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A Simplified Synthesis of Acridine and/or Lipid Containing Oligodeoxynucleotides

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Abstract: A simplified method has been developed for the synthesis of acridine and/or lipid containing oligodeoxynucleotides using a commercially available resin and reagents.

INTRODUCTION

Underivatized oligodeoxynucleotides (ODNs) have been shown to selectively inhibit the expression of mRNA both in whole cells and in cell free assays.[1] However, the rapid degradation of unmodified ODNs by nucleases frequently limits their effectiveness for *in vivo* use. To decrease ODN degradation by nucleases, several groups have synthesized ODNs with modified backbones (e.g., phosphorothioate ODNs and methylphosphonate ODNs). The phosphorothioate ODNs have been shown to bind to mRNA and arrest translation.[2] Yet, this laboratory[3] and other investigators[4,5] have found that phosphorothioate ODNs are relatively non-specific inhibitors of mRNA translation. Methylphosphonate ODNs display properties of facile cellular uptake and nuclease resistance.[6] Nevertheless, methylphosphonate ODNs have been shown to be inferior in their ability to inhibit mRNA translation in cell free assays using rabbit reticulocyte lysates.[7] ODNs have been tethered to both lipids and intercalators to facilitate increased cellular uptake and stability of the heteroduplex, respectively.[8,9] ODNs with a tethered 3'-acridine[10] or 3'-cholesterol[11] are resistant to degradation by exonucleases.

In order to study methotrexate resistance in whole cells, antisense ODNs complementary to the start codon of human dihydrofolate reductase mRNA were designed (Figure 1, **225** and **226**). Based upon previous work and the expected criteria for antisense ODNs as effective agents *in vivo*, the desired ODNs were substituted with thiourea-acridine and/or lipids as shown in Figure 1. Previous syntheses of ODNs substituted with acridine or cholesterol usually necessitated the preparation of specialized controlled pore glass solid support (CPG) resins, or the synthesis of multiple cholesterol intermediates before functionalization of the ODN.[8, 11-13] We now report a versatile and simplified method for the synthesis of substituted ODNs utilizing a commercially available CPG resin.

RESULTS AND DISCUSSION

3'-Cholesterol ODNs. The synthesis of 3'-cholesterol substituted ODNs is shown in Scheme 1. The synthesis columns were prepared in the usual manner using 3'-amino-modified C7 CPG resin, **1**, obtained from Glen Research (Sterling, VA). Initially, the 9-fluorenylmethyl carbamate (Fmoc) protecting the 3'-amino moiety was cleaved by treatment with 0.1 M 1,8-diazabicyclo[5.4.0.] undec-7-ene (DBU) in acetonitrile (CH_3CN) over a three minute period to give, **2**. The column and resin were then washed with CH_3CN and briefly dried under vacuum. The resin was then flushed back and forth with a 50 to 100 fold excess of cholesterol chloroformate in dry dimethylformamide (DMF) and pyridine (9/1 by volume) and then allowed to stand for 4 hrs at room temperature to yield the derivatized resin, **3**. The column and resin were then used on a DNA synthesizer (ABI 381A) using standard phosphoramidites and the manufacturer's recommended coupling cycles. Once the synthesis was complete, the ODNs were cleaved from the column support with concentrated

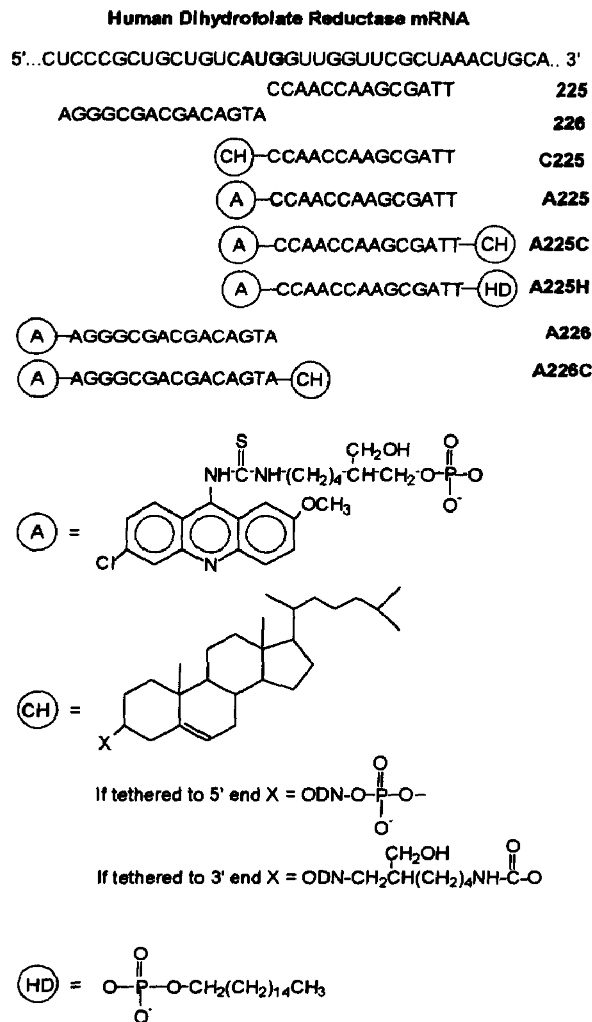
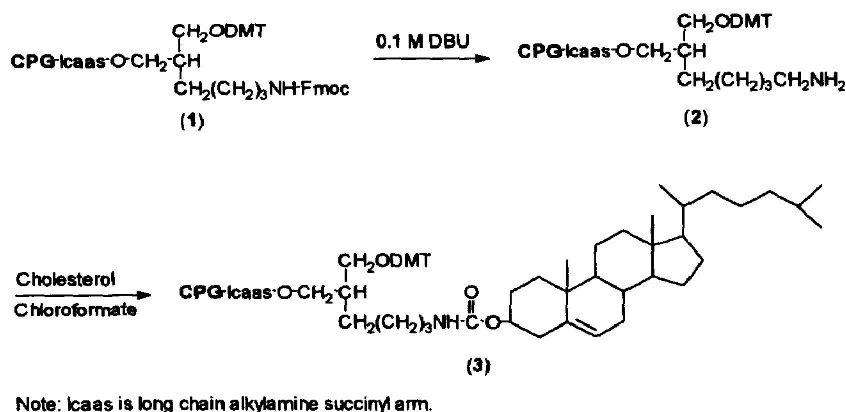


Figure 1. Overview of Acridine and/or Lipid Containing Oligodeoxynucleotides

aqueous ammonium hydroxide (NH_4OH) and deprotected with heating at 55 °C for 4 to 8 hrs. Analog **C225** and its corresponding shuffled derivative (CACCAACGAGCTAT) are representative of ODNs synthesized according to this procedure. Typical yields for a 1 μmole scale synthesis of HPLC-purified[14] ODNs bearing the carbamate cholesterol moiety ranged from 26-30%. The monomer coupling efficiency was comparable to unsubstituted CPG resin, with a small loss in yield due to hydrolysis of the cholesterol carbamate moiety upon deprotection with NH_4OH.

3'-Acridine-5'-Cholesterol Containing ODNs. The synthesis of 3'-acridine-5'-cholesterol or hexadecanol ODNs is shown in Scheme 2. The C7 CPG resin was used on the DNA synthesizer without prior modification and the ODNs were synthesized using standard phosphoramidites and coupling times. A 5'-end cholesterol or hexadecanol moiety was introduced by first converting cholesterol or hexadecanol to the corresponding phosphoramidite with 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite and the diisopropylammonium

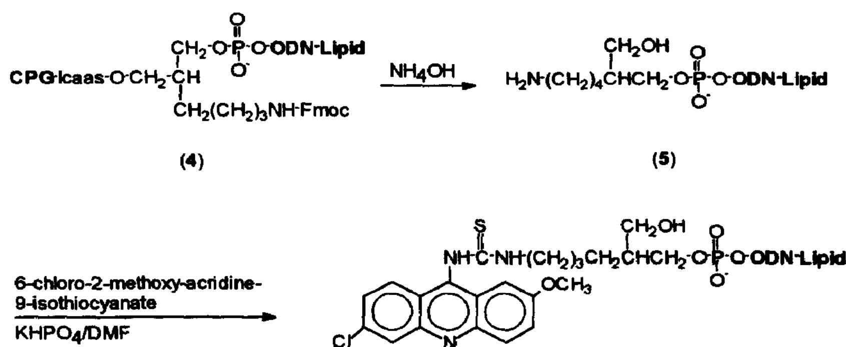
salt of tetrazole.[15] Without further purification, these analogs were taken up in an appropriate amount of CH_3CN, DMF and CH_2Cl_2 (1:1:1) to give a 0.1 M solution, and then used directly on the synthesizer to introduce the lipid as the last 5'-monomer, **4**. The phosphoramidites of cholesterol and hexadecanol were delivered via two extra coupling cycles on 0.2 μmole scale syntheses and three additional cycles for 1 μmole



Note: lcaas is long chain alkylamine succinyl arm.

Scheme 1. Synthesis of 3'-Cholesterol Oligodeoxynucleotides

scale syntheses with an additional wait step (15 sec) after each addition. The ODNs were then cleaved from the resin and deprotected in the usual manner with NH_4OH. Precipitation of the ODNs with NH_4OH and n-butanol (1:10 v/v) gave the semi-purified analog, **5**.[16] The crude ODNs were then dissolved in an equal volumes of DMF and 0.1 M $KHPO_4$ (pH 8.0) and stirred with a 20 fold excess of 6-chloro-2-methoxy-acridine-9-isothiocyanate[17] for 72 hrs. The reaction mixture was applied to a Sephadex G-25 column and eluted with 0.1 M triethylamine acetate (TEAA) to remove unreacted acridine-isothiocyanate. The appropriate fractions containing product were pooled, as determined by thin layer chromatography,[18] concentrated and purified by HPLC.[14] ODNs **A225C**, **A225H**, **A226C** and their corresponding shuffled derivatives were synthesized on a 1 μmole scale in this manner with yields ranging from 20-35%.



Note: lcaas is long chain alkylamine succinyl arm.

Scheme 2. Synthesis of 5'-Lipid-3'-Acridine Oligodeoxynucleotides

This method is also amenable for the synthesis of ODNs only substituted with a 3'-thiourea-acridine. The ODNs were synthesized in the usual manner, without a 5'-lipid, partially purified with NH_4OH/n-butanol precipitation and coupled to acridine thioisocyanate as previously described. ODNs **225A** and **226A** were synthesized by this method with an average yield of 17%. The poor solubility of acridine isothiocyanate in aqueous solutions contributes to the diminished yields.

CONCLUSION

The methods presented here allow for the simple synthesis of di- or mono-substituted ODNs via commercially available CPG resin. This methodology can be applied to the synthesis of either 3'-acridine or 3'-cholesterol mono-substituted ODNs and 3'-acridine-5'-lipid di-substituted ODNs with minimal synthetic manipulations, commercially available intermediates, and reproducible yields. This should further facilitate the development of ODNs functionalized to confer the pharmacological properties deemed necessary for whole cell and *in vivo* use.

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