Volume 16 Number 4 1988 Nucleic Acids Research

Synthesis of a thymidine phosphoramidite labelled with ^{13}C at C6: relaxation studies of the loop region in a ^{13}C labelled DNA hairpin

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Received January 7, 1988; Accepted January 21, 1988

ABSTRACT

A thymidine phosphoramidite labelled at C6 with ^{13}C has been synthesized, and incorporated into a synthetic oligonucleotide, d(CGCGT*T*GT*T*CGCG), which adopts a hairpin conformation. NMR relaxation measurements indicate that internal motion may be present in the loop region of the oligonucleotide. The relaxation behavior of a the C6 carbon in a model compound, N,N-1,3 dimethylthymine is examined in detail as a function of magnetic field strength to determine relative contributions of various mechanisms to the relaxation. The relaxation behaviour of the labelled carbons in the oligonucleotide is discussed in relation to these measurements.

INTRODUCTION

The study of synthetic oligodeoxynucleotides by NMR has progressed to the point where spectral assignments and structural information can be routinely obtained. Proton resonances can be assigned using the sequential assignment procedure on a combination of COSY and NOESY spectra[1]. Structural information is available by estimating interproton distances from the rate of NOE buildup. Quantitation of the NOE requires specification of a correlation time for the internuclear vector, a quantity whose determination is complicated by internal motions. Such internal dynamics are difficult to probe with proton NMR. Internal yardsticks can be used when two protons are fixed at a known distance to compare cross relaxation rates, but the general interpretation of proton T_1 and T_2 relaxation rates in terms of internal dynamics is difficult. Protons are relatively densely packed in biomolecules, and their relaxation rates more strongly reflect the number of nearest neighbors than they do the conformational dynamics of the molecule.

Carbon-13 relaxation is potentially a more powerful probe of dynamical processes. A carbon atom and a directly bonded hydrogen atom, separated by approximately 1Å, constitute a tightly coupled two spin system in which the relaxation is usually dominated by the dipolar interaction between them. Thus the measurement of carbon relaxation rates gives precise information about the motional characteristics of a particular C-H bond. The low natural abundance (1%) of carbon-13 is advantageous in that no carbon-carbon interactions need be considered, but is not advantageous from the standpoint of sensitivity. Often there are such limited amounts of samples readily available that natural abundance carbon spectra are difficult to obtain.

In particular, the measurement of several carbon relaxation parameters at several different temperatures and fields is extremely time consuming on samples of millimolar concentration. This difficulty can be overcome by specific isotopic enrichment of selected carbons, which is the approach we have taken.

A number of NMR studies on nucleic acids have employed ^{13}C NMR to explore internal dynamics[2,3,4,5]. Studies employing ^{13}C labelled nucleotides were mainly performed on tRNAs and require cell culture with the labelled bases, resulting in labelling of all similar sites in the molecule[6,7,8]. Recent advances in oligonucleotide synthesis have made possible the synthesis of large quantities of DNA oligomers of defined sequence. This methodology can be exploited to incorporate a synthetically labelled nucleotide at any position in a sequence.

We are presently investigating the solution structure of d(CGCGTTGTTCGCG) under conditions where it exists in a hairpin conformation. The molecule has four Watson-Crick C-G base pairs which constitute the stem region, and a single stranded loop region of five bases which are not base paired. Examination of the phosphorus and proton NMR spectra indicated that a great deal of conformational flexibility might exist in the loop region. To investigate the possibility of internal motions, we wished to have a specific carbon-13 probe in the loop region. We report here the synthesis of a thymidine phosphoramidite suitable for use in conventional solid phase DNA synthesis, and its incorporation into a synthetic oligonucleotide. The relaxation properties of the thymine C6 carbon in the labelled d(CGCGT*T*GT*T*CGCG) and the model compound, 1,3 dimethylthymine are also examined. The complete analysis of a multinuclear NMR study of d(CGCGTTGTTCGCG) will be published separately.[9]

RESULTS AND DISCUSSION

Synthesis

We chose the C6 carbon in the thymine ring as the site for labelling for a number of reasons. Internal motions of the deoxyribose carbons due to pseudorotational motion are always present and would complicate the observation of different mobility among residues. The C6 and the methyl C7 are the only protonated carbons in the ring moiety, and though it is chemically much easier to label the methyl carbon, the analysis of its relaxation properties is further complicated by the presence of the internal rotation of the methyl group. The C6 carbon and the H6 proton constitute a nearly ideal two spin system which reports on the motion of the plane of the base.

The syntheses of uracil, thymine and thymidine labelled with carbon-13 or carbon-14 at a number of positions have been reported by a number of investigators[10,11,12,13,14,15]. Although fragments of the chemistry presented here can be found scattered in the literature, we wish to present the synthesis of a ^{13}C labelled phosphoramidite from start to finish as a coherent unit. The C6 carbon can be labelled using ^{13}C cyanide as the isotopic source using route A as shown in Figure 1. The labelled cyanide was reacted with 2-bromopropionic acid (I) to give 2-(cyano-^{13}C)propionic acid (II), which was reacted with urea in the presence of acetic anhydride to afford

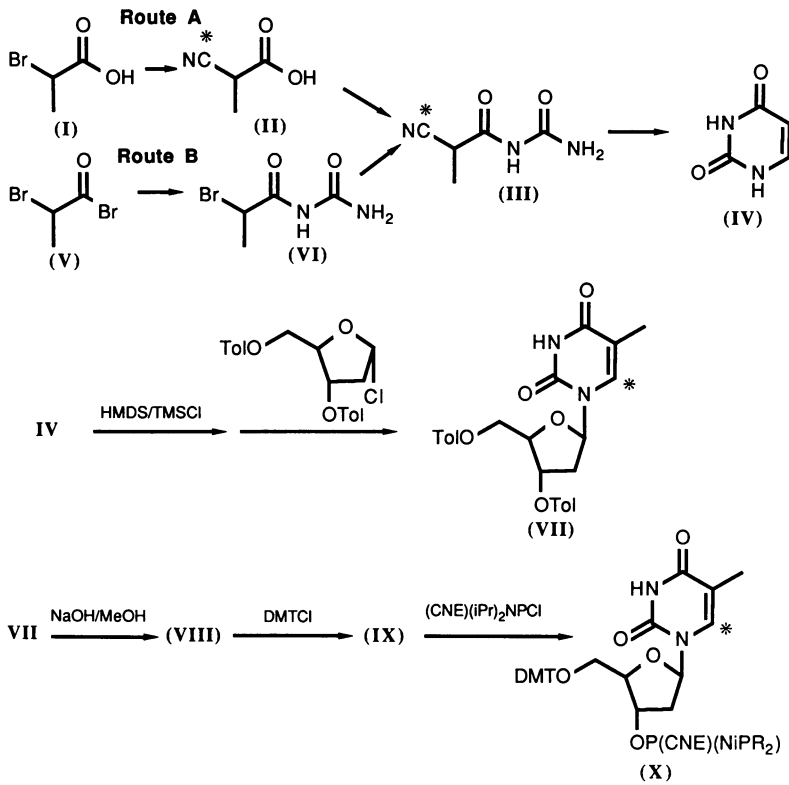

Figure 1. Synthesis of 5'-DMT-6-^{13}C-thymidine β-cyanoethyl phosphoramidite.

2-(cyano-^{13}C)propionyl urea (III). We found two drawbacks to this approach. First, the cyanopropionic acid is water soluble, and continuous extraction was required to recover the product in high yield. Also we found that the major product of the second reaction was acetyl urea, and we were unable to reproduce the yields reported in the literature[13,14]. Both problems are avoided by reversing the order of the first two steps, as shown in route B of Figure 1. The commercially available 2-bromopropionyl bromide (V) was reacted with urea[16], giving a solid 2-bromopropionyl urea (VI), which can be recrystallized from water, thus avoiding recovery of a low molecular weight carboxylic acid from the aqueous phase. The 2-bromo propionyl urea was then reacted with ^{13}C-cyanide to give 2-(cyano-^{13}C)propionyl urea (III). Though our reported yield for this intermediate is lower than that reported in the literature[13,14], Route B proved more efficient in our hands and is not optimized. In addition, incorporation of the isotopically enriched species one step later is advantageous from a labelling standpoint. The hydrogenation of the cyano-ureide (III) in acetic acid proceeded smoothly to afford 6-^{13}C-thymine (IV) in three steps (two steps from ^{13}C cyanide).

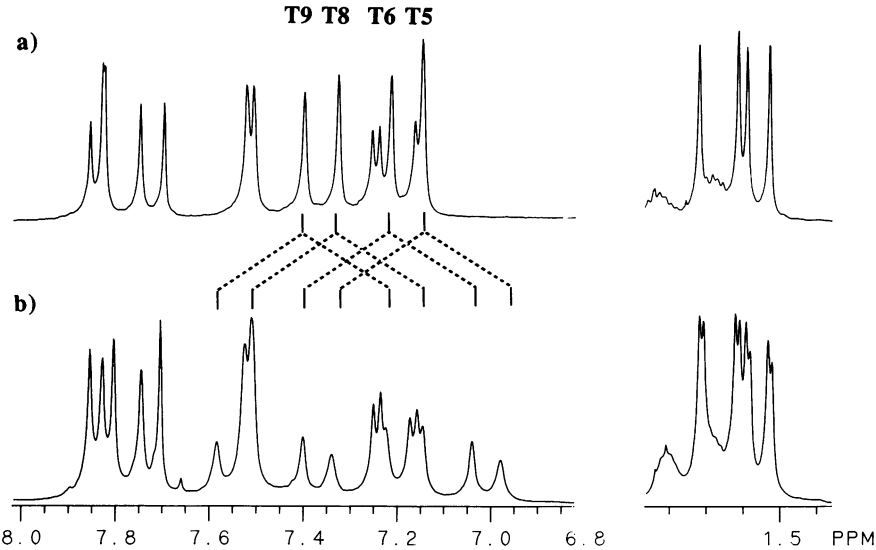

Figure 2. Expansion of the aromatic and methyl regions of the 500 MHz ^{1}H NMR spectrum of d(CGCGTTGTTCGCG) a) unlabelled, and b) labelled at thymidine C6 with ^{13}C. The doublet components of H6 due to coupling to C6 are indicated by dashed lines. There is a slightly different shift of the G13 H8 resonance at 7.8 ppm in the two samples, which is due to slight differences in the concentrations of oligonucleotide and buffer. Sample conditions were 70 mM NaCl, 35 mM phosphate buffer pH 7.0, 5 mM EDTA, at 25°C. Oligonucleotide concentration was a) 3 mM, and b) 7 mM.

The glycosylation of thymine to give thymidine has been reported previously[4,5,17]. The thymine was activated by conversion to its bis-trimethylsilyl derivative, and then reacted with 2-deoxy-3,5,-di-O-*p* -toluyl-D-erythro-pentosyl chloride[18] to afford 3,5-di-O-*p*-toluyl thymidine (VII). In most instances in the literature, the anomeric composition of the glycosylation products is not reported or is predominantly the undesired alpha anomer. A recent report suggested that use of chloroform as a nonpolar solvent would yield exclusively the beta anomer, by slowing down the rate of anomerization of the chloro-sugar[19]. This method was applied successfully to 6-^{13}C-thymine, and in practice we obtained 90% overall yield, with the favorable beta to alpha anomer ratio of 8:1. The desired anomer was easily isolated by repeated fractional crystallization from ethanol. The β-3,5-di-O-*p*-toluyl 6-^{13}C-thymidine was completely free from the α anomer as judged by proton and carbon NMR, and was isolated in 75% yield from thymine.

The 6-^{13}C-3',5'-ditoluyl thymidine was deprotected by treatment with sodium methoxide in methanol, and the crude 6-^{13}C-thymidine (VIII) tritylated directly to afford 6-^{13}C-5'-dimethoxytrityl thymidine (IX), which was purified by column chromatography. The phosphoramidite was prepared by reaction with β-cyanoethyl-N,N diisopropylamino phosphochloridite[20]. The overall yield of labelled thymidine phosphoramidite (X) was 5% based

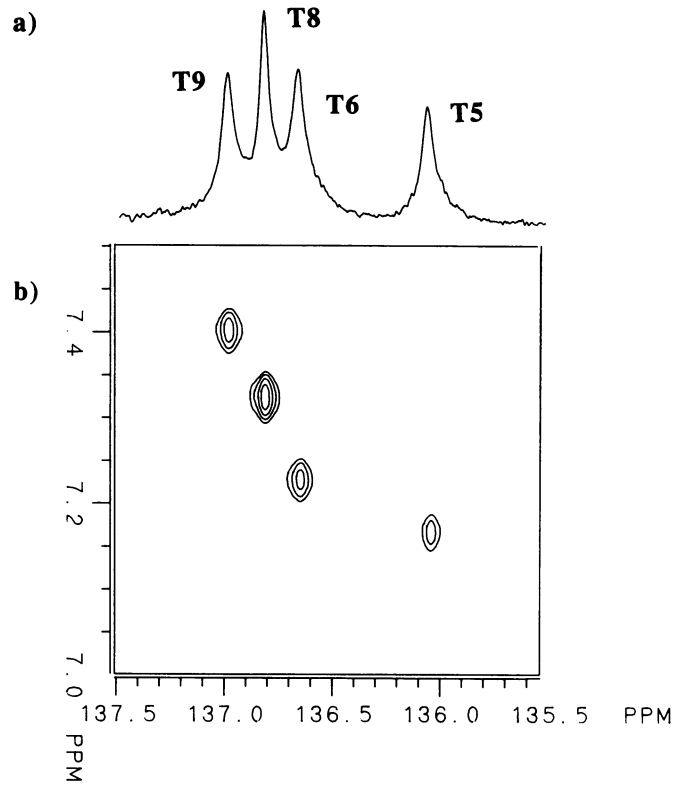

Figure 3. a) ^{13}C NMR spectrum of d(CGCGT*T*GT*T*CGCG) , 1000 aquisitions. b) heteronuclear correlation spectrum, 128 free induction decays were collected with 104 acquisitions each for a total aquisition time of 7.5 hrs. Apodization was a sine bell shifted in f2 by $\pi/4$, and an unshifted sine bell in f1. The spectrum is shown in absolute value mode.

on 2-bromopropionyl bromide, or 10% based on ^{13}C cyanide. This constitutes a seven step synthesis with only one chromatography of a labelled nucleotide in a form ready for conventional solid phase DNA synthesis.

^{13}C NMR Studies of the Hairpin

We have performed an intensive NMR investigation of the oligonucleotide d(CGCGTTGTTCGCG) which adopts a hairpin conformation[9]. Evidence from the ^{1}H and ^{31}P NMR suggested that conformational flexibility and internal motions might be present in the loop region. We used the labelled thymidine phosphoramidite to resynthesize the hairpin with a labelled thymine in all four loop positions. Expansions of the aromatic base proton and the methyl region of the proton NMR spectrum are shown for the unlabelled (2a) and labelled (2b) oligomers in Figure 2. The thymidine H6 protons are split by the C6 carbon-13 into doublets with J=177 Hz,

Table I. Measured relaxation parameters for thymidine C6 in d(CGCGT*T*G-T*T*CGCG), compared to the calculated parameters using the correlation time of 1.5 ns found for the stem region. The discrepancy reflects the effects of internal motions in the loop region.

	T_1 (ms)	NOE (%)	linewidth (Hz)
T5	209 ± 17	24 ± 1.0	8.8 ± .2
T6	234 ± 17	37 ± 1.6	11.2 ± .4
T8	228 ± 16	38 ± 1.5	7.0 ± .2
T9	210 ± 18	29 ± 1.4	9.4 ± .2
calculated	143	18	4.3

as are the methyl protons with J=6 Hz. The assignment of the thymidine H6 protons was obtained from a NOESY spectrum using the sequential assignment procedure[1,9]. The proton-decoupled carbon-13 NMR spectrum of d(CGCGT*T*GT*T*CGCG) is shown in Figure 3a, along with the heteronuclear correlation spectrum used to assign the carbon resonances, Figure 3b. The four C6 resonances of the thymidine residues are clearly resolved, and have been assigned as indicated by correlation to the previously assigned H6 resonances.

Two striking features can be observed in the one dimensional carbon spectrum. First, the peaks appear to have different intensities, and second, the linewidths are different. The measured values of T_1, the NOE, and linewidth for each of the four carbons are shown in Table I. The linewidths were estimated by fitting the spectrum to a sum of lorentzian lines by a non-linear least squares procedure. These results are consistent with different correlation times for some of the four thymidine residues. The spin systems are identical for each residue, therefore any differences in relaxation behavior is due to different motional characteristics of the plane of the base.

^{13}C NMR Studies of 1,3 dimethylthymine

In order to understand the complex molecular dynamics of the oligonucleotide, the details of the thymine C6 relaxation must be understood. The dominant relaxation mechanism is expected to be the dipolar relaxation due to H6, with a small contribution from the methyl protons. At high fields relaxation by chemical shift anisotropy must be considered, and two quadrupolar nitrogens are present in the thymine ring which may relax C6 through scalar coupling or by dipolar coupling. To determine the relative magnitudes of these contributions, a model compound, 1,3 dimethyl thymine was prepared, and T_1, T_2, and the NOE determined at 7.04, 9.35, and 11.74 T, (300,

Table II. Measured and calculated relaxation parameters for dimethylthymine C6 at different magnetic fields.

	11.74 T		9.34 T		7.04 T	
	observed	calc	observed	calc	observed	calc
T_1 (s)	1.54 ± .02	1.54	1.71 ± .01	1.61	1.65 ± .04	1.66
NOE	1.73 ± .03	1.73	1.80 ± .02	1.80	1.87 ± .03	1.86
T_2 (ms)	466 ± 6	440	459 ± 15	446	653 ± 52	450

400, 500 MHz ^{1}H) for the C6 carbon at 25°C. Dimethylthymine was chosen due to its reasonable solubility in chloroform, and the same sealed sample was used for all measurements. The results of the relaxation measurements are summarized in Table II. The value of the NOE is close to the theoretical maximum of 1.987 indicating that most of the relaxation is due to dipolar interaction with nearby protons. The NOE decreases at higher field as the contribution from chemical shift anisotropy becomes more important.

The magnitude of various possible contributions to the T_1 and T_2 relaxation as a function of magnetic field were calculated from standard expressions[21]. The calculations were performed using the following internuclear distances[22]: $r_{(C6\text{-}H6)}$=1.08Å, $r_{(C6\text{-}CH3)}$=2.8Å, $r_{(C6\text{-}N1)}$=1.38Å. The anisotropy of the chemical shift tensor was estimated from the value for toluene as $\Delta\sigma$=180 ppm[23]. The nitrogen quadrupole coupling constant and asymmetry parameter were take from pyrimidine and were 4.442 MHz and .3976, respectively[24]. The ^{13}C-^{14}N coupling constants were scaled from the measured ^{13}C-^{15}N coupling constants for pyrimidine[25]: $J_{C6\text{-}N1}$=1 Hz, $J_{C6\text{-}N3}$=3 Hz. The isotropic rotational correlation time was used as an adjustable parameter to fit the observed relaxation behaviour for the C6 carbon in dimethylthymine, and a value of 25 ps was found to give the best agreement. Increasing the value of $\Delta\sigma$ to 185ppm further improved the agreement between theory and experiment. Since the model compound was not ^{13}C labelled, a high concentration was required. It is likely that some aggregation is occuring which accounts for the long correlation time which we observe, however, the purpose of the model compound is to characterize the relative contributions to relaxation of C6 in an internally consitent fashion, for which it is well suited. The calculated relaxation parameters using τ_c = 25 ps and $\Delta\sigma$ = 185 ppm are also shown in Table II and are in good agreement with the measured values.

The contributions of various relaxation mechanisms at 11.74 T for dimethylthymine are shown in Table III. The two most important mechanisms for T_1 relaxation are the dipolar proton interaction dominated by H6 and chemical shift anisotropy; these two mechanisms contribute 98% of the relaxation. The calculated contribution of the dipolar interaction with the methyl protons represents an upper limit, since the internal rotation of the methyl group was not taken into

Table III. Calculated contributions of relaxation mechanisms for the C6 carbon in dimethylthymine and in the labelled 13mer, d(CGCGT*T*GT*T*CGCG) at 11.74 Tesla.

	dimethylthymine		d(CGCGT*T*GT*T*CGCG)	
	$1/T_1$ (s^{-1})	$1/T_2$ (s^{-1})	$1/T_1$ (s^{-1})	$1/T_2$ (s^{-1})
dipolar H6	.564	.564	5.08	9.80
dipolar CH_3	.010	.010	.09	.09
dipolar N1	.001	.002	.04	.06
CSA	.071	.083	1.77	3.73
Scalar^{II}	~0	1.622	~0	.04

account. The dominant mechanism for T_2 relaxation is scalar relaxation of the second kind due to interaction with the nitrogens.

Internal motion in the loop region

With an understanding of the relaxation behaviour for the C6 carbon in a small molecule, the relaxation parameters for thymine incorporated into the oligonucleotide were calculated and are compared in Table I with the measured values. The correlation time for the overall reorientation of the hairpin is estimated to be 1.5 ns, from the NOE buildup between the four sets of cytosine H6 and H5 protons in the stem region of the hairpin [9], and rotation is assumed to be isotropic. The calculated values clearly do not agree with the observed T_1 and NOE values for the thymidines in the loop region. Examination of the sensitivity of the calculated T_1 and NOE values to the correlation time indicates that the combination of observed T_1 and NOE data cannot be explained with a single correlation time. The observed NOE is larger than predicted, indicating the presence of internal motions which reduce the effective overall correlation time. Further evidence for the existence of internal motions is the observed difference in the linewidths for the various residues. T_2 was measured for all four resonances as ~70 ms, which would give a homogeneous linewidth of 4.5 Hz. The observed linewidths are much larger than expected from the calculated or measured T_2 values. The excess linewidths may be due to conformational inhomogeneity, but the important point is that the observed differences between residues are significant differences, regardless of their origin.

The contributions of various relaxation mechanisms in the labelled oligonucleotide were calculated and are show in Table III. The contributions relative to dimethylthymine have changed since the extreme narrowing condition no longer applies. Dipolar relaxation due to H6 and CSA dominate the T_1 relaxation, but the relative contribution of CSA is increased compared to

dimethylthymine. The contribution to T_2 from scalar relaxation is dramatically reduced due to a large increase in the rate of quadrupolar relaxation of the nitrogens, which lessens the effectiveness of scalar relaxation.

CONCLUSIONS

We have presented a complete and expedient synthesis of a thymidine phosphoramidite labelled at the thymine ring C6 with carbon-13. The synthesis includes improvements in the route to the thymine ring and the latest methodology for anomer selective glycosylation. We have incorporated the synthetically labelled phosphoramidite into a DNA oligonucleotide which forms a hairpin structure, demonstrating the feasibility of routine ^{13}C labelling. The contributions of various mechanisms to the relaxation of the C6 carbon in the model compound dimethylthymine have been investigated in detail, and agree well with the predicted behaviour. As expected, the relaxation is largely due to dipolar interaction with the H6 proton, with some contribution from chemical shift anisotropy. Analysis of the relaxation of the C6 carbons in the labelled oligonucleotide indicates that internal motions are required to explain the observed data. A detailed multinuclear NMR study of the solution structure and dynamics of the hairpin d(CGCGTTGTTCGCG) will be presented shortly[9]. The existence of internal motions in the loop region is an interesting part of this larger story, and this approach to selective labelling should find use in other applications.

MATERIALS AND METHODS

2-bromopropionyl bromide, deoxyribose, trimethylsilyl chloride, hexamethyl disilazane, potassium ^{13}C cyanide, platinum oxide, diiospropylethyl amine and dimethoxy trityl chloride were obtained from Aldrich Chemical Co. N,N diisopropylamino-O-cyanoethyl phosphochloridite was obtained from American Bionuclear. Ultra pure grade urea was obtained from Bethesda Research Laboratories.

Solvents were reagent grade and used without purification, except as follows. Anhydrous solvents were stored under nitrogen over activated 3A molecular sieves. Dimethyl sulfoxide was distilled at reduced pressure from calcium hydride. Chloroform was distilled from phosphorus pentoxide, and stored in the dark. Pyridine was distilled freshly from calcium hydride. Dichloromethane was distilled from calcium hydride. Tetrahydrofuran was distilled from sodium and benzophenone ketyl. Acetonitrile was distilled from phosphorus pentoxide.

Reagents were used without purification except as follows. 2,4,6 collidine and diisopropylethyl amine were freshly distilled from calcium hydride under nitrogen. Acetic anhydride, 2,6 lutidine, and dichloroacetic acid were freshly distilled prior to use. Methoxy di-p-toluyl erythropentoside was prepared according to standard procedure[18] and crystallization was induced from 20% ether in hexanes. The di-*p*-toluyl erythropentofuranosyl chloride was prepared from this freshly, and used within 3 days.

Routine NMR spectra were recorded on a 300 MHz Nicolet NMC-300 wide bore spectrometer, and chemical shifts were referenced to the residual solvent peak: chloroform = 7.26 ppm, dimethylsulfoxide = 2.49 ppm. Carbon-13 spectra were recorded at 75 MHz on a Nicolet NMC-300 spectrometer, at 100 MHz on a Varian XL-400 spectrometer, or at 125 MHz on a GE GN-500 spectrometer, and are referenced to the residual solvent peak: chloroform = 77.0 ppm, dimethylsulfoxide = 39.5 ppm, or to dioxane = 66.5 ppm as an external standard. Proton spectra of the deoxyoligonucleotide were taken at 500 MHz, and are referenced to d4-trimethylsilylpropionic acid as an internal standard. T_1 measurements were performed by the

inversion recovery method and T_2 measurements were made by the spin echo method.
(2-bromopropionyl) urea (VI): A three neck flask fitted with a mechanical paddle stirrer was charged with 25.0 g (.42 mol) urea, and 22.0 ml (45.3 g, .21 mol) 2-bromopropionyl bromide was added with vigorous stirring. After several minutes, the temperature began to rise and an ice bath was applied to avoid vigorous thermal decomposition. The solution became homogeneous, and then in several more minutes solidified into a solid light brown mass. Exothermic reaction continued for some time, and the reaction was stopped after 20 min. 600 ml boiling water was added in portions and the chunks broken up. The solution was cooled and filtered. The product was recrystallized from 3 l boiling water, filtered, and dried in an oven to afford 21.10 g product (51%). ^{1}H NMR [$CDCl_3$, δ 8.95(1H, br s, NH), 8.10(1H, br s, NH), 5.45(1H, br s, NH), 4.40(1H, q, CH), 1.85(3H, d, CH_3)].
(2-cyano-^{13}C-propionyl) urea (III): Potassium ^{13}C-cyanide (1.00g, 15.15 mmol) was dried in vacuo overnight at 85°C, then combined in a septum sealed flask with 2-bromopropionyl urea. Dimethyl sulfoxide (20 ml) was added via syringe, and the mixture stirred overnight under nitrogen. The solvent was removed on a Speed-vac concentrator, and the product crystallized in two crops from boiling water, affording 1.36 g (9.64 mmol) product (63%). ^{1}H NMR [DMSO, δ 10.51(1H,br s, NH), 7.42(2H,br s, NH), 3.92(1H, dq br, CH), 1.42(3H, t, CH_3)]. ^{13}C NMR [DMSO, δ 110.42 (CN)].
6-^{13}C-Thymine (IV): 500 mg platinum oxide in 10 ml 50% acetic acid was prereduced in an atmospheric hydrogenator, and 1.37 g cyanopropionyl urea in 25 ml 50% acetic acid added via the sidearm. The mixture was heated at 70°C for 5 hrs, by which time the uptake of hydrogen had ceased. The total consumption of hydrogen was 240 ml or 10.71 mmol. The mixture was filtered hot, concentrated to a syrup, and then crystallized in two crops from hot water yielding 561 mg (4.42 mmol) pure 6-^{13}C-thymine (46%). ^{1}H NMR [DMSO, δ 7.25(1H, d J=177 Hz, H6), 1.71(3H, d J=7 Hz, CH_3)]. ^{13}C NMR [DMSO, δ 137.55 (C6)].
3',5' ditoluyl-6-^{13}C-thymidine (VII): 421 mg (3.31 mmol) 6-^{13}C-thymine, 15 ml hexamethyldisilazane, and 15 ml trimethylsilyl chloride were refluxed under nitrogen overnight, during which time ammonium chloride sublimed in the condenser and the solution became homogeneous. The excess reagents were distilled off under aspirator pressure to afford the bis TMS derivative as a yellow oil. Chloroform, 5 ml, was added via syringe with a 2 ml rinse, and the combined solution was syringed into a solution of 1.29 g (3.31 mmol) 2-deoxy-3,5,-di-O-*p*-toluyl-D-erythro-pentosyl chloride freshly dissolved in 20 ml of chloroform. The mixture was stirred overnight, concentrated on a rotary evaporator, dissolved in 80 ml boiling ethanol, cooled very slowly, and then filtered. Two recrystallizations from 75 ml boiling ethanol afforded 740 mg pure β-3',5' di-*p*-toluyl-6-^{13}C-thymidine. The filtrates from the recrystallizations were combined and recrystallized from 30 ml boiling ethanol giving a second crop of 290 mg, for a total of 1.03 g (2.16 mmol) in 73% yield. ^{1}H NMR [$CDCl_3$, δ 9.42(1H, s, NH), 7.97(4H, m, toluyl), 7.31(1H, d J=178Hz, H6), 7.30(4H, m, toluyl), 6.51(1H, m, H1'), 5.67(1H, m, H3'), 4.75(2H, AB m, H5'/H5"), 4.65(1H, m, H4'), 2.53(2H, AB m, H2'/H2"), 2.43(6H, s, toluyl-CH_3), 1.64(3H, d J=7 Hz, thy-CH_3)]. ^{13}C NMR [$CDCl_3$, δ 134.60(C6)].
6-^{13}C-thymidine (VIII): 3',5' di-*p*-toluyl-6-^{13}C-thymidine (985 mg, 2.06 mmol) was dissolved in 25 ml absolute methanol, and 60 mg sodium methoxide added. After reflux for 3 hrs, TLC (10% methanol in dichloromethane) indicated some starting material remained, and 25 mg more sodium methoxide was added. After 4 more hrs reflux the reaction was complete. The solvent was removed on a rotary evaporator, and the product taken on crude.
5'-dimethoxytrityl-6-^{13}C-thymidine (IX): The crude thymidine was dried by coevaporation with two 25 ml portions of pyridine, then dissolved in 25 ml pyridine, and 800 mg dimethoxy chloride (DMTCl) added. After 8 hrs, the starting material was not consumed, and 400 mg more DMTCl was added, and the mixture stirred overnight. The solvent was removed by coevaporation from chloroform on a rotary evaporator, and the product chromatographed on a 5 x 15 cm bed of silica gel, eluting with 1.5 l of 1% methanol in dichloromethane, followed by 1 l of 2% methanol in dichloromethane. Product containing fractions were pooled and concentrated, giving a partially crystalline mass. The crystals were filtered and rinsed with 10 ml dichloromethane, and the filtrate then precipitated in 300 ml cold rapidly stirred hexanes. 480 mg of crystals were obtained along

with 302 mg of powder from the precipitation, giving a combined yield of 782 mg (69% from 3',5' di-*p*-toluyl-6-^{13}C-thymidine).

5'-dimethoxytrityl-6-^{13}C-thymidine ß-cyanoethylphosphoramidite (X): 670 mg (1.23 mmol) 5'-dimethoxytrityl-6-^{13}C-thymidine and 522 mg (.704 ml) diisopropylethylamine were dissolved in 10 ml dichloromethane. N,N diisopropylamino-ß-cyanoethyl phosphochloridite was added in 50 µl portions, the progress of the reaction being monitored by TLC (ethyl acetate:dichloro-methane:triethylamine 50:45:5). After a total of 500µl (1.9 eq) was added, the mixture was stirred 10 min, then poured into ethyl acetate and washed with saturated sodium bicarbonate. The organic layer was dried with sodium sulfate, filtered, and concentrated on a rotary evaporator. The residue was precipitated twice by dissolving in 10 ml dichloromethane and dropping into 100 ml ice cold hexanes. The product was filtered and dried in a vacuum dessicator, giving 660 mg pure product (70%).

1,3 dimethyl thymine: 500 mg (3.97 mmol) thymine, 1.094 g potassium carbonate (7.93 mmol), and 4.50 g (31.7 mmol) iodomethane were stirred in 15 ml DMSO under nitrogen overnight. The mixture was concentrated on a rotary evaporator to remove the excess iodomethane, and then poured into 50 ml water. The aqueous phase was extracted with two 50 ml portions of chloroform, and the organic layer washed with 100 ml of water. The chloroform layer was dried with sodium sulfate to afford 750 mg of crude crystals, the main impurity being DMSO. Recrystallization from ethanol afforded 125 mg pure dimethylthymine which was used for the NMR experiments. ^{1}H NMR [$CDCl_3$ sat. soln., δ 6.90(1H,s,H6), 3.09(3H, s, N-CH_3), 3.00(3H, s, N-CH_3), 1.597(3H, s, CH_3)]. ^{13}C NMR [$CDCl_3$ sat. soln., δ 163.35(C=O), 151.13(C=O), 138.87(C6), 108.34(C-CH_3), 35.90(CH_3), 27.09(CH_3), 12.20(CH_3)].

Preparation of NMR sample: A saturated solution of dimethylthymine in 0.6 ml deuterochloroform was prepared, and placed in an NMR tube, followed by 50 µl more deuterochloroform. The sample was partially degassed by two freeze-pump-thaw cycles and then sealed with a torch.

DNA Synthesis. The oligonucleotide was synthesized using the phosphoramidite chemistry on a home built, fully automated synthesizer. The 3' terminal base was linked to controlled-pore glass substituted with long chain alkyl amine at a loading of ~25 µmol/g. For routine DNA synthesis 10 µmol of resin bound nucleoside was used, with phosphoramidite and tetrazole concentrations of 100µmol/ml and 400 µmol/ml respectively. For labelled DNA synthesis, 20 µmol of bound resin was used, and the unlabelled phosphoramidite concentrations were increased to 200 µmol/ml, and the tetrazole concentrations was increased to 600 µmol/ml.

DNA purification. The oligonucleotide was cleaved from the solid support and deblocked by heating in concentrated ammonium hydroxide for 12 hrs in a sealed vial at 65°C. The resin was filtered off, the ammonia removed by rotary evaporation, and the crude product concentrated to dryness on a Speed-vac vacuum centrifuge. The trityl-oligomer was prepurified by reverse phase HPLC, detritylated by treatment with 80% acetic acid for 30 minutes, and then concentrated to dryness. A second round of HPLC was performed on the detritylated oligomer. Fractions are concentrated on a Speed-vac, and then desalted by loading on to a low pressure C-18 column, washing with water, then eluting the DNA with 20% acetonitrile, and finally concentrated on the Speed-vac to a white powder.

Preparation of NMR samples. The pure oligonucleotide is dissolved in the appropriate amounts of buffers made with D_2O, then concentrated on a Speed-vac to dryness. The sample is further exchanged by redissolving in 99.8% D_2O and concentrating to dryness three times, and finally dissolved in a freshly opened vial of 99.996% D_2O in a glove bag under nitrogen.

ACKNOWLEDGEMENTS

This work was supported by a grant from the National Institutes of Health (R01 GM27738). The 500 MHz NMR spectrometer was funded by grants from the National Science Foundation (DMB-8515942) and from the National Institutes of Health (1-510-RR02733-01). SGB is the recipient of a Presidential Young Investigator Award.

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