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Synthesis and properties of oligonucleotides containing 4-thiothymidine, 5-methyl-2-pyrimidinone-1-β-D(2'-deoxyriboside) and 2-thiothymidine

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ABSTRACT

Methods are given for the synthesis of derivatives of 4-thiothymidine (^{4S}T), 5-methyl-2-pyrimidinone-1-β-D(2'-deoxyriboside) (^{4H}T) and 2-thiothymidine (^{2S}T) suitable for incorporation into oligodeoxynucelotides by the cyanoethyl phosphoramidite method. ^{4H}T and ^{2S}T are incorporated with no base protection but the sulphur atom in ^{4S}T is protected with an S-sulphenylmethyl (-SCH_3) function. This can be removed with dithiothreitol after synthesis. These T analogues have been incorporated into GACGATATCGTC, a self-complementary dodecamer containing the Eco RV recognition site (underlined), in place of the two T residues within this site. Although pure dodecamers are obtained in each case the syntheses are not as efficient as those seen when normal unmodified bases are used mainly due to the chemical reactivity of ^{4S}T, ^{4H}T and ^{2S}T. Some of the chemical properties of oligonucleotides containing these bases (reactivity towards NH_3) as well as their physical properties (melting temperatures, U.V., fluorescence and circular dichroism spectra) have been determined and are discussed.

INTRODUCTION

The majority of the biological functions of DNA are dependent on proteins that recognise and bind to specific sequences of bases in the double helical structure. This effect is possible as the edges of each base pair in duplex DNA expose a unique array of hydrogen bonding groups either in the major or minor groove (1). Many proteins that usually bind long DNA polymers also shows similar interactions with short synthetic oligonucleotides providing the recognition sequence is present. In these cases the incorporation of modified bases into the recognition sequence is often useful for the study of the recognition process. Typically potential protein contact groups are deleted one at a time in a systematic method and the properties of the modified oligonucleotide with the protein under study determined. With investigations of this type the most difficult part is often the preparation of appropriate modified bases and their incorporation into oligonucleotides. With thymidine (T) the groups capable of interacting with proteins are shown in figure 1. Thus when T is incorporated into B-DNA the 4-keto oxygen atom and the 5-methyl group (both in the major groove) and the 2-keto oxygen atom (in the minor groove) are capable of interacting with proteins. A complete set of T analogues in which each potential contact has been altered is also shown in figure 1. The first of these, deoxyuridine (dU), has the 5-CH_3 group replaced by H. This removes a potential Van der Waals interaction with the protein. As this base (both as the free deoxynucleoside and in a form suitable for oligonucleotide synthesis) is commercially available there are no synthetic difficulties in incorporating it into oligomers. The second and third analogues 5-methyl-2-pyrimidinone-1-β-D (deoxyriboside) (^{4H}T) and 4-thiothymidine (^{4S}T) serve to probe the role of the 4-keto oxygen atom in T. This can

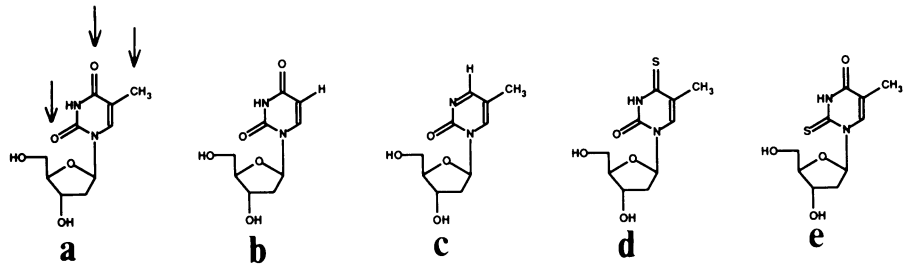

Figure 1 The structures of: (a) deoxythymidine, (b) deoxyuridine, (c) 5-methyl-2-pyrimidinone 1-β-D(2'-deoxyriboside), (d) 4-thiothymidine, (e) 2-thiothymidine. With deoxythymidine the 4-keto oxygen and 5-methyl group (in the major groove) and the 2-keto oxygen (in the minor groove) are capable of interacting with proteins when the base is incorporated into double stranded DNA. These sites have been arrowed.

form hydrogen bonds with appropriate amino acid side chains in proteins. With ^{4H}T all hydrogen bond forming ability to proteins is clearly lost. However ^{4H}T cannot form any Watson-Crick hydrogen bonds with its complementary A base in DNA and this could lead to double helix destabilisation or distortion. With ^{4S}T base pairing with A is still possible and the O → S change should also weaken hydrogen bonding capacity to proteins. The final analogue 2-thiothymidine (^{2S}T) probes the role of the 2-keto oxygen in T (again capable of hydrogen bonding with proteins) by converting this oxygen atom to sulphur. Although ^{4H}T (2), ^{4S}T (3) and ^{2S}T (4) have been synthesised the approaches given are designed to give the free deoxynucleosides rather than derivatives suitable for oligonucleotide synthesis. Of the three bases only ^{4H}T has previously been incorporated into oligonucleotides initially using outdated methods based on phosphotriester chemistry (5, 6). More recently oligonucleotides containing this base have been prepared by the more modern phosphoramidite approach (7). This paper presents methods for the preparation of ^{4H}T, ^{4S}T and ^{2S}T derivatives suitable for incorporation into oligonucleotides using the cyanoethyl phosphoramidite method (8). The incorporation of these derivatives into oligonucleotides is described. Furthermore some of the physical and chemical properties of the resulting oligonucleotides have been determined. Together with the available dU derivative these ^{4H}T, ^{4S}T and ^{2H}T compounds provide a full set of T analogues for the study of protein nucleic acid interactions.

MATERIALS AND METHODS

The following chemicals were purchased from Aldrich (Gillingham, Dorset) and used as supplied. Sodium methoxide (25% weight solution in CH_3OH), dimethoxytriphenylmethylchloride (dmt-Cl), Lawesson's reagent (p-methoxyphenylthionophosphinesulphide dimer), hydrazine hydrate, silver (I) oxide, silver acetate, methyltriphenoxyphosphonium iodide, methylmethanethiosulphonate, 2-cyanoethyl N,N-diisopropylchlorophosphoramidite and anhydrous dimethylformamide. Thymidine was obtained from Genofit (Geneva), anhydrous acetonitrile from Cruachem (Glasgow) and snake venom phosphodiesterase and alkaline phosphatase (molecular biology grade) from BCL (Lewes, East Sussex). All reagents for oligonucleotide synthesis were purchased from Applied Biosystems (Warrington, Cheshire). The following reagents were from BDH (Poole, Dorset) and were dried as stated. Tetrahydrofuran and 1,4-Dioxan (refluxed from

sodium/benzophenone until a deep purple colour was obtained and distilled), triethylamine and N,N-diisopropylethylamine (refluxed and distilled from calcium hydride), pyridine (refluxed and distilled from firstly ninhydrin and secondly KOH), methanol (dried using magnesium/iodine as stated in (9)), gaseous H_2S (passed over P_2O_5 immediately prior to use). 3'-O-acetylthymidine (10) and 3',5'-O-dibenzoyl thymidine (3) were prepared as described.

Reactions were routinely checked by thin layer chromatography (tlc) on aluminium backed silica gel tlc plates (5×7.5 cm) containing a 254 nm fluorescent indicator. Compounds were observed by illumination with 254 nm light. Flash chromatography (11) was performed using Merck silica gel 60 (0.040−0.063 mm particle size) and a positive nitrogen pressure of +0.5 atmospheres. Column eluates were monitored by tlc. ^1H NMR spectroscopy was carried out on a Brucker AM 360 spectrometer. Chemical shifts are given in ppm. The values obtained for the sugar protons have not been given in all cases but referred to as standard sugar spectrum. These were always similar to that recorded for 3',5'-O-dibenzyl 4-thiothymidine (full spectra can be obtained from BAC on application). Mass spectrometry was performed using a V.G. TS 250 spectrometer with a V.G. fast atom bombardment source operated in the positive ion mode. The atom gun used xenon and produced a beam of neutral atoms at 9−10 kV. Glycerol or nitrobenzylalcohol were used as matrices. Occasionally electron impact (EI) spectra were measured with this machine. Circular dichroism spectroscopy was undertaken using a Jasco J40-CS spectropolarimeter at 25°C. The oligonucleotide (10 μM) was dissolved in a 1ml volume of 25mM KH_2PO_4 pH 7.2 containing 100mM NaCl and 10mM $MgCl_2$. Melting temperature determinations were performed on a 10 μM concentration of oligonucleotide dissolved in 1.5ml of 50mM Hepes-KOH pH 7.5 containing 100mM NaCl and 10mM $MgCl_2$. The temperature was raised from 15 to 90°C at between 0.5 and 1°C per minute. The actual temperature of the solution was measured using a thermocouple in an adjacent cuvette. Both the temperature and absorbance outputs (from a Perkin-Elmer Coleman 572 spectrometer) were fed into a BBC model B microcomputer. The absorbance values were sampled every 40 msec and averaged when the temperature had risen by 0.1°C. The U.V. spectra of the modified bases were measured in H_2O (pH 7) using a Pye-Unicam SP8−400 spectrometer. Fluorescence spectra of ^{4H}T and oligonucleotides containing this base were measured in H_2O (pH 7) with a Perkin-Elmer 650S fluorescence detector fitted with a 150 B xenon power supply. High pressure liquid chromatography (hplc) was performed using a Varian 5000 liquid chromatograph and a UV 50 variable wavelength detector. The C18 reverse phase material Apex-I octadecysilyl (5μ particle size) packed into columns 25 × 0.45 cm supplied by Jones Chromatography (Llanbradach, Glamorgan) was used. Gradients were prepared from 0.1M triethylammonium acetate pH 6.5 containing 5% CH_3CN (hplc buffer A) and 0.1M triethylammonium acetate pH 6.5 containing 65% CH_3CN(hplc buffer B) at a flow rate of 1ml min^{-1}. Columns were run at room temperature for deoxynucleoside composition analysis and 50°C for oligonucleotide purification and analysis. Compounds were usually detected at 254 nm.

3',5'-O-dibenzoyl 4-thiothymidine

3',5'-O-dibenzoylthymidine (22.5g, 0.05 mol, dried by coevaporation from 3×50 ml anhydrous pyridine) was suspended in 500 ml of dry dioxan. Lawesson's reagent (18.3 g, 0.06 mol) was added and the mixture refluxed for 2 h. After this time tlc (CH_2Cl_2 98, CH_3OH 2) showed complete conversion of the starting material (Rf 0.2) to product (Rf 0.5). The mixture was cooled on ice and 25 ml H_2O added. After a further hour the

mixture was evaporated to dryness and subjected to standard work up procedure (dissolving in $CHCl_3$ and extraction with 5% $NaHCO_3$ (twice) and NaCl, followed by drying the $CHCl_3$ layer over Na_2SO_4 and evaporation of solvents). 22.1g (95%) of 3',5'-O-dibenzoyl-4-thiothymidine was obtained which was pure enough (>95% in the above system) to be used directly in subsequent steps. A small sample was recrystallised from ethanol to give a standard with satisfactory spectroscopic properties. ^1H NMR ($CDCl_3$) 10.1 (1Hs, N3H), 7.35 (1Hs, H6), 8.2−7.6 (10Hm benzoyl), 1.8 (3Hs CH_3). The sugar protons gave the following spectra 6.45 (1Ht H1'), 5.65 (1Hm, H3'), 4.9−4.5 (3Hm, H4', H5', H5"), 2.83 (1Hm, H2') 2.38 (1Hm, H2"). FAB-MS 467 (M + H^+, 100%), 325 (dibenzoyldeoxyribose$^+$, 90%), 144 (base + $2H^+$, 70%).

5'-O-dimethoxytrityl 4-thiothymidine

Unpurified 3',5'-O-dibenzoyl 4-thiothymidine (11.65g, 0.025 mol), obtained above, was converted to 4-thiothymidine with sodium methoxide as described (3). The product obtained was a yellow, very hygroscopic, solid. This solid gave satisfactory ^1H NMR, U.V. and FAB-MS spectra (values identical to those found for 4-thiothymidine obtained from 5'-dmt 4-thiothymidine and given below). Because of its hygroscopic nature no yield was determined and the product directly converted to its 5'- dmt derivative in the usual manner using dimethoxytritylchloride (12). After standard work up the product was purified by flash chromatography (25×4 cm column) using $CHCl_3$ containing 0.5% triethylamine as eluant. 9.1 g of product (a yield of 65% for the two steps from 3',5'-O-dibenzoylthymidine) was obtained. The product was pure by tlc (CH_2Cl_2 95, CH_3OH 5, Rf 0.75). ^1H NMR ($CDCl_3$) 7.7 (1Hs, H6), 7.48−6.8 (13Hm, dimethoxytrityl aromatic), 3.8 (6Hs, CH_3O) 1.68 (3Hs, CH_3) plus standard sugar spectrum. FAB-MS 303 (dmt$^+$, 100%).

5'-O-dimethoxytrityl 4(S-sulphenylmethyl) thiothymidine

5'-O-dimethoxytrityl 4-thiothymidine (2.8 g, 5 mmol) was dissolved in 100 ml of dry dioxan and dry triethylamine (3.5ml, 25 mmol) was added. Methylmethanethiosulphonate (1 ml, 10 mmol) was added and the mixture heated at 50°C for 15 h. Thin layer chromatography (ethyl acetate) showed partial conversion of starting material (Rf 0.7) to product (Rf 0.3). A further 1 ml of methylmethanethiosulphonate was added and after an additional 15 h tlc showed >95% reaction. The mixture was evaporated to give an oil and subjected to standard work up. The product was purified by flash chromatography (15×2 cm column) using $CHCl_3$ containing 0.5% triethylamine as eluant. 2.71g (89%) of product was obtained which was pure in the above tlc system. ^1H NMR ($CDCl_3$) 7.95 (1Hs, H6), 7.45−6.8 (13Hm, dimethoxytrityl aromatic), 3.8 (6Hs, CH_3O) 2.5 (3Hs, $SSCH_3$) 1.63 (3Hs, CH_3) plus standard sugar spectrum. FAB-MS 607 (M + H^+, 2%), 303 (dmt$^+$, 100%).

5'-O-dimethoxytrityl-5-methyl-2-pyrimidinone-1-β-D(2'-deoxyriboside)

5'-O-dimethoxytrityl-4-thiothymidine (2.8 g, 5 mmol) was converted to the 4-hydrazino derivative by refluxing in 100 ml C_2H_5OH containing 0.64 ml (20 mmol) hydrazine hydrate for 90 min (2). The crude product obtained after solvent evaporation and standard work up (CH_2Cl_2 95, CH_3OH 5, Rf 0.2) appeared >95% pure by tlc and gave satisfactory spectroscopic data. ^1H NMR ($CDCl_3$) 7.5−6.8 (14Hm, dimethoxytrityl aromatic plus H6), 3.76 (6Hs, CH_3O) 1.48 (3Hs, CH_3) plus standard sugar spectrum. FAB-MS 559 (M + H^+, 20%), 303 (dmt$^+$, 100%), 141 (base + $2H^+$, 15%). This hydrazino derivative was immediately converted to the 2-pyrimidinone by refluxing for two hours in 45 ml C_2H_5OH containing 5 ml triethylamine and 2.16 g (9.3 mmol) silver (I) oxide. The mixture was filtered through celite to remove Ag_2O, evaporated to dryness

and redissolved in 200 ml of $CHCl_3$. This solution was extracted with 2×200 ml of a 10% NaI solution, 2×200 ml of a 10% $Na_2S_2O_3$ solution and finally with 200 ml of saturated NaCl. After drying the organic layer (Na_2SO_4) the solvents were removed by evaporation. The product was purified by flash chromatography (15×2 cm column) using 500 ml of $CHCl_3$ containing 1% triethylamine and then 500 ml each of this solution containing in turn 1% and 3% CH_3OH. The product eluted in the 3% CH_3OH wash and after solvent evaporation 1.27 g (52%) was obtained. The product was pure by tlc (CH_2Cl_2 95, CH_3OH 5, Rf 0.6). ^1H NMR ($CDCl_3$) (8.42 1Hm, H4), 8.18 (1Hm, H6), 7.45−6.81 (13Hm dimethoxytrityl aromatic), 3.78 (6Hs, CH_3OF), 1.7 (3Hs, CH_3) plus standard sugar spectrum. FAB-MS 529 (M + H^+, 100%), 303 (dmt^+, 100%), 111 (base + $2H^+$, 20%).

2-thio-3'-O-acetylthymidine

3'-O-acetylthymidine (5 g, 17.6 mmol) was converted to its 5'-iodo, 5'deoxy derivative with methyltriphenoxyphosphonium iodide as described (13). Product purification was by flash chromatography (20×3 cm column) using 500 ml $CHCl_3$ and subsequently 500 ml $CHCl_3$ containing 2.5% CH_3OH as eluants. The product eluted with the second solvent and after evaporation of solvents 5.75 g (83%) of product was obtained. This was pure by tlc (CH_2Cl_2 95, CH_3OH 5, Rf 0.85). ^1H NMR ($CDCl_3$) 8.51 (1Hs, N3H), 7.62 (1Hs, H6), 2.15 (3Hs acetyl CH_3), 2.05 (3Hs base CH_3) plus standard sugar spectrum. EI-MS 394 (M^+). 4.6 g (1.7 mmol) of this 5'-iodo-5'-deoxy-3'-O-acetylthymidine was converted to the 2,5'-O-cyclo derivative by refluxing for 2 h in 150 ml dry CH_3CN containing 9.2 g (55 mmol) silver acetate (14). The mixture was cooled and passed through a Whatman No 1 filter to remove silver acetate. H_2S was bubbled through the resulting solution for 10 min to precipitate remaining silver. N_2 was then bubbled through the solution to remove excess H_2S. The mixture was filtered firstly through celite and then through 0.5μ teflon filters to remove precipitated silver sulphide. The mixture was evaporated to dryness and the product redissolved in 200 ml of $CHCl_3$ with gentle heating. Last traces of insoluble silver salts were removed by filtration through Whatman No 1 filters. The $CHCl_3$ solution was extracted with 4×200 ml H_2O and the water layers (into which the product partitioned) pooled and evaporated to give 2.58 g (83%) of product. This was pure by tlc (CH_2 95, CH_3OH 5 Rf 0.2). ^1H NMR (D_2O) 7.78 (1Hs, H6), 2.15 (3Hs acetyl CH_3), 1.92 (3Hs, base CH_3) plus standard sugar spectrum. EI-MS 266 (M^+) U.V. λ_{max} 250 nm (pH 7) U.V. literature λ_{max} 249−250 nm (pH7) (19). 2.4g (9mmol) of this 2,5'-O-cyclo-3'-O-acetylthymidine was converted to the 2-thio derivative with H_2S as described (4). On completion of the reaction tlc ($CHCl_3$ 95, CH_3OH 5) showed complete disappearance of starting material (Rf 0.2) and the appearance of several products with higher Rf values. N_2 was bubbled through the mixture for one hour to remove H_2S and solvents removed by evaporation. The resulting oil was dissolved in 100 ml of $CHCl_3$ and extracted with 3×100 ml of H_2O. The organic layer was dried over Na_2SO_4 and evaporated to give a solid. Thin layer chromatography (ethyl acetate) showed the presence of at least 4 compounds with Rf values 0.2 (the major product), 0.5 (the desired product), 0.7 and 0.95. The desired product was purified from these contaminants by flash chromatography (20×2 cm column) using ethyl acetate as eluant. Although the product obtained appeared pure by tlc using ethyl acetate, tlc using $CHCl_3$ 95, CH_3OH 5 showed two slight contaminants with Rf values of 0.44 and 0.48 (product Rf value 0.4). Final purification was achieved by recrystallisation from $CHCl_3$ (25 ml). 0.78 g of product (29%) was obtained which was pure by tlc using both the above solvent systems. ^1H NMR

($CDCl_3$) 9.45 (1Hs N3H), 7.92 (1Hs, H6), 2.14 (3Hs, acetyl CH_3) 2.00 (3Hs, base CH_3) plus standard sugar spectrum. FAB-MS 301 (M + H^+, 50%) 159 (3 -O-acetyldeoxyribose$^+$, 20%), 143 (base + 2H^+, 100%). UV λ_{max} 275 nm (H_2O, pH 7). UV literature λ_{max} 276 nm (H_2O, pH 7) (4).

5'-O-dimethoxytrityl-2-thiothymidine

This was prepared from 2-thio-3'-O-acetyl-2-thiothymidine by reaction with dmt-Cl (12) followed by deacetylation of the crude initial product with NH_3 (ethanol, pyridine, 35% aqueous NH_3: 1,1,1 for 20 h). The product was purified by flash chromatography (20×2 cm column) using $CHCl_3$ containing 1% CH_3OH and 0.5% pyridine as eluant. After solvent evaporation 0.9g (69%) of product, pure by tlc in the above system, was obtained. ^1H NMR ($CDCl_3$) 9.82 (1Hs N3H), 7.88 (1Hs, H6), 7.42−6.85 (13Hm, dimethoxytrityl aromatic), 3.85 (6Hs, CH_3O), 1.47 (3Hs, CH_3) plus standard sugar spectrum. FABS-MS 561 (M + H^+, 20%), 303 (dmt$^+$, 100%)

Protected deoxynucleoside 3'-O-(N,N,-diisopropylamino) 2-cyanoethylphosphinyl) derivatives

These were prepared from the appropriate protected deoxynucleosides on a 1 mmol scale as described (8). Yields of 65−85% were typical and all products were pure by tlc (ethylacetate 45, $CHCl_3$ 45, triethylamine 10 Rf values 0.7−0.9, both diastereomers visible).

4-thiothymidine, 4(S-sulphenylmethyl)thiothymidine, 5-methyl-2-pyrimidinone-1-β-D(2'-deoxyriboside), 2-thiothymidine

Pure samples of these four deoxynucleosides were prepared from their 5'-dmt derivatives by detritylation using 70% acetic acid. All four were pure by hplc (see below). 4-thiothymidine ^1H NMR (D_2O) 7.9 (1Hs, H6), 6.24 (1Ht, H1'), 4.39 (1Hm, H3') 3.95−3.7 (3Hm, H4', H5', H5"), 2.25 (2Hm, H2', H2") 2.08 (1Hs, CH_3). FAB-MS 259 (M + H^+, 20%) 143 (base + 2H^+, 100%), 117 (deoxyribose$^+$ 28%) UV λ_{max} pH 7 335 nm. UV literature λ_{max} pH 7 335 nm (3). 4-(S-sulphenylmethyl)thiothymidine ^1H NMR (D_2O) 8.21 (1Hs, H6), 6.19 (1Ht, H1'), 4.4 (1Hm, H3'), 4.04−3.66 (3Hm, H4', H5', H5"), 2.51 (3Hs, $SSCH_3$), 2.49−2.2 (2Hm, H2', H2"), 2.13 (3Hs, CH_3). FAB-MS 305 (M + H^+, 100%), 189 (base + 2H^+, 50%) 117 (deoxyribose$^+$, 18%). 5-methyl-2-pyrimidinone-1-β-D-2'-deoxyriboside ^1H NMR (D_2O) 8.55 (1Hm, H4), 8.3 (1Hm, H6) (these resonances collapsed to doublets J= 3Hz on irradiation of the resonance at 2.15). 6.21 (1Ht, H1') 4.42 (1Hm, H3'), 4.25−3.75 (3Hm, H4', H5', H5") 2.65−2.33 (2Hm, H2', H2"), 2.15 (3Hs, CH_3) UV λ_{max} pH 7 314 nm, UV literature λ_{max} pH 7 315 nm (15). Fluorescence λ_{max} pH 7 excitation 325 nm, emmission 385 nm. 2-thiothymidine ^1H NMR (D_2O) (7.91 1Hs, H6), 6.95 (1Ht, H1'), 4.45 (1Hm, H3'), 4.15−3.75 (3Hm, H4', H5', H5"), 2.7−2.2 (2Hm, H2', H2"), 1.95 (3Hs, CH_3). FAB-MS 259 (M + H^+, 35%), 142 (base + 2H^+, 100%) 117 (deoxyribose$^+$, 10%). UV λ_{max} pH6 276 nm, UV literature λ_{max} pH 5.5 276 nm (4).

Stability of $^{4SSCH_3}T$, ^{4H}T and ^{2S}T to the conditions of oligonucleotide synthesis

About 20 mg of the 5'-dmt derivatives of the above bases were dissolved in: a) 6.5% dimethylaminopyridine in tetrahydrofuran, b) 17.6% N-methylimidazole in tetrahydrofuran, c) 0.1M I_2 in tetrahydrofuran, pyridine, water: (93, 5, 2 v/v). Any decomposition of these bases was monitored by tlc ($CHCl_3$ 95, CH_3OH 5) over a period of 25 hours. About 20 mg of $^{4SSCH_3}T$, ^{4H}T or the 3'-O-acetylderivative of ^{2S}T were dissolved in either 3% dichloroacetic acid or 3% trichloroacetic acid in dichloromethane and decomposition over 25 h was monitored by tlc ($CHCl_3$ 90, CH_3OH 10).

Oligonucleotide Synthesis

All oligonucleotides were prepared using an Applied Biosystems 381A DNA synthesiser and standard 2-cyanoethyl phosphoramidite chemistry on a one μmol scale. Modified bases were dissolved in dry CH_3CN at the usual concentration of 0.1M and filtered through 0.5μ teflon filters prior to introduction at the fifth position (base X) on the machine. The standard one μmol scale synthesis programme was used with no changes being made because of the modified base. Syntheses were performed 'trityl on' and at the end of the chain assembly deblocking was performed using 35% aqueous NH_3 at 50°C for 3 h. When the $^{4SSCH_3}T$ base was present 0.1M dithiothreitol was included in the NH_3.

Oligonucleotide purification

Dmt containing oligonucleotides were purified by hplc using a linear gradient composed of 25 to 85% hplc buffer B over 20 min. The trityl containing oligonucleotides eluted at around 12 min. Fractions that contained product were pooled, evaporated to dryness and redissolved in 70% acetic acid (2 ml) for 90 min. The acetic acid was removed by evaporation followed by coevaporation from H_2O (3×5 ml). The resulting product was dissolved in 5 ml of H_2O and extracted with 3×10 ml of ether. The volume of the aqueous layer was reduced to 0.2−0.4 ml by evaporation. Final purification was by hplc using a linear gradient composed of 5 to 35% hplc buffer B over 20 min. The fractions that contained the desired product were pooled evaporated to dryness and the pure oligomer dissolved in 0.3 ml H_2O and stored frozen at −20°C.

Deoxynucleoside composition analysis

1−2 $O.D._{254}$ units of oligonucleotide was dissolved in 0.25 ml of 25 mM Hepes-NaOH pH 7.5 containing 5 mM $MgCl_2$ and 50 mM NaCl. Snake venom phosphodiesterase and alkaline phosphatase (20 μg of each) were added and the mixture incubated at 37°C for 1h. The product deoxynucleosides were analysed by hplc using 0% hplc buffer B for 5 min and then a linear gradient to 75% hplc buffer B in 20 min. The following elution times were observed: dC (3.6 min), $d^{5CH_3}C$ (4.6 min), dG (5.1 min), ^{4H}T (6.4 min), T (6.7 min), dA (9 min), ^{2S}T (13.7 min), ^{4S}T (15.6 min). For normal oligonucleotides and those containing ^{2S}T a single run monitored at 254 nm was performed. For oligomers containing ^{4S}T the first 12 min of the run was monitored at 254 nm (for dC, dG, T and dA) detection and the remainder at 335 nm (for ^{4S}T detection). With oligonucleotides containing ^{4H}T two analyses were performed. The first was monitored at 254 nm (for unmodified base detection) and the second at 315 nm (for ^{4H}T detection). The areas under each peak were determined by integration and the amounts of each base present determined using the following extinction coefficients (M^{-1} cm^{-1}): dC (6×10^3), $d^{5CH_3}C$ (5.1×10^3), dG (13.5×10^3) T (7×10^3), dA (14.3×10^3) all at 254 nm (16), ^{4H}T (5.3×10^3) at 315 nm (15) ^{4S}T (22.3×10^3) at 335 nm (3), ^{2S}T (6.3×10^3) at 254 nm (this was determined by multiplying the extinction coefficient at 267 nm (λ_{max}) of 16.6×10^3 (4) by the ratio of absorbance measured at 254 and 276 nm).

Stability of ^{4S}T, ^{4H}T, and ^{2S}T containing oligonucleotides to NH_3

About 10 OD_{254} units of oligonucleotides containing the above modified bases were dissolved in 1 ml aqueous (35%) NH_3. Samples were either left at room temperature or heated at 50°C. Aliquots (0.1ml) were withdrawn at times up to 25 h and the NH_3 removed by evaporation. The samples were redissolved in 0.1 ml H_2O and analysed by hplc using the solvent system utilised for the final purification of oligonucleotides. With the 25 h sample at 50°C, decomposition products were purified using this hplc system and identified by deoxynucleoside composition analysis as detailed above.

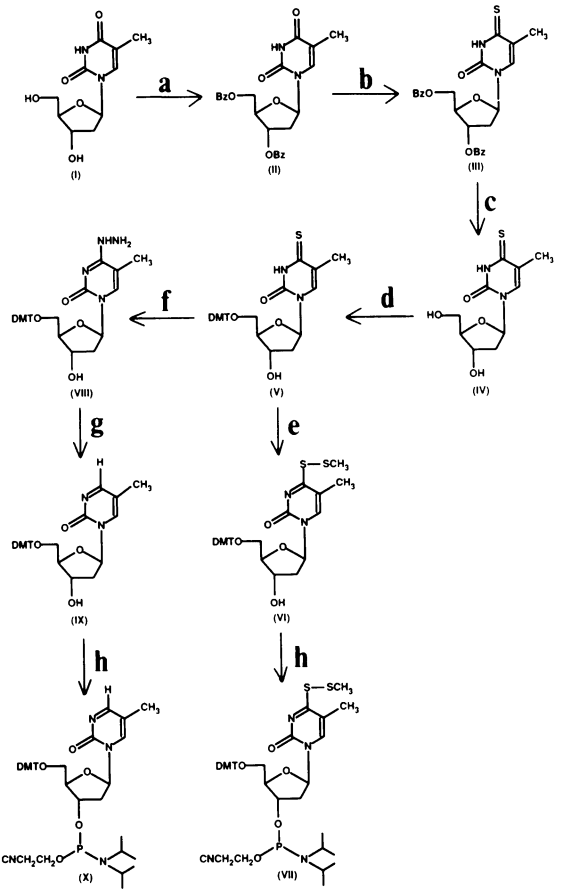

Figure 2 The synthesis of ^{4S}T (VII) and ^{4H}T (X) derivatives suitable for synthesis of oligonucleotides by the phosphoramidite approach from dT(I). Reagents: (a) benzoylchloride, (b) Lawesson's reagent, (c) sodium methoxide, (d) dmt-Cl, (e) methylmethanethiosulphonate, (f) hydrazine, (g) silver (I) oxide, (h) 2-cyanoethyl N,N-diisopropylchlorophosphoramidite.

RESULTS AND DISCUSSION

Our route to ^{4S}T and ^{4H}T derivatives suitable for incorporation into oligonucleotides is shown in figure 2. Both ^{4S}T (2, 3, 15) and ^{4H}T (2 ,15) have been previously synthesised but the methods given have been primarily aimed at producing the free deoxynucleosides. ^{4H}T has been introduced into oligonucleotides using the phosphtriester (5, 6) and more recently the phosphoramidite (7) methods. We have based our approaches on these methods but have modified them slightly to make more suitable for the preparation of (VII) and (X), ^{4S}T and ^{4H}T derivatives suitable of oligonucleotide synthesis by the most commonly used and efficient β-cyanoethylphosphoramidite method (8). The preparation of ^{4H}T from ^{4S}T (2) means that much of the synthesis of (VII) and (X) is via a common route resulting in a highly efficient overall pathway. The synthesis begins with the preparation of dibenzoyl

4-thiothymidine (III) from dibenzoylthymidine (II). This has previously been achieved using P_4S_{10} in either wet pyridine (3) or dioxan (2). We have found Lawesson's reagent (p-methoxyphenylthionophosphinesulphide dimer) far easier to use than P_4S_{10} and the product produced with it is pure enough after simple aqueous extraction for the next step in the pathway. We have found it highly advantageous to remove the dibenzoyl groups at this stage of the pathway to give 4-thiothymidine (IV). This can be simply achieved with sodium methoxide. The 4-thiothymidine (IV) obtained was very hygroscopic but had satisfactory spectroscopic properties. In our hands free deoxynucleosides have proven difficult to isolate as they cannot be purified by silica gel chromatography and are often poorly crystalline. It is more convenient to convert the crude 4-thiothymidine to its 5'-dmt (V) derivative with dmt-Cl and purify this compound by silica gel chromatography. At this stage the paths to ^{4S}T and ^{4H}T derivatives diverge. The sulphur atom in ^{4S}T is nucleophilic and reactive towards reagents such as CH_3I (2, 3). It is therefore expected to be reactive towards the phosphitylating agents used to prepare deoxynucleoside 3-phosphoramidites and tetrazole activated deoxynucleoside phosphoramidites during oligonucleotide synthesis. We have masked the nucleophilicity of this sulphur atom by converting it to an S-sulphenylmethyl (-SCH_3) derivative. The SCH_3 group can easily be added to dmt^{4S}T using the available reagent methylmethanethiosulphonate (17). Conversion of the thioketo group to the S-SCH_3 derivative is not ideal for the purposes of oligonucleotide synthesis as the disulphide bond has a certain lability to some of the reagents used (see below). Nevertheless it has the advantage of ease of introduction and removal (with dithiothreitol) and has yielded pure samples of the desired oligonucleotides containing ^{4S}T.

The conversion of ^{4S}T derivatives to ^{4H}T has been accomplished directly with Raney Ni (6, 15). This method is somewhat unreliable and variable yields have been reported probably due to competing reduction of the 5,6 pyrimidine double bond or irreversible absorption of the pyrimidines onto the metal catalyst. A better alternative involves the production of an intermediate hydrazino derivative followed by oxidation to ^{4H}T with Ag_2O (2). Similar reactions have been reported in the purine series for the production of 2-aminopurine (18). The conversion of dmt^{4S}T (V) to the 4-hydrazino derivative (VIII) with hydrazine hydrate proceeds smoothly and in high yields. The product produced is pure enough for direct use in the subsequent reaction. This hydrazinolysis on the dmt derivative is superior to that for the dibenzoyl derivatives as competing debenzoylation is not a problem. Furthermore we have found it unnecessary to convert the dmt^{4S}T to its 4-thiomethyl derivative with CH_3I (2). Treatment of (VIII) with Ag_2O furnishes the ^{4H}T derivative (IX) in reasonable yield.

The synthetic route to ^{2S}T derivatives suitable for oligonucleotide synthesis is shown in figure 3. This method was pioneered by Todd and coworkers (19) for the preparation of 2-thiouridine and later used by Faeber and Scheit to synthesise 2-thiothymidine (4). The preparation begins with the formation of 5'-iodo-5'-deoxy-3'-O-acetyl thymidine (XII) from 3'-O-acetyl thymidine (XI), a reaction most easily accomplished with methyltriphenoxyphosphoniumiodide (13). The 5'-iodo derivative (XII) is next converted to the 2,5'-O-cyclo derivative (XIII) with silver acetate (14, 20). This deoxynucleoside is soluble in H_2O and can be simply purified from contaminants (which are all organic solvent soluble) by aqueous extraction. The final step involves the conversion of this cyclo derivative to a 2-thiothymidine (XIV) derivative with H_2S. Only 30% yield of product was obtained and this is the only reaction in this publication that proceeds with an unsatisfactory yield. This reaction has long been associated with low yields and production

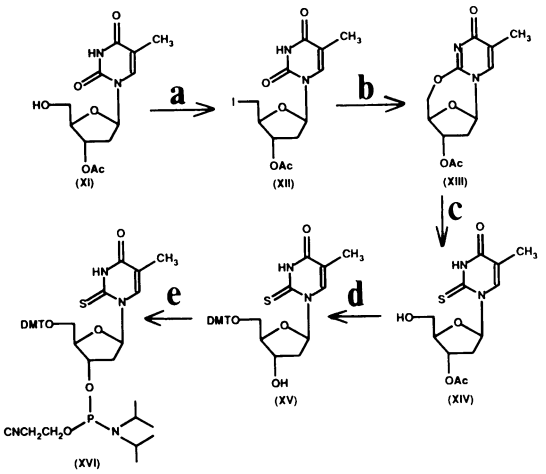

Figure 3 The synthesis of a ^{2S}T derivative (XVI) suitable for oligonucleotide synthesis via the phosphoramidite method from 3′-O-acetyl thymidine (XI). Reagents: (a) methyltriphenoxyphosphonium iodide, (b) silver acetate, (c) H_2S, (d) i dmt-Cl, ii NH_3, (e) 2-cyanoethyl N,N-diisopropylchlorophosphoramidite.

of side products (4, 19). In early work with the uridine series the major side products were identified as polysulphide dimers of 2-thiouridine (19) but this was later corrected to 5′-thiouridine (21). We have shown that the major product of the action of H_2S on (XIII) (the product with the lowest Rf value on tlc using ethylacetate) is in fact 5′-thio-5′-deoxy-3′-O-acetylthymidine (the identity of this product was confirmed by UV spectroscopy and FAB-MS). Although the desired 3′-O-acetyl 2-thiothymidine (XIV) is produced in low yield it can be purified by silica gel chromatography followed by crystallisation. Reaction of (XIV) with dmt-Cl followed by deacetylation with NH_3 gives the 5′-dmt ^{2S}T derivative (XV). It has long been recognised that the sulpur atom in ^{2S}T (or ^{2S}U) derivatives is much less reactive than that in ^{4S}T (^{4S}U) compounds (22) and in view of this low reactivity no further protection of derivative (XV) has been undertaken.

Compounds VI, IX and XV could be reacted with 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (8) to give derivatives suitable for oligonucleotide synthesis using the cyanoethyl phosphoramidite approach. Furthermore VI, IX and XV served as convenient sources for pure samples of ^{4SSCH_3}T, ^{4H}T and ^{2S}T by detritylation with acetic acid. The deoxynucleosides produced gave satisfactory UV spectra, ^{1}H NMR spectra and FAB-MS spectra confirming that the syntheses outlined in figures 2 and 3 had produced the desired bases.

The stability of ^{4SSCH_3}T, ^{4H}T and ^{2S}T towards the reagents used in oligonucleotide synthesis showed that all three bases were stable towards N-methylimidazole and dimethylaminopyridine solutions for at least 25h. Additionally ^{4H}T and ^{2S}T showed the same high stability towards I_2 solutions. Both ^{4H}T and ^{2S}T showed slight decomposition (about 10−15% after 25h) in 3% trichloroacetic acid in dichloromethane although no breakdown was seen after 2h. ^{4SSCH_3}T showed a slight instability towards I_2 solutions (50% decomposition in 25h, 5% breakdown in 2h) but a more appreciable lability in trichloroacetic acid of about 50% decomposition in 3h. The instability of all three bases (especially ^{4SSCH_3}T) towards some of the reagents used in oligonucleotide synthesis

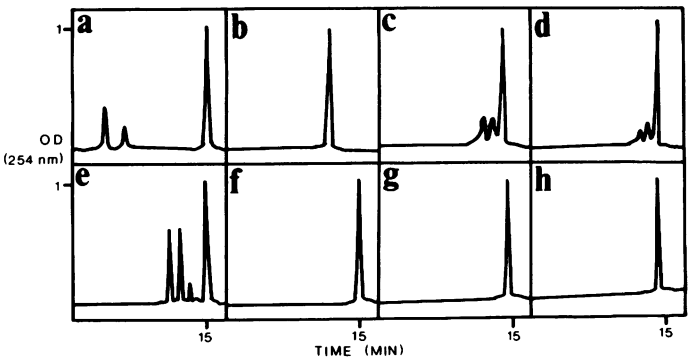

Figure 4 Hplc traces of (a) crude dmt GACGATA^{4H}TCGTC, (b) GACGATATCGTC, (c) GACGATA^{4H}TCGTC, (d) GACGATA^{2S}TCGTC, (e) GACGATA^{4S}TCGTC (b–e are the products obtained from the detritylation of the purified apppropriate dmt containing oligonucleotides. As can be seen (b) is pure, (c) and (d) have slight impurities and (e) is very impure), (f) GACGATA^{4H}TCGTC, (g) GACGATA^{2S}TCGTC, (h) GACGATA^{4S}TCGTC (f–h are the products obtained after a further hplc purification of c–e respectively). It should be noted that the gradient used for (a) (dmt purification) is different to (b)–(h) (completely deblocked purification).

probably results in the extra chromatographic steps (see below) needed to prepare pure products.

The derivatives of ^{4SSCH_3}T, ^{4H}T and ^{2S}T have been used to prepare analogues of the dodecamer GACGATATCGTC. This self complementary dodecamer contains the Eco RV recognition site GATATC (underlined). Six modified dodecamers have been prepared which contain a single T analogue (^{4S}T, ^{4H}T or ^{2S}T) at either of the two T positions within the recognition sequence. As a control the unmodified parent dodecamer has also been prepared. The individual trityl yields in all synthesis approached 100% and in particular no drop was seen on modified base addition. The final trityl group was retained for purification and after a brief NH_3 deblock (3 hours at 50°C) (containing dithiothreitol in the case of ^{4S}T) trityl specific purification was carried out. Figure 4 shows the chromatogram obtained with dmtGACGATA^{4H}TCGTC as an example. Most of the material elutes as full length sequences containing a trityl group. The chromatograms for the parent oligomer and those containing all the other modified T bases were similar. After trityl removal however the chromatograms of the individual oligonucleotides varied. Figure 4 shows that GACGATATCGTC is pure. Deoxynucleoside composition analysis showed the expected equivalent amounts of each of the 4 bases confirming that the correct sequence had been prepared. Oligonucleotides containing ^{4H}T or ^{2S}T contained small amounts of earlier eluting impurities. The traces outlined for ^{4H}T or ^{2S}T present in the second T site are shown but those found with these analogues at the first T site were identical. At present we do not know the nature of these impurities but they may be due to the slight lability of ^{4H}T/^{2S}T during synthesis to acid (see above) or during deblocking to NH_3 (see below). Nevertheless a further chromatographic purification step gives pure ^{4H}T and ^{2S}T containing products as shown in figure 4. Deoxynucleoside composition analysis of these purified products showed dC, dG, T dA and ^{4H}T (^{2S}T) to be present in the required ratios of 3,3,2,3,1 respectively. The yields of oligonucleotides containing ^{4H}T or ^{2S}T were about 30–40% compared to 60% for the unmodified dodecamer. Despite these approaches for

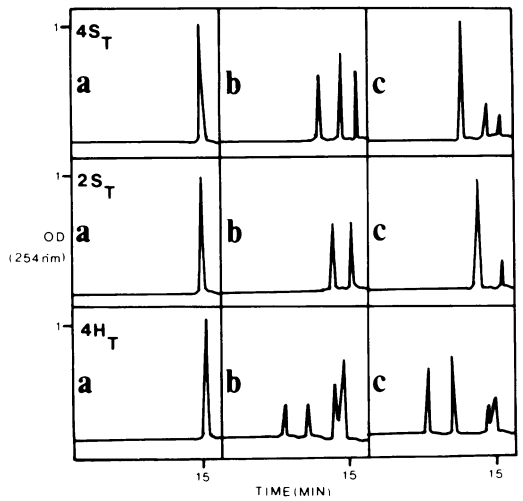

Figure 5 Hplc traces of the effect of concentrated aqueous NH_3 at 50°C on oligonucleotides containing ^{4S}T, ^{2S}T and ^{2H}T. For each oligonucleotide (GACGA^{4S}TATCGTC, GACGA^{2S}TATCGTC, and GACGA^{4H}TATCGTC) three hplc traces are shown: (a) at start of reaction; (b) about half-way through the reaction; (c) on near completion of the reaction. With ^{4S}T containing oligomers the ^{4S}T is converted to $^{5CH_3}dC$ with NH_3. The intermediate (visible in b and eluting just before the starting oligomer) is probably a disulphide linked dimer as this product is converted back to starting material on dithiothreitol addition. ^{2S}T containing oligomers are cleanly converted to polymers containing deoxyisocytidine with NH_3. With ^{4H}T oligomers an initial intermediate (visible in (b) and eluting just before the starting material) probably contains a 5,6 saturated pyrimidine due to NH_3 addition across the double bond. This cleaves to give the products shown in (c). Further details are given in the text and figure 6.

^{4H}T and ^{2S}T not being as clean as those for unmodified oligonucleotides they should be suited for the introduction of small numbers (1−3) of the modified bases into oligomers of medium length (up to 30 bases). Figure 4 shows that GACGATA^{4S}TCGTC elutes as three peaks of about equal amounts. The first of these is GACGATA^{5CH_3}CCGTC, the second GACGATATCGTC and the third the desired product GACGATA^{4S}TCGTC. Thus in the first peak the 4-thio function has been converted to a 4-amino group ($^{4S}T \rightarrow {}^{5CH_3}dC$) and in the second to a 4-keto group ($^{4S}T \rightarrow T$). Proof of this comes from the coelution of the first two peaks with standard oligonucleotides containing $^{5CH_3}dC$ or T at the appropriate position. Furthermore deoxynucleoside analysis shows both peaks 1 and 2 contain dC, dG, T and dA in ratios of 3,3,2,3 and 1 contains additionally one equivalent of $^{5CH_3}dC$ whereas 2 contains an extra equivalent of T. The desired product peak 3 can be purified by an extra chromatography step as shown in figure 4. After purification deoxynucleoside analysis shows the required ratios of bases dC(3), dG(3), T(2), dA(3), $^{4S}T(1)$. Yields of 10−15% were obtained about one fifth of that seen for unmodified oligonucleotides. Clearly this method will only be suitable for incorporation of a single ^{4S}T into short (10−15 bases) oligonucleotides. No differences in yields and purity of ^{4S}T containing oligonucleotides were observed if the sulphenylmethyl group was removed prior to or post NH_3 deblocking compared to the concomitant deblocking used here. Although ^{4S}T in oligonucleotides is sensitive to NH_3 leading to $^{5CH_3}dC$ the conditions we have used (3h at 50°C) for NH_3 deblocking should lead to far less $^{5CH_3}dC$ production than observed

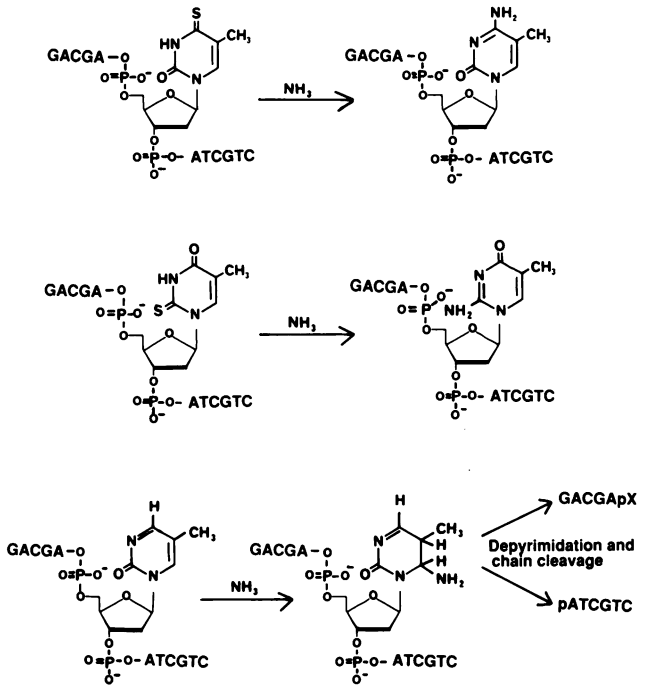

Figure 6 The mechanisms of NH_3 reaction with oligonucleotides containing ^{4S}T, ^{2S}T and ^{4H}T. With ^{4S}T and ^{2S}T a simple nucleophilic attack at the carbon bearing the sulphur occurs and results in replacement of the sulphur with an amino function. With the ^{4S}T disulphide dimers can also form. With ^{4H}T containing oligonucleotides the reaction is more complicated and probably involved initial attack of NH_3 at C6 leading to the saturated pyrimidine shown. These saturated pyrimidines undergo further reactions leading to depyrimidination and the formation of sugar derivatives capable of leading to chain cleavage via β-elimination mechanisms. In our case one product has a free 5′-phosphate but the product having a 3′-phosphate retains some part (as yet undetermined) of the sugar (labelled x above). Evidence for this is that NH_3 action on GACGA^{4H}TATCGTC gives a product (derived from the 5′-end) that contains one equivalent of dA and one of a dA derivative and not 2 equivalents of dA.

(see below). We feel that the main reason for ^{5CH_3}dC and T production in product oligonucleotides is due to the instability of the S-SCH_3 bond to H^+ and I_2 during synthesis. Partial cleavage of this bond would liberate the reactive ^{4S}T base. This could react with incoming tetrazole activated deoxynucleoside phosphoramidites to give initially ^{4S}T-S-phosphite adducts. Decomposition of these with the capping agent could give rise to T. A similar reaction is observed with the 6-keto group of dG (23, 24). Alternatively these may be oxidized to ^{4S}T-S-phosphate adducts which react with NH_3 during the deblocking yielding the observed ^{5CH_3}C. We are actively investigating alternative protecting groups for ^{4S}T.

We have evaluated the stability of oligonucleotides containing ^{4S}T, ^{4H}T and ^{2S}T to NH_3. Figure 5 shows that GACGA^{4S}TATCGTC is converted to GACGA^{5CH_3}CATCGTC by NH_3 at 50°C. Product identification was by coelution with a standard synthetic dodecamer containing ^{5CH_3}C and the correct deoxynucleoside ratios obtained on enzymatic analysis (dC(3), dG(3), T(2), dA(3), d^{5CH_3}C(1), ^{4S}T(0)). The intermediate formed during the

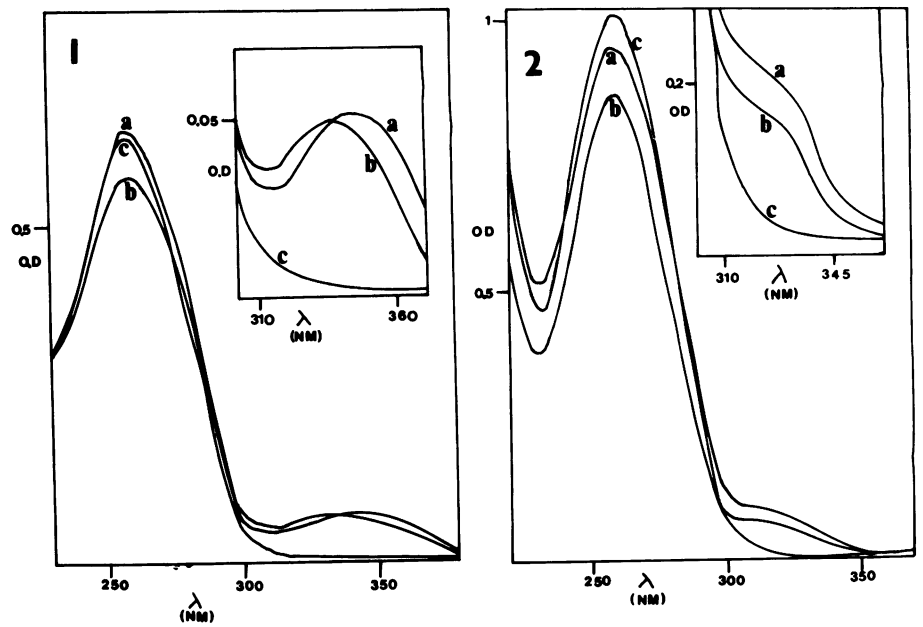

Figure 7 The UV spectra of oligonucleotides containing ^{4S}T and ^{4H}T. The ^{4S}T spectra are shown in diagram 1 and are: (a) GACGA^{4S}TATCGTC, (b) GACGATA^{4S}TCGTC and (c) GACGATATCGTC (control). The ^{4H}T spectra are shown in diagram 2 and are: (a) GACGA^{4H}TATCGTC, (b) GACGATA^{4H}TCGTC and (c) GACGATATCGTC (control).

reaction is probably an intramolecular disulphide caused by -S-S- bridge formation between two ^{4S}T containing oligomers. This can be reduced to starting material with dithiothreitol as expected or be further converted to ^{5CH_3}C with NH_3. The formation of ^{5CH_3}C from ^{4S}T with NH_3 is a well documented reaction (3). Figure 5 shows that GACGA^{2S}TATCGTC is converted to a single product with NH_3. We suspect this contains 2'-deoxy-5-methylisocytidine (2-amino-5-methyl-4-pyrimidinone-1-β-D-2'-deoxyriboside) in place of ^{2S}T and arises from simple nucleophilic displacement of the sulphur atom by NH_3. Nucleophilic displacements at C2 of pyrimidines that have an appropriate C2 substituent (for example ammoniolysis of 2-methoxyuridine derivatives) have been previously observed (20). Deoxynucleoside composition analysis of the product showed dC(3), dG(3), T(2), dA(3) as expected but no trace of ^{2S}T indicating reaction at this site. A fifth peak (between dC and dG) which could be 2'-deoxy5-methylisocytidine was observed but as we have no standard we cannot confirm this. The reaction of GACGA^{4H}TATCGTC with NH_3 is more complicated as shown in figure 5. An intermediate is initially formed which breaks down to two products. We suspect that the intermediate arises from NH_3 addition across the 5,6 double bond giving a saturated pyrimidine. The base lability of ^{4H}T derivatives has been noted but very few details are available (6, 15). It is known that strong nucleophiles such as hydrazine or hydroxylamine can attack pyrimidine bases at C6 leading to derivatives with a saturated 5,6 double bond (25). This reaction does not usually occur with NH_3. However presumably with ^{4H}T derivatives the replacement of the 4-keto oxygen with hydrogen activates the double bond to nucleophilic attack allowing

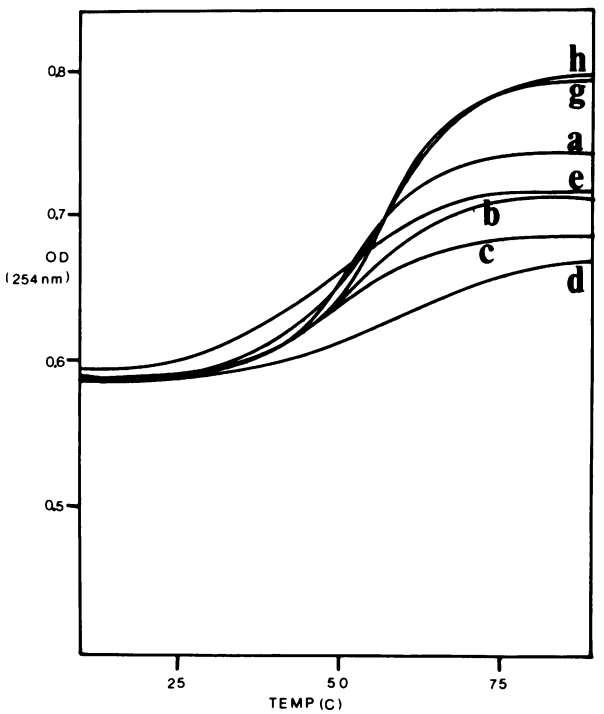

Figure 8 The melting temperature profiles of (a) GACGATATCGTC (53°C), (b) GACGA^{4S}TATCGTC (52°C), (c) GACGATA^{4S}TCGTC (52°C), (d) GACGA^{4H}TATCGTC ((50°C), (e) GACGATA^{4H}TCGTC (50°C), (f) GACGA^{2S}TATCGTC (56°C) and (g) GACGATA^{2S}TCGTC (56°C). The figures in brackets represent the melting temperatures obtained from differential plots of the above data.

reaction with NH_3. Saturated pyrimidines are known to be susceptible to depyrimidination reactions leading to loss of the heterocycle and the production of derivatives prone to chain cleavage (25). This depyrimidination and cleavage gives rise to the two end products. Analysis of these products shows that one contains dC(2), dG(1), T(2) and dA(1) whereas the other has dC(1), dG(2), T(0), dA(1) and a dA derivative (not cleavable by nucleases). This accords with the mechanism given in figure 6 where the reactions of NH_3 with oligomers containing ^{4H}T, ^{4S}T and ^{2S}T is given. The half-lives of oligonucleotides containing ^{4S}T, ^{4H}T and ^{2S}T to NH_3 at 50°C were respectively about 25h, 35h and 40h. This suggests minimal decomposition should occur during the 3h at 50°C used for NH_3 deblocking of the bases after synthesis. With ^{4S}T about 8% conversion to ^{5CH_3}C was seen in this time and much less was observed with the other 2 bases. No reaction at room temperature was seen with oligonucleotides containing ^{4H}T or ^{2S}T after 24 h. With ^{4S}T containing oligomers about 5% reaction was seen under these conditions. This would suggest that a slight improvement in the synthesis could be attained using very NH_3 labile base protecting groups that are removed in 3h at room temperature with NH_3 (26).

The UV maximum of the ^{4S}T base and ^{4H}T base are 335 nm and 315 nm respectively (3, 15). This is well separated from the 260 nm maximum observed in oligodeoxynucleotides

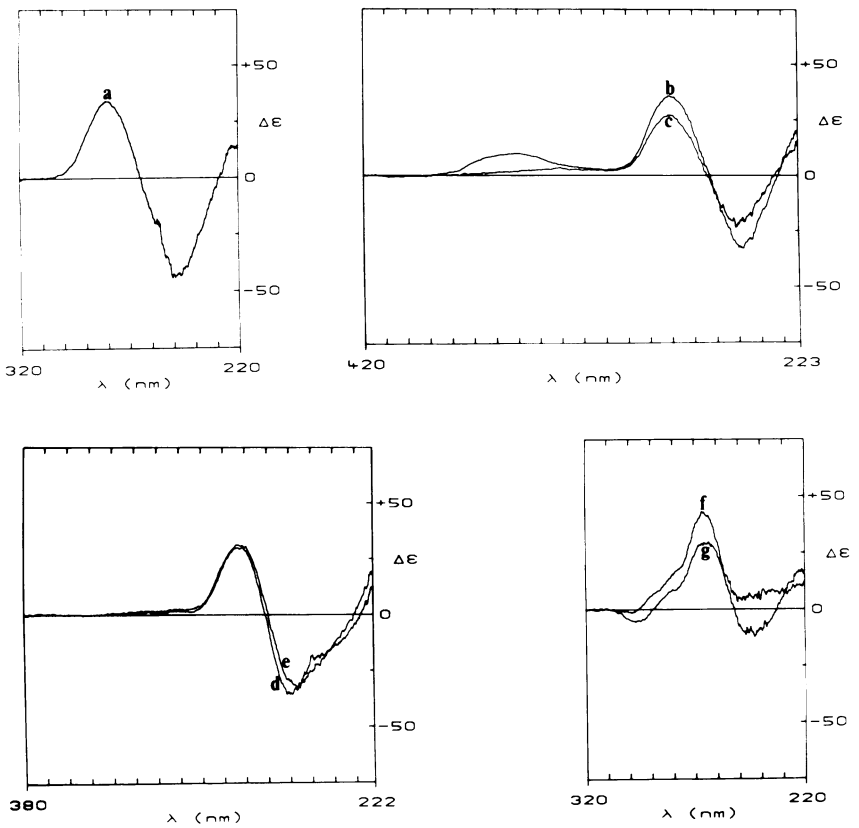

Figure 9 The CD spectra of (a) GACGATATCGTC, (b) GACGA^{4S}TATCGTC, (c) GACGATA^{4S}TCGTC, (d) GACGA^{4H}TATCGTC, (e) GACGATA^{4H}TCGTC, (f) GACGA^{2S}TATCGTC and (g) GACGATA^{2S}TCGTC.

and DNA. As shown in figure 7 the ^{4S}T chromophore can clearly be seen in oligonucleotides that contain this base. Interestingly GACGA^{4S}TATCGTC and GACGATA^{4S}TCGTC show slightly different λ_{max} values of 345 nm and 335 nm respectively. Figure 7 shows that the ^{4H}T chromophore can also be seen in oligonucleotides as a shoulder at 315 nm. ^{4H}T is also fluorescent and free ^{4H}T has excitation and emission maxima of 325 and 385 nm respectively (in H_2O, pH 7). Under the same conditions GACGA^{4H}TATCGTC showed maxima at 323 and 390 nm whereas GACGATA^{4H}TCGTC had values of 326 and 392 nm. Fluorescence yields were also reduced to 45% and 82% for the two oligomers respectively compared to 100% seen with the free nucleoside.

As it is planned to use oligonucleotides containing ^{4S}T, ^{4H}T and ^{2S}T to study protein nucleic interactions it is important to see what effects these bases have, on structure, when incorporated into GACGATATCGTC. To assess the effects these bases have on duplex stability we have determined the melting temperatures of oligonucleotides containing ^{4S}T, ^{4H}T and ^{2S}T and compared them to the result obtained with the parent unmodified

oligomer. These results are shown in figure 8. All the oligonucleotides exhibit a double to single strand transition with melting points at around 50°C. This suggests that they should all be double stranded at 25°C, a temperature suited to enzymatic assays. It should be noted that the exact shape of each melting curve varies with the particular oligonucleotides but as yet we have not further analysed (by for example studying melting curves as a function of oligonucleotide concentration) this data. Perhaps the most surprising aspect is that ^{4H}T, which cannot form either of its normal Watson-Crick hydrogen bonds with a complementary dA, does not cause appreciable helix destablisation. Finally we have measured the circular dichroism spectra of the dodecamers containing the modified bases and these are presented in figure 9. As expected the parent oligomer GACGATATCGTC shows a typical B-DNA spectrum with a negative peak at 250 nm and a positive one at 280 nm (27). This indicates that this dodecamer forms a double helix with a B-DNA structure. The oligomers that contain ^{4S}T also show typical B-DNA spectra with negative and positive peaks at 250 and 280 nm respectively. Interestingly the 340 nm chromophore due to ^{4S}T also contributes to the CD spectra and this contribution is well resolved form those due to the other bases. With GACGA^{4S}TATCGTC a fairly large positive peak is seen at 355 nm whereas with GACGATA^{4S}TCGTC a much less intense peak at 330 nm is observed. At present we do not know the reason for these wavelength maxima and intensity differences. Oligonucleotides that contain ^{4H}T show B-DNA like spectra with a negative peak at lower wavelengths and a positive towards higher. However the positions of the peaks, particularly that at the lower wavelength, are displaced slightly when compared to the parent oligonucleotide. These spectral changes may be due to slight distortions away from B-DNA structure due to ^{4H}T being unable to base pair with A residues on complementary strands. A very weak positive peak can be seen at around 310 nm (especially for GACGATA^{4H}TCGTC) which could be due to the ^{4H}T chromophore which absorbs in this region. However any contributions to the CD spectrum due to ^{4H}T would probably be better detected by fluorescence CD spectroscopy using the intrinsic fluorescence of this base. Figure 9 also shows that the oligonucleotides that contain ^{2S}T do not have a B-DNA like CD spectrum. These spectra with a positive peak at 365 nm are more reminiscent of A-DNA (27). However we are unable to say at present whether these oligonucleotides are actually of the A-form or a distorted B-type helix. We are presently using ^{1}H NMR spectroscopy to further elucidate these structures.

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