. Galluppi, *J. Am. Chem. Soc.*, **105**, 661 (1983).
11) T. Tanaka and T. Oishi, *Chem. Pharm. Bull.*, **33**, 5178 (1985).
12) T. Tanaka and R. L. Letsinger, *Nucleic Acids Res.*, **10**, 3249 (1982).
13) T. Dörper and E.-L. Winnacker, *Nucleic Acids Res.*, **11**, 2575 (1983).