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SYNTHESIS OF ^{14}C-RADIOLABELED OLIGONUCLEOTIDES WITH A NOVEL PHOSPHORAMIDITE REAGENT

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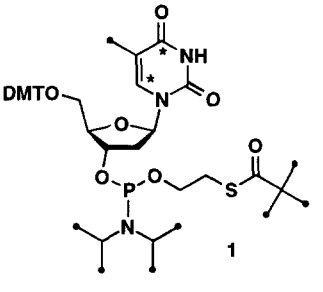
Abstract: A novel solid-phase synthesis of 5'-radiolabeled oligonucleotides is described. The labeling reaction is carried out by the phosphoramidite method with the aid of [4,6-di-^{14}C]-5'-dimethoxytritylthymidine building block **1**. The feasibility of the method is demonstrated by preparation of 3'-phosphorylated dodecathymidylate phosphorothioate containing radiolabeled nucleoside at the 5'-terminus.

Labeling with radioactive isotopes provides an efficient tool for studying pharmacological properties of antisense oligonucleotides. As with other classes of drug compounds, this novel class of therapeutics[1] requires high sensitivity radiodetection for evaluation of distribution of antisense agents in tissues and assessment of their metabolic fate.[2] Several methods to introduce ^{35}S at the internucleosidic thiophosphate[3] or ^{3}H or ^{14}C at the base moiety of synthetic oligonucleotides[4,5] have been reported. Among these labels, ^{14}C offers the highest specific activity and the longest half life. Considering catabolism of nucleic acids,[6] labeling with ^{14}C at the C-2 position of thymidine[4,5] results in formation of $^{14}CO_2$ which is cumbersome to trap and analyze. On the other hand, labeling at either the C-4 or C-6 position leads to β-aminoisobutyric acid as the metabolite which is much more convenient to analyze.

We report in this communication a novel method for ^{14}C labeling of synthetic oligonucleotides. The incorporation of radioisotope is achieved with the aid of phosphoramidite reagent **1** that is derived from [4,6-di-^{14}C]thymidine and thus possesses high specific activity. Phosphoramidite **1** features the *S*-pivaloyl 2-mercaptoethyl group which recently has been reported as an enzyme-labile phosphate protecting group.[7] As the first step in our study of bioreversible protection of the internucleosidic (thio)phosphate moiety we applied **1** to the preparation of phosphorothioate oligonucleotides, taking advantage of its lability in basic conditions.



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Synthesis of phosphoramidite reagent **1** is presented in Scheme 1. First, *bis*(diisopropylamino)chlorophosphine, **2**, was reacted with *S*-pivaloyl-2-mercaptoethanol, **3**, to give bisamidite **4**. Phosphorochloridite **2** (1.0 mmol in 2.5 mL of CH_2Cl_2) was added to an anhydrous solution of **3** (1.0 mmol) and ethyl-*N*,*N*-diisopropylamine (1.1 mmol) in CH_2Cl_2 (1.0 mL) at –30 °C for 5 min. The progress of the reaction was monitored by ^{31}P NMR spectroscopy which, after 30 min at room temperature, showed complete

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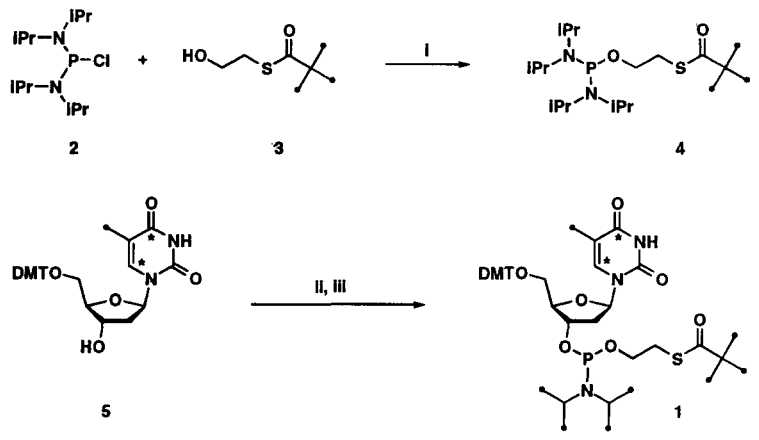


Scheme 1 i: $EtN(iPr)_2/CH_2Cl_2$; ii: 1*H*-tetrazole/MeCN/CH_2Cl_2; iii: aq $NaHCO_3$.

disappearance of **2** (141.3 ppm) and formation of bisamidite **4** (125.3 ppm; 70–75% yield based on integral intensity) along with several other products. No substantial decomposition of **4** was observed after 3 h. However, attempts to isolate the product by brief aqueous workup led to more complicated mixtures. Therefore, bisamidite **4** was used in the next step as the crude reaction mixture.

It was found in preliminary experiments that 50% excess of **4** is sufficient for complete conversion of unlabeled 5'-*O*-dimethoxytritylthymidine (DMT-T) when 10 to 50 μmol of the nucleoside is used. Thus, to obtain radiolabeled phosphoramidite **1**, [4,6-di-^{14}C]-DMT-T (**5**; 40 μmol; specific activity 25 Ci mol^{-1}) was treated with crude **4** (60 μmol) in the presence of 1*H*-tetrazole (32 μmol) for 40 min at room temperature followed by quenching of the reaction with aqueous $NaHCO_3$ and extraction of **1** with benzene.

To achieve the proper purification of **1**, hydrolytic stability of its unlabeled analog was determined. Half-life of nonradioactive **1** (**1a**) in 80% aq MeCN was found to be 58 h, which permits safe isolation of **1** in aqueous media. Accordingly, crude **1** was purified by RP HPLC (Figure 1).[8] Collected fractions were diluted with water, extracted with benzene, dried over Na_2SO_4 and evaporated to give **1** (31.5 μmol, 78.8% yield).[9]

Oligonucleotide **6** was assembled on an ABI 380B DNA Synthesizer using commercial 3'-Phosphate CPG (Glen Research; 20 μmol), phosphoramidite chemistry, and 3*H*-1,2-benzodithiol-3-one 1,1-dioxide[10] as the sulfur-transfer reagent. To prove homogeneity of **6**, a small sample of the solid support (*ca.* 0.1 μmol) was deprotected with ammonia, and the content of full length material was determined to exceed 90% by CE, ion-exchange HPLC and ES MS.

Incorporation of radiolabeled phosphoramidite into synthetic oligonucleotides differs from that of standard phosphoramidites. In the latter case a coupling yield of 99+% is mandatory. In contrast, the former requires incorporation of the expensive radiolabeled phosphoramidite to the highest degree whereas the coupling yield *i.e.*, the yield of actual chain elongation reaction of the support-bound oligonucleotide is not as important. Accordingly, conditions for coupling of **1a** to CPG-bound $(Tp)_{11}$ phosphorothioate oligonucleotide, **6**, were optimized.

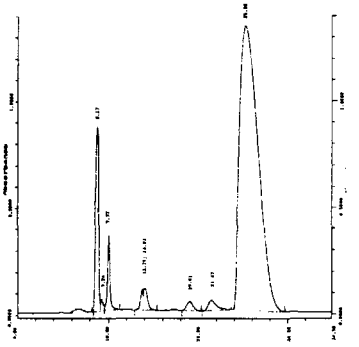
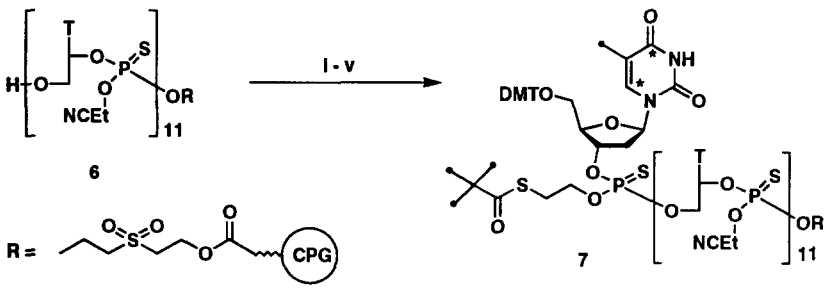


Figure 1. RP HPLC profile of crude **1**.[8]



Scheme 2 i: **1a** (1 equiv.)/1*H*-tetrazole; **ii:** **1** (2 eqiv.)/1*H*-tetrazole; **iii:** sulfurization;[10] **iv:** **1a** (5 eqiv.)/1*H*-tetrazole; **v:** sulfurization.[10]

In optimal condition, coupling procedure with **1** consisted of 3 main steps (Scheme 2). First, **6** (10 μmol) was dried by treatment with a mixture of **1a** (10 μmol) and 1*H*-tetrazole (0.45 M; 700 μL) for 1 min. This step was designed to result in a very low coupling yield (5 to 10%) due to the low concentration of the phosphoramidite. However, it efficiently consumes any traces of water and other nucleophiles left on the solid support that were not removed by washing with acetonitrile and extended drying *in vacuo*. After removal of solution by filtration, the solid support was ready for immediate labeling with radioactive phosphoramidite **1**. A solution of **1** (0.2 M in MeCN; 20 μmol) was delivered to the solid support followed by 1*H*-tetrazole solution (0.45 M in MeCN; 250 μL), and the reaction mixture was gently shaken for 20 min. The step was completed by washing the support with MeCN and performing the sulfurization reaction. As determined by DMT assay, coupling efficiency of **1** was 83% (41% yield with respect to **1**).

Finally, the solid support was coupled with **1a** (50 μmol) and excess 1*H*-tetrazole in order to obtain uniform full-length support-bound oligonucleotide **7**. After sulfurization and extensive washing with MeCN, the product was treated with concentrated ammonia for 2 h at room temperature. Under these conditions, oligonucleotide was released from the solid support, and cyanoethyl and S-pivaloyl 2-mercaptoethyl protecting groups were simultaneously removed. Finally, the mixture was heated for 2 days at 60 °C which completes the release of the 3'-terminal thiophosphate monoester. Crude **8** was purified by RP HPLC (Figure 2). Two major peaks on Figure 2 represent *S*- and *R*-P diastereomers at the 5'-terminal nucleoside-3'-phosphorothioate residue. 5'-DMT protection was cleaved with 80% aq AcOH for 30 min. The product was converted to its sodium ionic form and desalted by RP HPLC as reported previously to give **9** in 60% yield (specific activity 0.1 μCi AU^{-1}).[11] The labeled oligonucleotide **9** was analyzed by MALDI-TOF mass spectrometry and anion exchange HPLC monitoring both the UV-absorbance and radioactivity (Figure 3).[12] Both UV- and radioactive

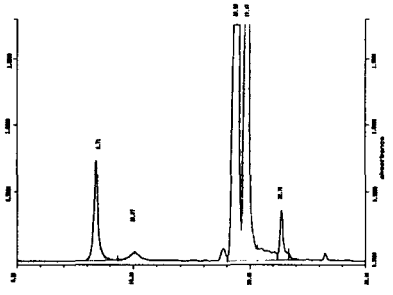
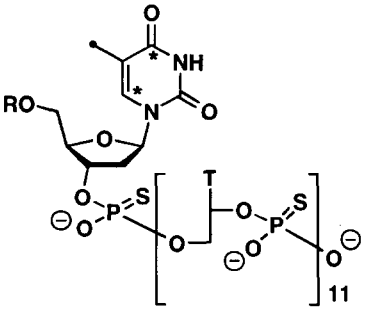


Figure 2. RP HPLC profile of crude **8**.[8]



8: R = DMT; 9: R = H

fractions were found to coelute with authentic sample of unlabeled $(Tp)_{12}$ phosphorothioate synthesized by the routine method. Moreover, HPLC profiles of **9** showed no unbound radioactive label.

In summary, the present method allows for efficient incorporation of ^{14}C radioisotope into synthetic oligonucleotides using a novel phosphoramidite. The results of alternative use of phosphoramidite **1** in the synthesis of chemically modified antisense oligonucleotides that possess a neutral backbone will be reported in due course.

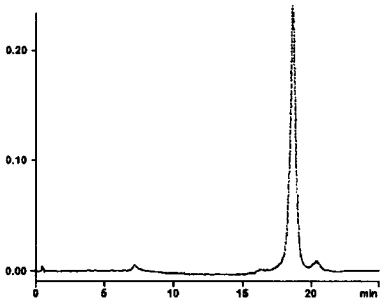


Figure 3. Ion Exchange HPLC of purified **9**.[12]

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8. Analysis and isolation of **1** was performed on a Delta Pak 15 μm C18 300Å column (7.8 × 300 mm), using isocratic elution with 50% aq MeCN for 10 min, then 75% aq MeCN for 25 min at a flow rate 5 mL/min. Purification of **8** was carried out on the same column using 0.1 M NH_4OAc as buffer A, 0.05 M NH_4OAc in 75% aq MeCN as buffer B, and a linear gradient from 15 to 80% B in 30 min at a flow rate 5 mL min^{-1}. Desalting was performed by injecting on to the same column, then washing with 0.1 M NaOAc (10 min), water (10 min) and eluting **9** as a sodium salt with 50% aq MeCN (20 min) at a flow rate 5 mL min^{-1}.
9. Compound **1**: ^{31}P NMR (CD_3CN): δ 148.69, 149.11; UV (80% aq MeCN): λ_{max} = 266 nm, ε = 17300 L mol^{-1}; specific activity 21 Ci mol^{-1}.
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12. MALDI/TOF MS: 3861.19 (found); 3861.14 (calcd.). Analysis of **9** by anion-exchange HPLC was done on a Resource™ Q - 1 mL column (Pharmacia) using 0.02 M aqueous Tris as buffer A, 5M NaBr as buffer B, and a linear gradient from 0 to 30% B in 25 min.