A Practical Method for the Synthesis and Purification of ^{14}C Labeled Oligonucleotides

Henri M. Sasmor, Douglas J. Dellinger#, Paul C. Zenk* and Larry P. Lee*

ISIS Pharmaceuticals, Carlsbad, California 92008, U.S.A.
#MetaForm Pharmaceuticals, Boulder, Colorado 82006 U.S.A.
*Oread Laboratories Inc., Lawrence, Kansas 66047, U.S.A.

Summary

A practical method for the solid-phase synthesis and purification of ^{14}C labeled oligonucleotides is described. A streamlined procedure for the synthesis of 5'-O-dimethoxytrityl-2-(^{14}C)-thymidine 3'-(2-cyanoethyl-N,N-diisopropyl) phosphoramidite from commercially available 2-(^{14}C) labeled thymidine nucleoside is detailed. Methodology for the manual synthesis, isolation, and purification of a high specific activity oligonucleotide phosphorothioate 20-mer (ISIS 2105) are elaborated.

Key words: ^{14}C thymidine, oligonucleotide, phosphoramidite, phosphorothioate

Introduction

The rapid transition of antisense oligonucleotides from research tools [1], to potential therapeutic uses [2], to clinically administered experimental drugs [3] has generated the need to produce such molecules with high specific activity radioisotopic labels for drug metabolism and pharmacokinetic studies. The simple chemical composition of nucleotides provides several atoms where radioisotopes can be utilized (^{3}H, ^{32}P and ^{14}C) and the extensive use of phosphorothioate molecules in the antisense arena provide an additional site for radio label incorporation in the form of ^{35}S [4,5]. Post-synthetic radioisotopic labeling of oligonucleotides has been accomplished by enzymatic [6,7] and chemical methodologies [8,9], both techniques allowing for the

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generation of high specific activity molecules. Enzymatic techniques provide unique, site specific incorporation while post-synthetic chemical techniques provide a more homogenous incorporation, each method and isotope having advantages and disadvantages.

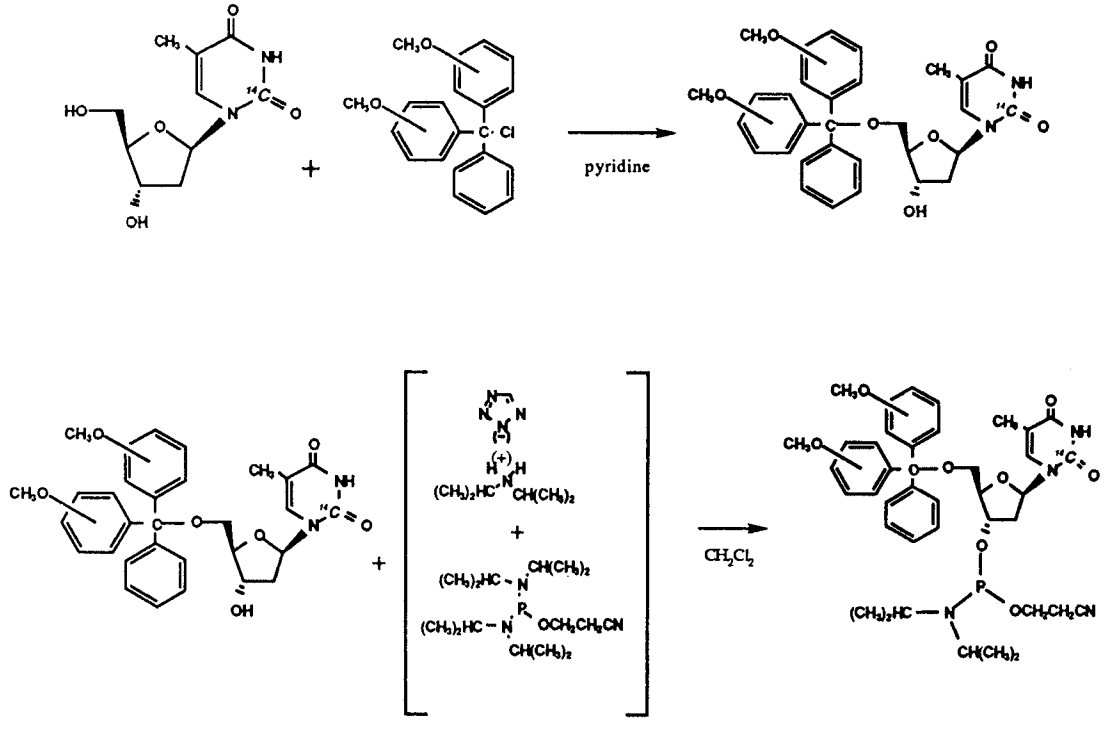
^{32}P incorporation is most easily accomplished enzymatically, generating site specific, high activity probes useful for *in vitro* laboratory experiments. Use of this method and radionuclide, however, is limited by the requirement of enzyme-substrate recognition [6,7], limited number and site of incorporation, and short-lived product in *in-vivo* experimentation [10]. The uniform incorporation of a ^{3}H label, by $^{3}H_2O$ exchange [8] or catalyzed exchange [11,12], can produce oligonucleotides with high specific activities but with low detectability. The low detectability and inherent exchange rates limit the *in vivo* usefulness of ^{3}H oligonucleotides generated by exchange to short time frame experiments and greatly limits their use in drug metabolism studies. The ^{35}S labeling of phosphorothioate oligonucleotides, during phosphoramidite synthesis [9,13] or post-synthetically using H-phosphonate chemistry [14], can generate homogeneously labeled, stable, high specific activity oligonucleotides [15]. The most severe limitations of this labeling methodology are those imposed by the obvious requirement that the product contain sulfur and the high excess/lower yielding chemistries required to generate product [14,16,17].

The cost and time required to chemically synthesize and purify antisense oligonucleotides, in practice, limits radioisotopic replacements to the relatively long lived isotopes (≥ 30 day half life) of hydrogen, sulfur and carbon. Considering the limitations of ^{3}H and ^{35}S and our need to produce millimolar quantities of a high specific activity radio labeled phosphorothioate deoxyoligonucleotide for pharmacokinetic and drug metabolism studies, we were interested in determining if ^{14}C could effectively be used to radio label synthetic oligonucleotides. This has been accomplished by converting a commercially available radiolabeled nucleoside to its protected nucleoside phosphoramidite and then incorporation of this synthon during oligonucleotide synthesis. Our choice of the 2-(^{14}C)-thymidine nucleoside was based on three factors: the sequence composition of our deoxyoligonucleotide phosphorothioate in Phase II clinical trials (ISIS 2105; 9 of the 20 nucleotides are

thymidine residues), commercial availability of the starting material, and the minimal number of synthetic steps necessary to produce the phosphoramidite synthon.

Results and Conclusions

Our synthetic efforts addressed two issues: the two-step conversion of 2-(^{14}C)-thymidine to the protected nucleoside phosphoramidite synthon as shown below,



and the use of this synthon to incorporate ^{14}C into an oligonucleotide product via solid phase phosphoramidite chemistry. Synthesis of the ^{14}C nucleoside phosphoramidite was examined at three specific activities; a tracer level (0.43 mCi/mmol), at the maximum specific activity attainable with this lot of ^{14}C nucleoside (56.3mCi/mmol), and at an intermediate level (9.7 mCi/mmol). The phosphoramidites from these three experimental productions were utilized to synthesize a 20-mer phosphorothioate oligonucleotide (ISIS 2105) at the 25 μmole, 2 μmole and 53 μmole scales, respectively. Streamlined synthetic methodologies for converting the ^{14}C labeled nucleoside to the phosphoramidite and for manipulating the labeled oligonucleotide were established (as detailed in Experimental) in an effort to minimize loss of ^{14}C nucleoside and limit radioactive contamination of glassware, equipment, and environment.

Our initial tracer level synthetic effort was designed to identify potential practical problems, to validate the process, equipment and methodology designed, and to determine the overall feasibility of the synthesis effort. The streamlined process for the production of the amidite synthon generated less than 2 liters of ^{14}C contaminated organic waste and less than 400 grams of contaminated silica gel solid waste. The two-step conversion of the nucleoside to the ^{14}C-thymidine phosphoramidite was accomplished with a 55% overall molar yield compared to 70-95% yields reported in the literature for the production of thymidine phosphoramidite.

The oligonucleotide synthesis protocol (expected to incorporate only 10% of the starting material) generated ~0.25 liter high level radioactive waste (the 90% excess unincorporated synthon) and ~2 liter low level radioactive organic waste (all other synthesis steps except that noted above). The yield of the crude synthetic product (~2600 AU_{260}) was typical for a phosphorothioate of this length (20-mer). An average stepwise coupling efficiency of 97% (the ^{14}C-thymidine incorporations were not measured independently) was calculated based on the percent full length product (~55%) in the crude reaction. This compares to ≥98% average stepwise coupling efficiencies achieved using automated processes involving the same chemistry. The HPLC purification and final deprotection protocols resulted in ~0.75 liters of ^{14}C contaminated aqueous waste. The HPLC purified final product (2.3 μmole, 8.7 μCi) was less than routinely achieved for synthesis at this scale (10% versus 30-35% of the crude synthesis product), however this can be accounted for by the higher purity of the final

^{14}C labeled product (93-95% full length product compared with only ≥90% typically recovered). The specific activity of the final product (3.79 μCi/μmol) was within 2% of theoretical based on the specific activity of the starting material (~0.43 mCi/mmol).

Our second synthesis addressed the production of a very high specific activity 2-(^{14}C)-thymidine phosphoramidite (56.3 mCi/mmol) at the 1.73 mmol scale. The overall yield for the two-step process was approximately 9%, (35% and 25% stepwise respectively). The low yield in the first reaction step could potentially be due to instability of the DMT protected product (decomposition at a labile bond) a radiolysis problem occasionally encountered in carrier free, high specific activity synthesis [18]. However, TLC analysis of the isolated phosphoramidite gave no evidence of instability. More likely it was failure of the tritylation reaction to go to completion, due to incomplete dehydration of the reaction mixture, or decomposition to starting materials on the silica column. A methanol wash of the silica column recovered ~55 mCi of starting material (2-(^{14}C)-thymidine). The phosphitylation reaction required additional reagents and time to go to completion, again indicative of non-anhydrous reaction conditions. The recovery of the protected nucleoside phosphoramidite gave only a 25% stepwise yield (112 mg., 8.5 mCi). Our most plausible explanation for this low yield was loss of material due to the small scale of the reaction and large column used for chromatography. In this later case the remaining ^{14}C was not recoverable from the silica column nor were any ^{14}C-labeled or UV absorbing side products detected by TLC.

The yield of crude oligonucleotide product from a 2 μmole synthesis, utilizing the high specific activity amidite, was approximately 25% the expected yield for an automated synthesis at this same scale. Additionally, the purity of the crude synthetic product was lower than routinely achieved (<50% full length). This may have resulted from low amidite purity (confirmed only by TLC comparison to standards), radiolytic degradation, or simply loss introduced by equipment, the small scale and the modified synthesis scheme utilized (see Methods). The final product (after HPLC and final deprotection) had a full length integrity of only ~72%. As with the previous synthesis, the product specific activity (476 μCi/μmole) was within 6% of theoretical (507 μCi/μmol) based on the specific activity of the starting material (56.3 μCi/μmol).

Our third, intermediate specific activity phosphoramidite synthesis utilized the ~55 mCi starting material recovered from the high specific activity phosphoramidite

synthesis (methanol column wash), an additional 10 mCi of 2-(C^{14})-thymidine nucleoside (56.3mCi/mmol), and 5.6 mmol carrier thymidine. At this scale and specific activity, the two step conversion to the phosphoramidite was successful (69% overall yield). The purity of the phosphoramidite product was confirmed by both TLC and ^{31}P NMR. The average stepwise coupling yield during oligonucleotide synthesis was calculated to be >98% (based on full length product in the crude reaction mixture) and 35% of the crude oligonucleotide synthetic product was recovered as final product. The specific activity of the purified oligonucleotide was 90 μCi/μmol, again within 3% of the predicted specific activity. The 1.68 mCi ^{14}C recovered in the final product represents a 37% yield of theoretical from the initial ^{14}C nucleoside.

We have demonstrated that ^{14}C can be successfully incorporated into deoxy-oligonucleotide phosphorothioates, at specific sites, and with predictably defined specific activities. Yield improvements in converting the nucleoside to the protected phosphoramidite can be achieved by further optimizing the reaction conditions and scale. Incorporation of phosphoramidite synthons at lower mole excess could also further improve the overall yield. While this report focuses on use of 2-(^{14}C)-thymidine nucleoside, the methodology developed is applicable for the production of any radio labeled nucleoside phosphoramidites and their subsequent utilization in the production of labeled oligonucleotides. Similarly, while the equipment and the described manual synthesis procedures are applicable for this situation, the entire process is amenable to the automated synthesis of oligonucleotides on any commercially available instrument.

Materials, Equipment and Methods

Phosphoramidite synthesis reagents *Reagents for the synthesis of the* ^{14}C-thymidine phosphoramidite were either purchased from commercial sources or synthesized following published procedures. Thymidine was purchased from Aldrich Chemical Company, Milwaukee, WI, as were the following solvents: anhydrous acetonitrile (SureSeal), anhydrous toluene (SureSeal), anhydrous methanol (SureSeal), triethylamine, hexane, and ethyl acetate. Dimethoxytritylchloride was purchased from Aldrich and re-crystallized from Hexane/Acetylchloride. Pyridine and anhydrous dichloromethane (SureSeal) were purchased from Aldrich and re-distilled over CaH. 2-Cyanoethyl-*bis*-(N,N-diisopropyl)-phosphorodiamidite and the diisopropyl amine

tetrazolide salt were prepared following literature protocols [19]. 2-(^{14}C)-Thymidine (56.3 mCi/mmol) was purchased from Sigma Chemical Company.

DNA synthesis reagents: Reagents and materials for the solid phase synthesis of DNA were purchased from commercial sources as follows. 5'-O-dimethoxytrityl-[dAbz, dCbz and dGibu] 3'-β-cyanoethyl nucleoside phosphoramidites were supplied by Milligen Corporation, Milford MA. Trichloroacetic acid/CH_2Cl_2 (3% w/v), 0.5 M tetrazole/acetonitrile, acetic anhydride/lutidine/tetrahydrofuran (1:1:8, Cap solution A), and 1-methyl imidazole/tetrahydrofuran (3:7, Cap solution B) were purchased from Applied Biosystems Inc., Foster City CA. Control pore glass (CPG) solid support derivatized with the 3' base [5'-O-dimethoxytrityl-dCbz] (CPG-dC) was purchased from CPG Inc., Fairfield, NJ. 3*H*-1,2-benzodithiole-3-one-1,1-dioxide (Beaucage Reagent) was purchased from R.I. Chemical Inc., Costa Mesa, CA. Concentrated NH_4OH and pharmaceutical grade sodium acetate were purchased from J.T.Baker Inc. HPLC grade methanol was from Aldrich Chemical Company. Glacial acetic acid was supplied by Malinkrodt. Sterile water (WFI) for both sample preparation and HPLC solvents was either from Kendall McGaw or Abbot Laboratories.

Equipment: Synthesis of the ^{14}C labeled nucleoside phosphoramidite and the oligonucleotide were performed in a fume hood fitted with a vacuum/gas manifold to allow work under inert/dry atmosphere, a rotary evaporator and a Teflon head, diaphragm vacuum pump. A 24/40 vacuum adapter with an extra-coarse sintered glass frit inserted in the neck was constructed to prevent aspiration of ^{14}C solids into the vacuum line. Chromatography of the protected nucleoside and phosphoramidite were performed by gravity elution in open-top columns rather than the more standard medium pressure conditions. The manual synthesis of the oligonucleotide was carried out in a sintered glass funnel/receiver flask setup based upon a Merrifield apparatus. An extra long drip tube was constructed on the synthesis funnel to prevent aspiration of solvent into the vacuum line. A set of two receiver flasks were used; one to collect the high-level ^{14}C waste during the ^{14}C phosphoramidite coupling step(s) and a second to collect the other low-level waste.

The ^{14}C labeled oligonucleotide was purified by reverse phase HPLC on a Waters Bondapak HC18 HA, 8x100 mm column in the Radial-Pak cartridge

configuration. Analysis of the synthetic oligonucleotide products utilized standard laboratory instrumentation. UV scans (quantitation) were measured on either a Cary13 or Hewlett Packard UV/Vis Spectrophotometer. Analytical separation of oligonucleotide polymers was by electrophoresis on 20% denaturing polyacrylamide gels (20 cm gels) and separated oligonucleotide products visualized by staining with Stains-all dye (Sigma Chemical Company). Quantitation of the gel separated products was either by laser scanning densitometry of the dye stained gels (Molecular Dynamics Scanning Densitometer) or autoradiographic imaging for determination of radiochemical purity (Molecular Dynamics Phosphorimager). Quantitation of radioactivity was by counting in an aqueous scintillant. ^{1}H NMR of the ^{14}C thymidine and ^{31}P NMR of the phosphoramidite were run on a 300 MHz General Electric QE Plus Spectrophotometer (Oread Laboratories). ^{31}P NMR of the phosphoro-thioate oligonucleotide was run on a 500 MHz Bruker AMX Spectrophotometer at the University of Missouri, Columbia, Missouri.

5 mmol (~2 mCi Tracer) Synthesis

5'-O-dimethoxytrityl-2-(^{14}C)-Thymidine: Thymidine (1.3 g., 5.4 mmol) was weighed into a 200 mL round bottom reaction flask. An aqueous solution of 2-(^{14}C)-thymidine (~2 mCi, 10 mg, 0.044 mmol) was transferred into the reaction flask. The nucleoside was dissolved in anhydrous pyridine (60 mL) and made anhydrous by three successive co-evaporation's with pyridine, *in-vacuo*, on a rotary evaporator. The anhydrous nucleoside was dissolved in anhydrous pyridine (60 mL) and re-crystallized dimethoxytritylchloride (2.4 g., 7.0 mmol) was added to the reaction flask. The reaction was stirred under nitrogen at room temperature for 30 minutes and monitored by silica plate TLC (Merck). The reaction was quenched with anhydrous methanol (20 mL) and the pyridine removed by concentration *in-vacuo*. The concentrate was diluted with CH_2CL_2 (100 mL, 0.5% triethylamine (TEA)) and transferred to the sand cap of an ~15 cm bed height silica gel column (5 cm X 55 cm column, 230-400 mesh flash grade silica (Aldrich)) pre-equilibrated with CH_2CL_2 (0.5% TEA). The reaction mixture was allowed to run onto the silica gel, the reaction flask rinsed with 3 X 10 mL volumes of column solvent and these rinses transferred to the top of the column and eluted onto the silica gel. This rinse procedure ensured that both the flask and the column sand-cap were completely rinsed of the reaction mixture. The column was then eluted using a

two step gradient: CH_2CL_2 (1000 mL, (0.5% TEA)); CH_2CL_2:CH_3OH, 95:5 (1000 mL, (0.5% TEA)).

Column elution was monitored by periodically sampling the elutant stream with a capillary tube and spotting onto a fluorescent silica gel TLC plate observed under UV light. UV absorbing spots were monitored for β^- particle emission using a field meter. UV absorbing material eluted from the column in three distinct fractions, only the third fraction of which exhibited β^- particle emission. In order to minimize radioactive contamination only two 500 mL Erlenmeyer flasks and a 500 mL Kjeldahl (round bottom) flask were used to collect column fractions. The first UV absorbing material eluting from the column was determined to be the excess dimethoxytrityl reagent (TLC). The second UV absorbing material to elute from the column was residual pyridine from the reaction, the appearance of which was the signal to begin collecting column fractions in the Kjeldahl flask. The third UV absorbing sample exhibited β^- emission. After each 100 mL volume was collected, column flow was stopped and the solvent removed on the rotary evaporator. The product eluted in approximately 600 mL total volume. Residual pyridine in the final product (observed on TLC) was removed by two successive co-evaporation's from anhydrous toluene. The resulting viscous oil was dried to a white foam *in-vacuo* (4.23 g., 144% theoretical yield).

5'-O-dimethoxytrityl-2-(^{14}C)-Thymidine 3'-(2-cyanoethyl-N,N-diisopropyl-amino phosphoramidite: The 5'-O-dimethoxytrityl-2-(^{14}C)-thymidine foam was dissolved in freshly distilled CH_2Cl_2 (100 mL). Diisopropylamine tetrazolide salt (0.46 g., 2.7 mmol) was added to the flask followed by 2-Cyanoethyl-*bis*-(N,N-diisopropyl)-phosphorodiamidite (3.25 g., 10.8 mmol). The reaction flask was purged with nitrogen, stirred at room temperature, and monitored by TLC. The phosphitylation reaction was observed to be complete after 2 hours. The reaction mixture was concentrated to ~25 mL *in-vacuo* and transferred to an ~20 cm pre-equilibrated silica column (previously described). The reaction flask was rinsed with 3 X 25 mL ethyl acetate and chromatography performed as previously described with a hexane:ethyl acetate, 1:1 (0.5% TEA) solvent system. Under these conditions the phosphoramidite is the first UV absorbing material to elute from the column. The product was collected in a single Kjeldahl flask by periodically removing solvent (total volume collected was ~600 mL). The isolated product was verified by TLC using commercially available non-radioactive

phosphoramidite standards but showed a residual amount of unreacted starting material (<5%). The phosphoramidite was confirmed by ^{31}P NMR. The product was dried to a glass overnight *in vacuo* (2.26 grams, ~3.03 mmol, 55% overall yield).

Oligonucleotide Synthesis: DNA was manually synthesized (25 μmole scale) utilizing the deoxynucleoside phosphoramidite approach. CPG-dC (560 mg, 45 μmol/g) was transferred to the described manual synthesis funnel. The four step synthesis cycle (and associated anhydrous solvent and inert gas washes) consisted of:

i) trichloroacetic acid detritylation,

ii) 5 minute coupling step (10 X phosphoramidite/40 X tetrazole),

iii) 2 X 3 minute sulfur oxidation reaction, and

iv) 2 X 2 minute capping reaction (Cap A:Cap B, 1:1)

Sulfur oxidation, to afford the phosphorothioate linkage, was effected by utilizing 0.055M 3*H*-1,2-benzodithiole-3-one-1,1-dioxide in acetonitrile instead of aqueous iodine as the oxidizing reagent.

After completion of the 19 cycles of the solid-phase synthetic process, the CPG was dried in a stream of inert gas and transferred to a 10 mL conical bottom reaction vial (Pierce). The 5'-O-dimethoxy-trityl protected crude product was cleaved from the solid support by three successive room temperature treatments with concentrated NH_4OH (10 mL, 90 minute incubation/treatment). Each NH_4OH fraction was transferred to a sealed reaction vial and further oligonucleotide deprotection afforded by an 18 hour incubation at 55° C. The deprotection reactions were cooled to room temperature and the NH_4OH removed by reducing the samples to near dryness *in vacuo*. The samples were resuspended in sterile water (7 mL total volume). The yield of crude product was ~2600 AU_{260}. Analysis of the crude synthetic product, by 20% denaturing polyacrylamide gel electrophoresis, indicated ~55% trityl-on full length product.

HPLC Purification: The ~2600 AU_{260} crude material was split into 2 samples (1200 and 1400 AU_{260}, respectively) and each sample purified by reverse phase HPLC (maximum load on the 8 X 100 mm HAC18 HA reverse phase column, for this specific oligonucleotide sequence, was previously determined to be 1900-2000 AU_{260}). The HPLC column initial conditions were 70% H_2O (Solvent A), 20% MeOH (Solvent B), 10% 2 M NaOAc (pH7.2) (Solvent C). The solvent flow rate was 5 mL/min. The

preparative loading of the diluted crude sample, in a large volume (52 mL), was afforded by direct introduction of the sample, over a 15 minute period, through the fourth solvent port of the quaternary solvent delivery system rather than through the injection port. This allowed for a slower, preparative loading of the sample and eliminated the need for multiple injections and frequent sample handling. The column was then washed for 10 minutes at initial conditions.

The dimethoxytrityl-on product was eluted from the column with a linear MeOH gradient, from 20 - 50%, over 60 minutes. The UV absorption profile of the HPLC eluant was monitored at 260 nm (Figure 1) and the gradient collected in 1 minute fractions (5 mL/fraction). The radiochemical profile of the gradient matched the UV absorption profile (Figure 1) and the product elution profile was analyzed by 20% denaturing polyacrylamide gel electrophoresis of samples across the major UV absorption peak (Figure 2). The total UV absorbance recovered, in the major peak of the 2 separate gradients, was 40% (45% and 36% respectively). Radiochemically, 83% of the input radioactivity was recovered in the major peak.

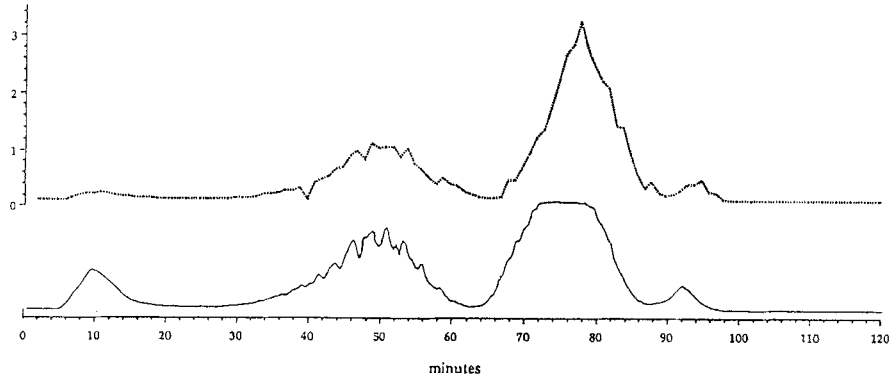


Figure 1: Lower trace is the UV absorption profile of HPLC column eluant for ^{14}C-labeled oligonucleotide purification as described. Sample loading was initiated at t=0 minutes, the methanol elution gradient initiated at t=25 minutes. The plateau in the trace between 70 and 80 minutes reflects saturation of the detector. The upper trace (dotted) is the radiochemical profile of the gradient. A 10 µL sample of each HPLC column fraction was counted in 10 mL scintillation cocktail and the total cpm's plotted.

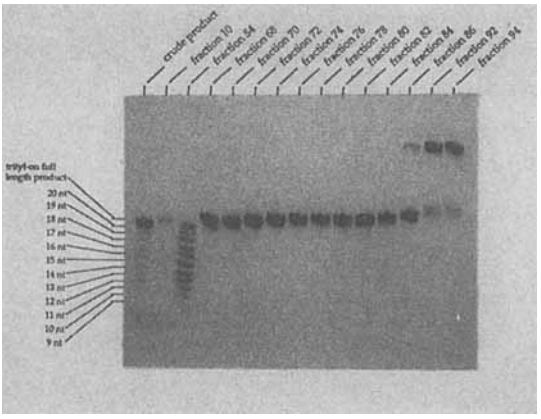


Figure 2: Samples from the HPLC fractions (corresponding to the time profile in minutes) were precipitated with ethanol and ~3µg (0.1 AU_{260}) evaluated by 20% denaturing PAGE. Separated products were visualized by developing the gel with Stains-all dye and photographed. The gel lanes are marked with the corresponding fraction number (minutes) from the elution gradient (Figure 1). The first sample is the crude synthetic product.

Final Purification and Analysis: The volume of each HPLC fraction was reduced to less than 900 µL *in vacuo*. The 5'-O-dimethoxytrityl group was acid deprotected by adjusting the aqueous sample to [3.5 M] CH_3COOH and incubated for 30 minutes at room temperature. The DNA product was precipitated directly out of the aqueous acid solution by addition of 2.5 X volume absolute ethanol (cold). The precipitation process was carried out for 60 minutes at -70 °C. The precipitate was collected by centrifugation and resuspended in 50 µL sterile water. The resuspended samples were adjusted to 0.3M NaOAc (pH7.2) and the product reprecipitated with 3 X volume absolute ethanol (cold). This 2nd precipitation was allowed to stand overnight at -20 °C. The product was collected by centrifugation, gently rinsed with 70% ethanol and the final product resuspended in sterile water. 85% - 99% of the starting radioactivity was recovered through the double precipitation procedure.

Each HPLC fraction was spectrophotometrically quantitated, analyzed for full length integrity by 20% denaturing polyacrylamide gel electrophoresis and laser scanning densitometry and pooled to give two final fractions representing the two separate purification's.

Purification/ Fraction	% full length	µmoles recovered	total µCi	specific activity
1	93 %	1.53	5.7	3.72 µCi/µmol
2	95	0.76	3.0	3.95

1.7 mmol (98 mCi High Specific Activity) 2-(^{14}C)-thymidine Synthesis

5'-O-dimethoxytrityl-2-(^{14}C)-Thymidine: An aqueous suspension of 2-(^{14}C)-thymidine (~98 mCi, 420 mg, 1.7 mmole) was transferred into a 200 mL round bottom reaction flask and the shipping vial rinsed 3 times with anhydrous pyridine. Additional anhydrous pyridine (75 mL) was added to the reaction flask and the water co-evaporated with the pyridine once *in-vacuo*. The nucleoside was dissolved in anhydrous pyridine (75 mL), re-crystallized dimethoxytritylchloride (0.863 g., 2.55 mmol) was added to the reaction flask and the tritylation reaction (30 minute) and silica gel chromatography performed as previously described with the following two modifications: residual pyridine was removed prior to chromatography (rather than afterwards) by co-evaporation with anhydrous toluene (50 mL) and the reaction flask was rinsed 2 X 10 mL CH_2Cl_2 and 1 X 10 mL CH_3OH. The chromatographed product was dried to a white foam *in-vacuo* (0.35 grams, 0.6 mmol, 35% yield).

5'-O-dimethoxytrityl-2-(^{14}C)-Thymidine 3'-(2-cyanoethyl-N,N-diisopropyl-amino phosphoramidite: The 5'-O-dimethoxytrityl-2-(^{14}C)-thymidine foam was dissolved in distilled CH_2Cl_2 (80 mL). Diisopropylamine tetrazolide salt (0.055 g., 0.3 mmol) was added followed by 2-Cyanoethyl-*bis*-(N,N-diisopropyl)-phosphoro-diamidite (0.36 g., 1.2 mmol). The reaction flask was purged with nitrogen and stirred at room temperature. At 2 hours TLC analysis indicated that the reaction was not complete. Additional tetrazolide salt (0.03 g.) and phosphorodiamidite (0.20 g.) were added and the reaction stirred overnight. The reaction mixture was transferred directly to the silica column and chromatographed as previously described. The product was dried *in-vacuo* (112 mg, ~0.151 mmol,~25% yield).

Oligonucleotide Synthesis: DNA was manually synthesized at the 2 µmol scale (44 mg solid support) as previously described with the following modification. The minimum volume necessary to saturate the small quantity of CPG utilized for this synthesis was determined to be ~350 µL. The 112 mg of ^{14}C-thymidine phosphoramidite was resuspended in 320 µL anhydrous acetonitrile [~0.5 M]. All ^{14}C-

thymidine amidite coupling reactions entailed the addition of 32 μL ^{14}C-thymidine amidite + 320 μL tetrazole [0.45 M].

The 5'-O-dimethoxytrityl protected crude product was cleaved from the CPG as previously described with 2 concentrated NH_4OH treatments (2 mL each) and deprotected 18 hr's at 55 °C. A third NH_4OH treatment (2mL) of the CPG was performed for 18 hours at 55° C. The yield of crude product was 58.4 AU_{260} (14.6 AU_{260}, 19.0 AU_{260} and 24.8 AU_{260} from the three NH_4OH samples, respectively).

HPLC Purification: The three samples were pooled and purified by reverse phase HPLC as previously described. The solvent flow rate was 2 mL/min. The AU_{260} product elution profile and gradient radiochemical profile matched as previously seen. The major peak consisted of 14 X 2 mL fractions (from 83-96 minutes in the gradient) and constituted 41% of the total UV absorbance, 70% of the total radioactivity.

Final Purification and Analysis: The detritylation and precipitation of the final product was carried out as previously described. After work up the 14 samples were pooled into 2 fractions and precipitated a third time: the center of the peak, 87-91 minutes, constituted Fraction 1, the leading edge of the major peak (83-86 minutes) and the trailing edge (92-96 minutes) were combined to give Fraction 2. The radioactivity remaining in the ethanol supernatants from the third precipitation (Fractions 1B and 2B) was quantitated to estimate unrecovered product.

Fraction	% full length	nanomoles recovered	total μCi	specific activity
1A	73%	10.92	5.19	0.475 mCi/μmol
1B	-	-	1.27	
2A	72	9.08	4.33	0.477
2B	-	-	2.44	

6.6 mmol (65 mCi) Preparative Synthesis

5'-O-dimethoxytrityl-2-(^{14}C)-Thymidine: A solution of 2-(^{14}C)-thymidine (~65 mCi, 279 mg., 1.1 mmol) was transferred to a 250 mL Kjeldahl flask and carrier thymidine (1.37 grams, 5.5 mmol) added. The nucleoside was made anhydrous by multiple co-evaporation's with anhydrous pyridine as previously described. The tritylation reaction was performed with a 1.5 molar excess of re-crystallized dimethoxytritylchloride (3.4 grams, 10.0 mmol), reacted 45 minutes, and

chromatographed as previously described. The resulting viscous oil was co-evaporated with anhydrous toluene to remove the residual pyridine. The final foam product was desiccated overnight. The recovered material again exceeded theoretical.

5'-O-dimethoxytrityl-2-(^{14}C)-Thymidine 3'-(2-cyanoethyl-N,N-diisopropyl-amino phosphoramidite: The 5'-O-dimethoxytrityl-2-(^{14}C)-thymidine was converted to the phosphoramidite as previously described with one modification. The reaction was performed in anhydrous acetonitrile (100 mL) instead of CH_2Cl_2. A 1.6 molar excess of 2-Cyanoethyl-*bis*-(N,N-diisopropyl)-phosphorodiamidite (3.38 g., 11.25 mmol) was used, 0.5 equivalents diisopropyl amine tetrazolide salt (593 mg, 3.45 mmol), and the reaction stirred overnight at room temperature. After chromatography the isolated product was dried to a foam *in vacuo* (3.54 grams, ~4.75 mmol, 69% overall yield). The phosphoramidite product was confirmed by TLC and ^{31}P NMR.

Oligonucleotide Synthesis: DNA was manually synthesized (53 μmole scale) as previously described. The 5'-O-dimethoxytrityl protected crude product was cleaved from the CPG support in concentrated NH_4OH (3 X 10 mL). After deprotection and NH_4OH removal, the crude product was resuspended in sterile water with a yield of ~8000 AU_{260}. 20% denaturing polyacrylamide gel electrophoresis analysis of the crude product indicated ~73% trityl-on full length product.

HPLC Purification: The ~8000 AU_{260} crude material was split into 4 X ~2000 AU_{260} samples and each sample purified by reverse phase HPLC as previously described. The solvent flow rate was 4 mL/min. HPLC fractions that correlated in the four separate purification's (as determined by polyacrylamide gel analysis and gradient elution time) were pooled to give 22 separate fractions. The product yield in the major UV absorbing peak was 42% of the starting AU_{260} material, 76% of the total counts, with an estimated yield of ~20.4 μmoles (by UV absorbance).

Final Purification and Analysis: Detritylation and ethanol precipitation were as previously described. The final precipitated products were rinsed with 70% ethanol and resuspended in sterile water. Fractions were analyzed for full length integrity by 20% denaturing polyacrylamide gel electrophoresis and scanning laser densitometry and pooled to give three final fractions varying in their full length integrity. Fractions 1 and 2 (4.7 and 12.7 μmoles, respectively) had an average full length and radiochemical purity of 87-88%, as determined by laser scanning densitometry and ^{14}C

phosphorimaging. Fraction 3 (1.1μmoles) was ~84% full length and radiochemical purity. The yield of final product was 18.5 μmoles (35% yield of the 53 μmole DNA synthesis scale) with a specific activity of ~90 μCi/μmole. The phosphorothioate content was determined to be 97.6% by ^{31}P NMR.

Fraction	% full length	μmoles recovered	total mCi	specific activity
1	88 %	4.7	0.436	92 μCi/μmol
2	87	12.7	1.144	90
3	84	1.1	0.100	91

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