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Solid-Phase Synthesis and Side Reactions of Oligonucleotides Containing *O*-Alkylthymine Residues†

Henryk Borowy-Borowski and Robert W. Chambers*
Department of Biochemistry, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada
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ABSTRACT: As part of our studies on the molecular mechanism of mutation [Chambers, R. W. (1982) in *Molecular and Cellular Mechanisms of Mutagenesis* (Lemontt, J. F., & Generoso, W. M., Eds.) pp 121–145, Plenum, New York and London], we wanted to prepare specific oligonucleotides carrying O^2- or O^4-alkylthymidine residues. Since *O*-alkylthymine moieties are known to be alkali labile, side reactions were expected during the deprotection procedures used for synthesis of oligonucleotides on a solid support by the classical phosphoramidite method. We have studied these side reactions in detail. Kinetic data show the deprotection procedures displace most *O*-alkyl groups at rates that make these procedures inappropriate for synthesis of most oligonucleotides carrying *O*-alkylthymine moieties. We describe alternative deprotection procedures, using readily accessible reagents, that we have used successfully to synthesize a series of oligonucleotides carrying several different *O*-alkylthymine moieties. The oligonucleotides synthesized are d(A-A-A-A-G-T-alkT-T-A-A-A-A-C-A-T), where alk = O^2-methyl, O^2-isopropyl, O^4-methyl, O^4-isopropyl, and O^4-*n*-butyl. This work extends the previously described procedure for the chemical synthesis of oligonucleotides carrying an O^4-methylthymine moiety [Li, B. F., Reese, C. B., & Swann, P. F. (1987) *Biochemistry 26*, 1086–1093] and reports the first chemical synthesis of an oligonucleotide carrying an O^2-alkylthymine. The oligonucleotides synthesized have a sequence corresponding to the minus strand that is complementary to the viral strand at the start of gene G in bacteriophage ΦX174 replicative form DNA where the normal third codon has been replaced with the ocher codon, TAA.

When active carcinogens react with DNA, stable adducts form. Some of these adducts have been shown to produce mutations in vivo, and attention has focused on the possible role of these mutations in cancer. Using a combination of chemical and biochemical techniques, it is now possible to introduce these carcinogen–DNA adducts at preselected positions in biologically active DNA so that the mutagenic activity of the adduct and the effect that DNA repair has on that activity can be studied directly in living cells. The approach we have been using requires synthesis of an oligonucleotide having a predetermined sequence and carrying the

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carcinogen to be studied attached to a particular group in the oligonucleotide, which can be incorporated into biologically active DNA (Bhanot et al., 1979; Chambers et al. 1985). While chemical synthesis of oligonucleotides carrying normal purine and pyrimidine residues is now routine, synthesis involving modified bases is not. Two problems exist. First, the purine and pyrimidine rings have several reaction centers, and one does not always get the desired product in usable yields by simple addition reactions. Second, serious side reactions often occur during deprotection of oligonucleotides carrying modified nucleotides (Borowy-Borowski & Chambers, 1987).

As part of our studies on the molecular mechanism of mutation (Chambers, 1982), we wanted to prepare specific oligonucleotides carrying O^2- or O^4-alkylthymidine residues. It has been known for some time that these compounds are both acid and alkali labile. Since classical chemical synthesis of oligonucleotides involves both acidic and basic deprotection steps, side reactions were expected. In this paper we present a detailed study of the side reactions occurring during deprotection of the final oligonucleotide carrying O^2- or O^4-methyl, -ethyl, -isopropyl, or -*n*-butyl groups. On the basis of this information we have developed alternative procedures for removing the protecting groups. We have used these procedures to synthesize several oligonucleotides carrying O^2- or O^4-alkylthymine residues.

Experimental Procedures

Unless otherwise noted, all reagents and solvents were from commerical sources and were used without further purification. Unless specified otherwise, the conditions for column chromatography, TLC, HPLC, and nucleoside analysis were as described previously (Borowy-Borowski & Chambers, 1987). The NMR spectra were recorded on a Nicolet 360 NB spectrometer at the Atlantic Region NMR Center, Dalhousie University. The chemical shifts are given downfield from tetramethylsilane as internal standard in Me_2SO-d_6. The peaks were assigned as described previously (Borowy-Borowski & Chambers, 1987).

Direct Alkylation of 5′-O-(Dimethoxytrityl)thymidine. (A) General Procedure with Diazoalkanes. The reactions with 5′-*O*-(dimethoxytrityl)thymidine were carried out on 4–6-mmol scale as described previously for 5′-*O*-(dimethoxytrityl)-deoxyguanosine (Borowy-Borowski & Chambers, 1987). For analysis the trityl groups were removed with 80% acetic acid and the crude mixture of alkylthymidines was examined by reverse-phase HPLC. The average product distributions were as follows: (with diazomethane) O^2- 6%, N^3- 87%, O^4- 7%; (with diazoethane) O^2- 20%, N^3- 60%, O_4- 20%; (with diazobutane) O^2- 22%, N^3- 56%, O^4- 22%.

The *O*-ethyl- and *O*-*n*-butyl-5′-*O*-(dimethoxytrityl)thymidine derivatives required for oligonucleotide synthesis were isolated as follows. The reaction mixture was applied to a silica gel column (3 cm × 30 cm). The column was washed with ethyl acetate (100 mL) followed by methanol in ethyl acetate (2% for *O*-ethyl, 1% for *O*-butyl). Impure 5′-*O*-(dimethoxytrityl)-O^4-alkylthymidine was obtained. Pure 5′-*O*-(dimethoxytrityl)-O^2-alkylthymidine was eluted with 5% methanol in ethyl acetate. Final purification of 5′-*O*-(dimethoxytrityl)-O^4-alkylthymidine was achieved by rechromatography of the impure product on a silica gel column (3 cm × 25 cm) eluted first with chloroform (100 mL) followed by methanol in chloroform (2% for *O*-ethyl, 1% for *O*-butyl). Suitable fractions were pooled and concentrated under reduced pressure. The products were precipitated with cold hexane. The yields and the characterization of the products by ^{1}H NMR are as follows.

5′-*O*-(Dimethoxytrityl)-O^2-ethylthymidine: 12.5% yield after crystallization from ethyl acetate; mp 189–190 °C; δ 1.29 (t, 3, J = 7.0 Hz, $-CH_3$), 1.48 (s, 3,5-CH_3), 2.22–2.37 (m, 2, H2′,2″), 3.19–3.21 (m, 2, H5′,5″), 3.73 (s, 6, $ArOCH_3$), 3.92 (m, 18 H4′), 4.30 (m, 1, H3′), 4.33 (q, 2, J = 6.9 Hz, OCH_2–), 5.39 (bs, 1,3′OH), 6.13 (t, 1, J = 6.4 Hz, H1′), 6.87–7.41 (m, 13, Ar), 7.62 (s, 1, H6).

5′-*O*-(Dimethoxytrityl)-O^4-ethylthymidine: 19% yield, amorphous powder; δ 1.29 (t, 3, J = 7.0 Hz, $-CH_3$), 1.54 (s, 3,5-CH_3), 2.11–2.30 (m, 2, H2′,2″), 3.22–3.23 (m, 2, H5′,5″), 3.73 (s, 6, $ArOCH_3$), 3.93 (m, 1, H4′), 4.30 (m, 1, H3′; q, 2, J = 7.0 Hz, OCH_2), 5.34 (d, 1, J = 3.2 Hz, 3′OH), 6.17 (t, 1, J = 6.3 Hz, H1′), 6.88–7.38 (m, 13, Ar), 7.80 (s, 1, H6).

5′-*O*-(Dimethoxytrityl)-O^2-*n*-butylthymidine: 12% yield, amorphous powder; δ 0.91 (t, 3, J = 7.3 Hz, CH_3), 1.39 (m, 2, $-CH_2CH_3$), 1.48 (s, 3, 5-CH_3), 1.66 (m, 2, $-CH_2CH_2CH_3$), 2.24–2.35 (m, 2, H2′,2″), 3.20 (m, 2 H5′,5″), 3.73 (s, 6, $ArOCH_3$), 3.92 (m, 1, H4′), 4.28 (t, 2, J = 6.5 Hz, OCH_2–), 4.31 (m, 1, H3′), 5.40 (d, 1, J = 3.3 Hz, 3′OH), 6.12 (t, 1, J = 6.4 Hz, H1′), 6.87–7.49 (m, 13, Ar), 7.62 (s 1, H6).

5′-*O*-(Dimethoxytrityl)-O^4-*n*-butylthymidine: 16% yield, amorphous powder; δ 0.91 (t, 3, J = 7.3 Hz, CH_3), 1.39 (m, 2, $-CH_2CH_3$), 1.54 (s, 3,5-CH_3), 1.66 (m, 2, $-CH_2CH_2CH_3$), 2.11–2.31 (m, 2, H2′,2″), 3.23 (m, 2, H5′,5″), 3.73 (s, 6, $ArOCH_3$), 3.93 (m, 1, H4′), 4.25 (t, 2, J = 6.5 Hz, OCH_2–), 4.30 (m, 1, H3′), 5.34 (bs, 1, 3′OH), 6.17 (t, 1, J = 6.3 Hz, H1′), 6.88–7.49 (m, 13, Ar), 7.80 (s, 1, H6).

(B) Alkylation with Isopropyl Bromide. 5′-*O*-(Dimethoxytrityl)thymidine (5 mmol) was treated with fresh Ag_2O and isopropyl bromide in the dark at 45 °C essentially as described for thymidine (Singer et al., 1983). Overalkylation at the 3′-position (identified by ^{1}H NMR, data not shown) to give the diisopropyl derivative posed a problem. To avoid this, reaction was frequently monitored by HPLC analysis and stopped (3–4 h) when a small amount of unreacted material remained. The product distribution in crude reaction mixture, analyzed by HPLC after removal of the trityl group, was as follows: O^2- 31%, O^4- 69%. No *N*-isopropyl derivative was detected.

The crude reaction mixture was first purified by flash chromatography on a silica gel column (3 cm × 20 cm) eluted with 5% methanol in chloroform. The appropriate fractions were combined, concentrated, and rechromatographed on a silica gel column (3 cm × 30 cm) eluted with 5% methanol in ethyl acetate. The products were precipitated from pooled concentrated fractions with cold hexane yielding 5′-*O*-(dimethoxyoxytrityl)-O^2-isopropylthymidine (45%) and 5′-*O*-(dimethoxytrityl)-O^4-isopropylthymidine (20%). For additional characterization small samples were detritylated (80% acetic acid) and purified by preparative TLC (10% methanol in chloroform). Pure isomers were dissolved in 2-propanol and concentrated to induce crystallization. The molar extinction coefficients calculated from the ultraviolet spectra and the melting point for O^2-isopropylthmidine (mp 171–172 °C) were similar to those reported by Singer et al. (1983); the melting point for O^4-isopropylthymidine differed significantly (160–162 °C) from that (99–101 °C) reported previously (Singer et al., 1983).

The identity of the purified products were checked further by ^{1}H NMR as follows. O^2-Isopropylthymidine: δ 1.30 [d, 6, J = 6.2 Hz, $CH(CH_3)_2$], 1.78 (s, 3,5-CH_3), 2.14 (m, 2, H2′,2″), 3.55–3.61 (m, 2 H5′,5′), 3.80 (m, 1, H4′), 4.24 (m, 1, H3′), 5.08 (t, 1, J = 5.1 Hz, 5′OH), 5.21 [m, 1, –OCH-$(CH_3)_2$], 5.30 (d, 1, J = 4.0 Hz, 3′OH), 6.07 (t, 1, J = 6.6 Hz, H1′), 7.81 (s, 1, H6).

O^4-Isopropylthymidine: δ 1.29 [d, 6, J = 6.2 Hz, CH-$(CH_3)2_i$, 1.85 (s, 3,5-CH_3), 1.98–2.21 (m, 2, H2′,2″), 3.56–3.64 (m, 2, H5′,5″), 3.81 (m, 1, H4′), 4.23 (m, 1, H3′), 5.07 (t, 1, J = 5.1 Hz, 5′OH), 5.21–5.26 [d, 1, 3′OH; m, 1, $-OCH(CH_3)_2$], 6.14 (t, 1, J = 6.5 Hz, H1′), 8.01 (s, 1, H6).

Alkyl Exchange. (A) Kinetics. Solutions (0.1%) of each O-alkyl derivative (see Tables I and III) in the appropriate anhydrous alcohol were made 10% with 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU) and left to stand at 25 or 50 °C in sealed tubes. The disappearance of starting material was followed as a function of time by analytical, reverse-phase HPLC. When present, the trityl groups were removed with 80% acetic acid before analysis. The data, obtained by measuring peak areas, were plotted as "fraction starting material remaining" by using a statistical program and a microcomputer. The pseudo-first-order rate constant of each exchange reaction and the standard error were obtained from the fitted curve. The half-lives were calculated from the rate constants. The values are given in Tables I and III.

(B) Preparation of 5′-O-(Dimethoxytrityl)-O^2-methylthymidine and 5′-O-(Dimethoxytrityl)-O^4-methylthymidine. 5′-O-(Dimethoxytrityl)thymidine (5 mmol) was alkylated with fresh Ag_2O and isopropyl bromide as described above. The crude reaction mixture containing O^2-isopropyl- and O^4-isopropylthymidines was filtered to remove silver salts, evaporated to dryness, and taken up in anhydrous methanol (25 mL). DBU was added to make a 10% solution, and the exchange reaction was carried out at 50 °C for 7 days. The reaction mixture was applied to a silica gel column (3 cm × cm). The column was washed with ethyl acetate (100 mL), and 5′-O-(dimethoxytrityl)-O^4-methylthymidine was eluted with 5% methanol in ethyl acetate. The yield after precipitation from cold hexane was 45%. The remaining 5′-O-(dimethoxytrityl)-O^2-methylthymidine was eluted from the column with 10% methanol in ethyl acetate. The yield after crystallization from ethyl acetate was 20%; mp 190–192 °C. The identities of these compounds were confirmed by ^{1}H NMR as follows.

5′-O-(Dimethoxytrityl)-O^4-methylthymidine: δ 1.54 (s, 3,5-CH_3), 2.13–2.29 (m, 2, H2′,2″), 3.22 (m, 2, H5′,5″), 3.73 (s, 6, ArOCH), 3.84 (s, 3, $-OCH_3$), 3.93 (m, 1, H4′), 4.30 (m, 1, H3′), 5.35 (bs, 1, 3′OH), 6.18 (t, 1, J = 6.3 Hz, H1′), 6.88–7.41 (m, 13, Ar), 7.81 (s, 1, H6).

5′-O-(Dimethoxytrityl)-O^2-methylthymidine: δ 1.49 (s, 3, 5-CH_3), 2.24–2.34 (m, 2, H2′,2″), 3.20 (m, 2, H5′,5″), 3.73 (s, 6, $ArOCH_3$), 3.86 (s, 3, $-OCH_3$), 3.92 (m, 1, H4′), 4.32 (m, 1, H3′), 5.40 (bs, 1, 3′OH), 6.12 (t, 1, J = 6.4 Hz, H1′), 6.87–7.38 (m, 13, Ar), 7.62 (s, 1, H6).

Stability of O^2- and O^4-Alkylthymidine in Concentrated Ammonia. Solutions of O^2- and O^4-alkylthymidine (0.1%) in concentrated ammonia (0.1 mL) were kept at 50 °C in sealed tubes. At regular time intervals reaction was stopped by evaporation of ammonia under reduced pressure. The residue was taken up in a known volume of H_2O and analyzed by HPLC. The pseudo-first-order rate constants, their standard errors, and the half-lives were obtained as before. A similar analysis was carried out on d(A-A-A-A-G-T-m^{4}T-A-A-A-A-C-A-T). The results are given in Table II.

The products of the reaction of concentrated ammonia with O^4-alkyl series were thymidine (R_t 12.3 min) and 5-methyldeoxycytidine (R_t 9.0 min). The latter was identified by its ultraviolet spectra (Dekker & Elmore, 1951). The ratio of these products was 1:3 and was independent of the alkyl group. In the O^2-alkyl series the products were thymidine and 5-methyldeoxyisocytidine (R_t 8.7 min). The latter was identified by its ultraviolet spectra (Kowollik & Langen, 1968). The ratio of these products was 1:1 and was independent of the alkyl group.

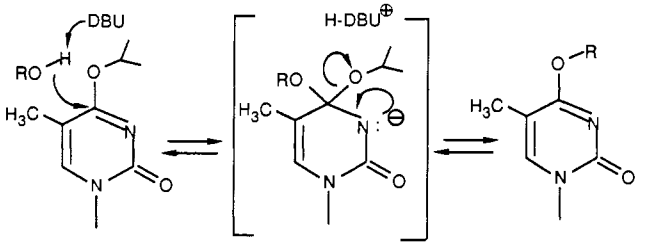
Stability of O^2- and O^4-Alkylthymidine during Thiophenoxide Treatment. Solutions of O^2- and O^4-alkylthymidines (0.1%) in 0.3 mL of thiophenol/triethylamine/dioxane (1/1/2 v/v/v) were incubated under standard conditions employed during deprotection of synthetic oligonucleotides (45 min at 25 °C), and a sample was taken for analysis. Reactions were stopped by addition of 1 mL of benzene followed by extraction with H_2O (2 × 0.1 mL). Combined water phases were extracted with benzene (3 × 0.5 mL), evaporated to dryness, and redissolved in H_2O for analysis. The products were measured by HPLC.

Synthesis of the Oligonucleotides. (A) General Procedure. The syntheses were carried out manually with homemade equipment assembled from commercially available components. Oligonucleotides with the sequence d(A-A-A-A-G-T-T*-T-A-A-A-A-C-A-T) (where T* = O^4-methyl, O^4-isopropyl, O^4-n-butyl or O^2-methyl, O^2-isopropyl) and d(T-m^{2}T-T) were synthesized on a 1–5-μmol scale according to the phosphoramidite method as described elsewhere (Tanaka & Letsinger, 1982; Borowy-Borowski & Chambers, 1987). The 5′-O-(dimethoxytrityl)-O-alkylated thymidines were converted to methoxyphosphoroamidite derivatives following published procedures (Beaucage & Caruthers, 1981; Köster et al., 1984). The yield was 70–80% after precipitation from cold hexane (−70 °C) and lyophilization from benzene. The estimated purity was >90% as judged by reverse-phase HPLC on a 4.6 mm × 250 mm column (Lichrosorb RP-18, 10-μm particle size) with a gradient of acetonitrile (40–80% during 10 min) in 0.05 triethylammonium acetate, pH 8.0.

After the condensation steps, the oligonucleotide was partially deprotected with triethylammonium thiophenoxide in dioxane during 45 min at 25 °C. It was then released from the solid support and deacylated with DBU/methanol (0.5 mL) under the conditions given in Table IV. After deprotection, the solid support was removed by centrifugation. The pellet was washed with H_2O (2 × 0.1 mL). Combined supernatant and washings were immediately extracted with benzene (5 × 1 mL) to remove DBU. Acetic acid (0.05 mL, 80%) was added to give pH ≈ 6.5, and the solution was concentrated under reduced pressure. The crude mixture of the oligonucleotides were purified by gel electrophoresis followed by reverse-phase HPLC using a Beckman RPSC column (4.6 × 75 mm) with a 3–15% gradient of acetonitrile in 0.1 M triethylammonium acetate over 20 min. The elution times for d(A-A-A-A-G-T-T*-T-A-A-A-A-C-A-T) were as follows: T* = T, 15 min; m^{2}T, 15.6 min; m^{4}T, 15.9 min; ip^{2}T, 16.7 min; ip^{4}T, 17.8 min; b^{4}T, 19.0 min. The elution time for d(T-T-T) was 7.2 min; that for d(T-m^{2}T-T) was 8.8 min. Each of these products was characterized by its ultraviolet spectrum and by its nucleoside content.

(B) Synthesis of d(A-A-A-A-G-T-m^{2}T-T-A-A-A-A-C-A-T) by Alkoxy Exchange at the Oligonucleotide Level. d(A-A-A-A-G-T-ip^{2}T-T-A-A-A-A-C-A-T) was synthesized as described above. After purification it was treated with 10% DBU in methanol at 50 °C for 4 days. The resulting mixture was fractionated by reverse-phase chromatography using isocratic elution with 6% acetonitrile in 0.1 M triethylammonium acetate, pH 7.0 (gradient elution as described above did not give an adequate separation). The elution times were as follows: d(A-A-A-A-G-T-T-T-A-A-A-AC-A-T), 11.0 min; d(A-A-A-A-G-T-m^{2}T-T-T-A-A-A-AC-A-T), 15.6 min; d(A-A-A-A-G-T-ip^{2}T-T-T-A-A-A-A-C-A-T), 37.8 min. The

Scheme I: DBU-Catalyzed Alkoxy Exchange[a]



[a] R = Me, Et, etc.

Table I: DBU-Catalyzed Displacement of O^2- and O^4-Isopropyl Groups by Other Alcohols

	$k \times 10^5$ (s^{-1})	SE $\times 10^5$	$t_{1/2}$[b]
10% DBU/MeOH, 50 °C			
O^2-isopropylthymidine	1.1	0.18	17.4 h
O^4-isopropylthymidine	0.5	0.01	38.3 h
10% DBU/EtOH, 50 °C			
O^2-isopropylthymidine	0.04	0.0006	20 days
O^4-isopropylthymidine	0.01	0.002	80 days
O^4-*n*-butylthymidine	0.1	0.005	8 days

[a] Calculated from pseudo-first-order plots; SE = standard error. [b] Calculated from the rate constants.

product distribution determined from peak areas is given in the text. The desired product was isolated and characterized by its ultraviolet spectrum and its nucleoside content: theory, $C_{1.00}G_{1.00}T_{3.00}A_{9.00}m^2T_{1.00}$; found, $C_{0.98}G_{1.01}T_{2.94}A_{9.04}m^2T_{1.02}$.

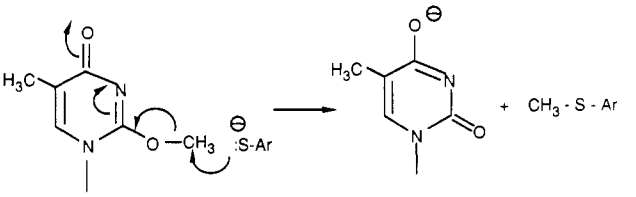
Results and Discussion

Synthesis of 5′-O-(Dimethoxytrityl)-O-alkylthymidines. Incorporation of an *O*-alkylthymidine into an oligonucleotide by the classical solid-phase, phosphoramidite method requires preparation of 5′-*O*-(dimethoxytrityl)-*O*-alkylthymidines (Matteucci & Caruthers, 1981). Treatment of thymidine with diazomethane gives primarily 3-methylthymidine (Farmer et al., 1973, Lawley et al., 1973, Kuśmierek & Singer, 1976). We obtained similar results with 5′-*O*-(dimethoxytrityl)thymidine, and an alternative route to the 5′-*O*-(dimethoxytrityl)-*O*-methylthymidines had to be found.

In previous studies we showed that alkoxy exchange in O^6-alkylguanine moieties is catalyzed by DBU in methanol (Borowy-Borowski & Chambers, 1987). Since it has been shown that the isopropyl groups of *O*-isopropylthymidine exchange with sodium methoxide in dry methanol to give the desired *O*-methylthymidines (Singer et al., 1983), it appeared that DBU should catalyze the exchange of various alcohols with an *O*-isopropyl group as shown in Scheme I for the O^4 derivative. A similar reaction should occur with O^2-isopropylthymidine. In methanol the exchange proceeded smoothly with 5′-*O*-(dimethoxytrityl)-O^2-isopropylthymidine and 5′-*O*-(dimethoxytrityl)-O^4-isopropylthymidine ($t_{1/2}$ = 24.8 and 45.5 h, respectively) to give the corresponding *O*-methyl derivatives in good yield. This provided the intermediates needed for synthesis of oligonucleotides containing *O*-methylthymidine residues.

In principle, this exchange reaction could provide a general synthesis of various *O*-alkylthymidines from a single intermediate (Scheme I, R = Et, *n*-Bu, etc.). This would be particularly attractive if the exchange could be carried out at the oligonucleotide level. To examine this possibility, we measured the rate of exchange at the nucleoside level. Some representative data are shown in Table I. Although the exchange occurred at usable rates in methanol, the reaction was too slow in other alcohols to be useful. Since the slow rate might be due to the branched chain, we examined the exchange of O^4-*n*-butyl in ethanol. Although the rate was significantly faster than with the corresponding isopropyl derivative, it was still too slow to be useful. Therefore, it does not appear that this approach can be used as a general method. However, exchange in methanol provides an alternative synthesis of *O*-methylthymidines from the readily available *O*-isopropyl derivatives. The other *O*-alkylthymidines are available by a direct synthesis with the appropriate diazoalkane. For example, diazoethane and diazobutane gave usable yields of the desired O^2- and O^4-alkyl derivative (see Experimental Procedures).

Scheme II: Removal of O^2-Methyl Groups by Thiophenoxide Ions



Dealkylation during Deprotection of Oligonucleotides. The classical phosphoramidite method yields a protected oligonucleotide attached to a solid support. Removal of the protecting groups involves two different processes. First, the methyl groups protecting the internucleotide bonds are removed with thiophenol and triethylamine in dioxane (Matteucci & Caruthers, 1981). Second, the partially deprotected product is treated with concentrated ammonia to release it from the solid support and remove the acyl groups used to protect the exocyclic amino groups. Since the deprotection conditions were expected to produce side reactions, we examined the effect of these conditions on model nucleosides in order to assess the magnitude of the problem.

When we treated O^2-methylthymidine with thiophenol and triethylamine in dioxane under the conditions used to remove internucleotide protecting groups (25 °C for 45 min) only 35% of the o^2-alkylthymidine survived; 65% was converted to thymidine. In contrast to the results with O^2-methylthymidine, O^2-ethyl-, isopropyl-, and *n*-butylthymidines were stable under these conditions. Under the same conditions 97% of the methyl group in O^4-methylthymidine survived; with O^4-ethyl, *n*-butyl, or isopropyl no loss of the alkyl group was detected.

These results suggest that the O^2-methyl group is removed by direct displacement of the methyl group as shown in Scheme II. The alternative is addition of thiophenoxide to the pyrimidine ring followed by elimination of the alkoxide. Initially this would produce the thiophenoxide adduct by a mechanism similar to that shown in Scheme I. We found no evidence for such an intermediate.

This side reaction appeared to be a very serious problem in the synthesis of oligonucleotides carrying O^2-methylthymine moieties. To test this, the blocked trinucleoside diphosphate $d(Tp_mm^2Tp_mT)$ was prepared in the usual manner. The internucleotide methyl groups were removed with thiophenol and triethylamine under standard conditions. From the nucleoside data we expected a 35% yield of $d(T\text{-}m^2T\text{-}T)$; we obtained only 15%.

To assess the side reactions occurring during the second deprotection step, we measured the loss of alkyl groups from both O^2- and O^4-alkylthymidines in concentrated ammonia at 50 °C (Matteucci & Caruthers, 1981). The kinetic data are given in Table II.

The data in Table II and under Experimental Procedures illustrate several points:

(1) There was no significant difference between the rate constants for loss from O^2 and O^4 for a given alkyl group.

Table II: Loss of the *O*-Alkyl Group from O^2- and O^4-Alkylthymidine in Concentrated Ammonia at 50 °C

compound	$k \times 10^5$ (s^{-1})	SE × 10^5	$t_{1/2}$ (h)
O^2- or O^4-methylthymidine	16.0	0.09	1.2
O^2- or O^4-ethylthymidine	8.1	0.2	2.4
O^2- or O^4-*n*-butylthymidine	6.7	0.19	2.9
O^2- or O^4-isopropylthymidine	2.0	0.17	9.6
d(A-A-A-A-G-T-m^{4}T-T-A-A-A-A-C-A-T)	13.4	0.4	1.4

Table III: Alkoxy Exchange Catalyzed by 10% DBU in Methanol at 25 °C

	$k \times 10^6$ (s^{-1})	SE × 10^6	$t_{1/2}$ (h)
O^2-isopropylthymidine	1.8	0.1	106
O^4-isopropylthymidine	0.8	0.02	240
O^2-ethylthymidine	18.4	0.7	10.4
O^4-ethylthymidine	13.1	0.1	14.6
O^2-*n*-butylthymidine	13.9	0.5	13.8
O^4-*n*-butylthymidine	9.3	0.1	20.6

(2) The O^4-alkylthymidines gave 5-methyldeoxycytidine by ammonolysis; the O^2-alkylthymidines gave 5-methyldeoxyisocytidine (see Experimental Procedures). Formation of these products establishes the addition–elimination mechanism shown in Scheme III. Thymidine was also formed. There are two possible routes to this side product. The first involves hydrolysis by the mechanism shown in Scheme III. The second involves displacement at the alkyl carbon. We cannot distinguish between these two mechanisms with our current data.

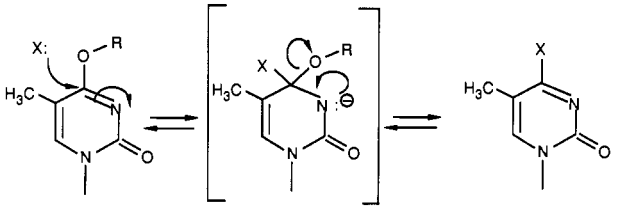
(3) The ratio of 5-methyldeoxycytidine to thymidine was 3:1 from the O^4-alkyl derivatives and 1:1 from the O^2-alkyl derivatives regardless of the alkyl group involved. These product ratios result from the primary reaction since we have shown that 5-methyldeoxycytidine and 5-methyldeoxyisocytidine are stable in concentrated ammonia at 50 °C for at least 6 h. This result indicates that the partitioning between ammonolysis and hydrolysis is different for O^2- and O^4-alkylthymine residues even though the overall rate for removing a given alkyl group is the same for O^2- as O^4-alkylderivatives.

(4) *O*-Isopropyl is the only group with a sufficient half-life to give any significant amount of oligonucleotide product under the standard deprotection conditions.

(5) The half-life for an O^4-methylthymidine moiety in an oligonucleotide is only slightly longer than that for the corresponding nucleoside. Furthermore, we have demonstrated that the mechanism shown in Scheme III operates at the oligonucleotide level by isolating d(A-A-A-A-G-T-m^{5}C-T-A-A-A-A-C-A-T) (data not shown) after treating d(A-A-A-A-G-T-m^{4}T-T-A-A-A-A-C-A-T) with concentrated ammonia under the conditions given in Table II.

Clearly, the standard conditions used for release of oligonucleotides from the solid support and removal of the acyl protecting groups are not suitable for synthesis of oligonucleotides carrying *O*-alkylthymine residues. Similar problems have been encountered with oligonucleotides carrying O^6-alkylguanine moieties. In this case, substitution of the hindered amine, DBU, in dry methanol for ammonia provided a satisfactory solution (Kuzmich et al., 1983; Borowy-Borowski & Chambers, 1987). From our nucleoside data (Table I) we could anticipate that DBU-catalyzed deprotection in methanol would provide satisfactory results with *O*-alkylthymidines when the alkyl group is methyl. In methanol, exchange with *O*-ethyl, isopropyl, or *n*-butyl groups was expected. Some representative kinetic data are shown in Table III. Under conditions that remove the acyl protecting groups completely, exchange with the *O*-alkyl group creates a serious problem. The obvious solution is to carry out deprotection in the homologous alcohol. Because of low solubility, this could not be done in the alcohol alone, but a mixture of alcohol and tetrahydrofuran gave satisfactory results.

Scheme III: Side Reactions Produced by Concentrated Ammonia[a]



[a] R = alkyl; X = NH_3 or OH^-.

Synthesis of Oligonucleotides Containing O-Alkylthymidine Residues. The information described above was used to develop methodology for the synthesis of several oligonucleotides carrying an *O*-alkylthymine moiety. For most of this work d(A-A-A-A-G-T-alk*T-T-A*-A-A-A-C-A-T) was synthesized. This corresponds to a minus strand sequence at the start of gene G in bacteriophage ΦX174. The sequence *TTA* is complementary to an ocher codon, TAA, in the viral DNA. The potential use of modified thymidine residues in ΦX DNA carrying such a sequence to study the factors that affect mutations frequencies in vivo has been described (Chambers, 1982).

A series of blocked oligonucleotides having the sequence d(bzA-bzA-bzA-bzA-ibG-T-alkT-T-bzA-bzA-bzA-anC-bzA-T) were synthesized on a solid support by using the appropriate *O*-methyl *N*,*N*-diisopropylphosphoramidite derivative of 5′-*O*-(dimethoxytrityl)-*O*-alkylthymidine. The product was treated first with thiophenol and triethylamine in dioxane under standard conditions to remove the internucleotide methyl groups and then with DBU/alcohol under the conditions given in Table IV to release the oligonucleotide from the solid support and remove the acyl protecting groups. The resulting mixture was worked up as described under Experimental Procedures and purified by electrophoresis on a polyacrylamide gel. The band corresponding to a 15-mer was analyzed by reverse-phase chromatography. The approximate distribution

Table IV: Deprotection of d(bzA-bzA-bzA-bzA-ibG-T-alkT-T-bzA-bzA-bzA-bzA-anC-bzA-T) with DBU/Alcohol To Give d(A-A-A-A-G-T-alkT-T-A-A-A-A-C-A-T)

alkyl group	time	oligonucleotide product distribution		
O^4-methyl	3 days[a]	...-m^{4}T-..., 80%		+ ...-T-..., 20%
O^2-methyl	2 days[a]	...-m^{4}T-..., 16%[c]		+ ...-T-..., ,84%[c]
O^4-isopropyl	3 days[a]	...-m^{4}T-..., 16%	+ ...-ip^{4}T-..., 73%	+ ...-T-..., 11%
O^2-isopropyl	2 days[a]	...-m^{2}T-... (16%)[d]	+ ...-ip^{2}T-..., 84%	+ ...-T-..., (16%)[d]
O^4-*n*-butyl	5 days[b]	...-b^{4}T-..., 83%		+ ...-T-..., 17%

[a] The initial product was treated with thiophenol and triethylamine in dioxane for 45 min at 25 °C. The product was released from solid support and deacylated with 10% DBU in anhydrous methanol at 25 °C for the times specified. [b] As in footnote *a* but DBU step in 1-butanol/tetrahydrofuran (60/40 v/v) at 50 °C. [c] The product ratio was determined by nucleoside analysis of the oligonucleotide mixture obtained after purification by gel electrophoresis. [d] ...-m^{2}T-... and ...-T-... cochromatographed (reverse-phase HPLC) under the conditions used in this particular experiment so the distribution of products in this peak is unknown. Later conditions for separating these products were found (see Experimental Procedures).

of products is given in Table IV. The data illustrate the several points:

(1) Deprotection in methanol gave good yields of the O^4-methyl (80%), O^4-isopropyl (73%), and O^2-isopropyl (84%) derivatives but a poor yield of the O^2-methyl derivative. The 16% yield of 15-mer carrying an O^2-methylthymine residue was essentially the same as obtained with d(T-m^{2}T-T) (15%). Since we have shown that both O^2-methylthymidine and O^4-methylthymidine are stable in 10% DBU/methanol at 25 °C for 2 days, the poor yield of d(A-A-A-A-G-T-m^{2}T-T-A-A-A-A-C-A-T) is due primarily to dealkylation by thophenoxide and not to methoxide generated by DBU.

(2) Deprotection of the oligonucleotide carrying an O^4-*n*-butylthymine moiety in DBU/methanol converted the O^4-*n*-butyl moiety to the O^4-methyl derivative, but deprotection in a mixture of DBU in *n*-butyl alcohol and tetrahydrofuran gave a satisfactory yield (83%) of the desired product.

(3) Some dealkylation invariably occurs during the removal of acyl groups with DBU/alcohol. We believe this occurs by the mechanism shown in Scheme III and is due to traces of moisture, but we cannot rule out the possibility that some of the dealkylation is due to direct displacement.

(4) A methyl group can be introduced at the oligonucleotide level by exchange with an O^4-isopropyl group. However, the rate is very slow. Even at 50 °C for 5 days in 10% DBU/methanol, we obtained only 27% d(A-A-A-A-G-T-m^{4}T-T-A-A-A-A-C-A-T) from d(A-A-A-A-G-T-ip^{4}T-T-A-A-A-A-C-A-T). This yield is less than can be obtained by incorporating 5′-*O*-(dimethoxytrityl)-O^4-methylthymidine into the oligonucleotide in the usual manner.

Oligonucleotides carrying O^2-methylthymine moieties were not accessible by the direct approach we were using because most of the O^2-methyl is lost during removal of the internucleotide protecting group. However, in this case the alkoxy exchange is fast enough to be practical, and we used it to synthesize d(A-A-A-A-G-T-m^{2}T-T-A-A-A-A-C-A-T) from d(A-A-A-A-G-T-ip^{2}T-T-A-A-A-A-C-A-T). First, the protecting groups were removed in the usual manner; the isopropyl group is stable under these conditions. After purification of d(A-A-A-A-G-T-ip^{2}T-T-A-A-A-A-C-A-T), the isopropyl group was exchanged for methyl with DBU in methanol. The desired product was isolated and characterized as described under Experimental Procedures.

The synthesis of d(A-A-A-A-G-T-ip^{2}T-T-A-A-A-A-C-A-T) by the direct route and d(A-A-A-A-G-T-m^{2}T-T-A-A-A-A-C-A-T) by the exchange route represent the first chemical syntheses of oligonucleotides containing an O^2-alkylthymine moiety. Unfortunately, exchange of O^2-isopropyl for another alkyl groups does not represent a general method for synthesis of oligonucleotides carrying an O^2-alkylthymine moiety because the exchange rate is too slow to be useful with alcohols other than methanol. However, the 5′-*O*-(dimethoxytrityl)-O^2-alkylthymidines other than methyl are accessible in acceptable yields by direct synthesis, and they can be incorporated into the oligonucleotide in the usual manner. The only remaining difficulty is displacement of the O^2-alkyl group during removal of the internucleotide protecting group (Scheme II). The magnitude of the difficulty depends on the particular alkyl group involved. It seems likely that this side reaction can be minimized, and perhaps avoided, by using an *O*-(cyanoethyl) as the internucleotide protecting group (Sinha et al., 1983, 1984). We have not investigated this directly, but we did explore deprotection of d(ibGp$_{ce}$ibGp$_{ce}$T) under conditions where the *O*-alkylthymidine groups are stable. We found *O*-(cyanoethyl) groups are removed under the conditions required to remove the other acyl protecting groups (e.g., 10% DBU/methanol at 25 °C for 2 days; shorter times were not investigated). Thus, complete deprotection and release from the solid support can be done in a single step. Small amounts of dT and d(G-T), due to incomplete coupling, were detected, but the synthesis was very clean and a good yield of d(G-G-T) was obtained. Therefore, the cyanoethyl protecting group appears advantageous not only for normal nucleosides but particularly for modified nucleosides.

The data presented here indicate that oligonucleotides containing any *O*-alkylthymine moiety can be synthesized by modifying procedures used in the classical phosphoramidite procedure. Another route to oligonucleotides carrying O^4-alylthymine residues has been described. It involves a DBU-catalyzed exchange of methoxide with the 4-(3-nitro-1,2,4-triazolo) group of 4-(3-nitro-1,2,4-triazolo)-3′,5′-bis-*O*-(methoxyacetyl)thymidine to give the blocked O^4-methylthymidine (Li et al., 1987). While this approach gives satisfactory results, the key intermediate, 3′,5′-bis-*O*-(methoxyacetyl)thymidine, is designed for a particular synthetic route developed by Reese and Skone (1984). Many laboratories will find this approach difficult since the key intermediate is not commercially available. Furthermore, the synthesis described by Reese's laboratory gives only the O^4-alkyl derivative, and so far only O^4-methyl has been reported. Nevertheless, this approach has proven useful in making milligram amounts of oligonucleotides carrying an O^4-methylthymine moiety for studying base pairing by NMR (Kalnik et al., 1988a,b).

An enzymatic approach has been used to incorporate O^4-methylthymidylate residues into biologically active DNA (Hall & Saffhill 1983; Singer et al., 1986a). This provides an appealing alternative to the chemical approach. It has several limitations, however. First, the *O*-alkylthymidine triphosphates must be synthesized. This is a four-step procedure involving synthesis of O^4-isopropylthymidine, phosphorylation, alkoxy exchange, and conversion of O^4-alkyl-d-TMP to its triphosphate derivative (Singer et al., 1986b). Second, the rates of incorporation are slow compared to those of d-TMP itself, and the resulting modified DNA is difficult to characterize rigorously. The enzymatic approach has been used successfully to prepare modified DNA for site-directed mutagenesis studies (Preston et al., 1986, 1987), and it will continue to have appeal for certain experiments of this kind. Nevertheless, the chemical approach is more general and can be adapted to scales suitable for either biological or physicochemical studies.

Finally, it should be noted that the DBU-catalyzed alkoxy exchange has been used to introduce a ^{14}C and ^{13}C label at the *O*-alkyl carbon (Li et al., 1987), and it can probably be extended to other isotopes (^{3}H, ^{15}N, ^{17}O, ^{18}O). In principle, these labels, which could be useful in DNA probes and in physicochemical studies, can be introduced as shown in Scheme II at the oligonucleotide level.

Oligonucleotides carrying an *O*-alkylthymidine moiety have assumed new importance since the recognition that repair of *O*-alkylpyrimidines in mammalian cells differs significant from that found in *Escherichia coli* (Brent et al., 1988). Incorporation of *O*-alkylthymidine residues into specific sites in appropriate DNAs via the appropriate oligonucleotide represents an attractive approach for studying repair of this kind of DNA damage in animal cells.

Registry No. d(A-A-A-A-G-T-m^{4}T-A-A-A-A-C-A-T), 117827-88-0; d(A-A-A-A-G-T-m^{4}T-T-A-A-A-A-C-A-T), 117827-89-1; d(A-A-A-A-G-T-ip^{4}T-T-A-A-A-A-C-A-T), 117827-90-4; d(A-A-A-A-G-T-b^{4}T-T-A-A-A-A-C-A-T), 117896-01-2; d(A-A-A-A-G-T-m^{2}T-A-A-A-A-C-A-T), 117895-99-5; d(A-A-A-A-G-T-ip^{2}T-A-A-A-A-C-A-T), 117896-00-1; d(T-m^{2}T-T), 117775-92-5; d(bzA-bzA-

bzA-bzA-ibG-T-m^{4}T-T-bzA-bzA-bzA-bzA-anC-bzA-T), 117827-91-5; d(bzA-bzA-bzA-bzA-ibG-T-m^{2}T-T-bzA-bzA-bzA-bzA-anC-bzA-T), 117827-92-6; d(bzA-bzA-bzA-bzA-ibG-T-ip^{4}T-T-bzA-bzA-bzA-bzA-anC-bzA-T), 117896-02-3; d(bzA-bzA-bzA-bzA-ibG-T-ip^{2}T-T-bzA-bzA-bzA-bzA-anC-bzA-T), 117896-03-4; d(bzA-bzA-bzA-bzA-ibG-T-b^{4}T-T-bzA-bzA-bzA-bzA-anC-bzA-T), 117896-04-5; thymine, 65-71-4; 5′-*O*-(dimethoxytrityl)thymidine, 40615-39-2; 5′-*O*-(dimethoxytrityl)-O^2-ethylthymidine, 117775-84-5; 5′-*O*-(dimethoxytrityl)-O^4-ethylthymidine, 117775-85-6; 5′-*O*-(dimethoxytrityl)-O^2-*n*-butylthymidine, 117775-86-7; 5′-*O*-(dimethoxytrityl)-O^4-*n*-butylthymidine, 117775-87-8; 5′-*O*-(dimethoxytrityl)-O^2-isopropylthymidine, 117775-88-9; 5′-*O*-(dimethoxytrityl)-O^4-isopropylthymidine, 117775-89-0; O^2-isopropylthymidine, 87539-53-5; O^4-isopropylthymidine, 87539-54-6; 5′-*O*-(dimethoxytrityl)-O^4-methylthymidine, 117775-90-3; 5′-*O*-(dimethoxytrityl)-O^2-methylthymidine, 117775-91-4; O^2-methylthymidine, 37085-48-6; O^4-methylthymidine, 50591-13-4; O^2-ethylthymidine, 59495-21-5; O^4-ethylthymidine, 59495-22-6; O^2-*n*-butylthymidine, 82543-40-6; O^4-*n*-butylthymidine, 82543-41-7.

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