Antisense pro-drugs: 5'-ester oligodeoxynucleotides

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Received April 11, 1994; Revised and Accepted June 22, 1994

ABSTRACT

Oligonucleotides bearing a terminal lipophilic group attached through a biodegradable ester bond should be useful as antisense pro-drugs with improved cellular uptake. The synthesis of 5'-ester oligonucleotides is, however, problematic due to lability of the ester bond during aqueous ammonia treatment that is commonly used for the deprotection of synthetic oligonucleotides. The synthesis of 5'-palmitoyl oligodeoxynucleotides was accomplished in good yield by the use of a combination of base-labile tert-butylphenoxyacetyl amino protecting groups (t-BPA), the oxalyl-CPG anchor group, and ethanolamine (EA) as a deprotecting reagent.

INTRODUCTION

The inhibition of gene expression utilizing synthetic oligonucleotides and their analogs has attracted great interest in recent years [1]. The promise of antisense therapeutics have been clearly demonstrated [2,3] and several clinical trials are underway [4,5].

There is, however, a major limitation in the application of oligonucleotides as potential universal drugs, that is the low ability of these polyanionic molecules to traverse cell membranes [6]. Because of this limitation hydrophobic nonionic backbone-modified analogs, such as methylphosphonates, were developed, in order to penetrate membrane bilayers due to their lipophilicity [7]. Nevertheless, the problem of cellular uptake does exist in the case of such polyanionic analogs as phosphorothioates and phosphorodithioates, as well as their co-polymers.

It has been shown that cellular uptake of native oligonucleotides and polyanionic analogs may be improved by the attachment to these molecules of terminal lipophilic groups, such as cholesterol, that are known to interact specifically with cell membranes [8—16]. But this approach may have a substantial drawback, in that the lipophilic groups of these conjugated oligomers may remain attached to cell membranes, and this anchoring may make the antisense molecules unavailable for binding to their mRNA targets.

To address this potential problem MacKellar et al. [8] proposed the attachment of a lipophilic moiety through a tetramethylguanidine bridge with normal phosphodieste bonds which are degradable by nucleases in cells and serum. Similarly, Oberhauser and Wagner used a biodegradable disulfide bond to attach a cholesterol moiety on 2'-O-methyl-oligoribonucleotides [14].

Another attractive possibility to realization of the so-called 'pro-drug' approach for antisense oligonucleotide analogs would be to attach the lipophilic moiety through an ester bond, that is known to be easily hydrolyzed by intracellular esterases [17]. This is not, however, a trivial synthetic task, as the ester bond is very sensitive to ammonia treatment that is usually used for oligonucleotide deprotection. An alternative is to derivatize an unprotected oligonucleotide with the terminal phosphonomoester group using the carbodiimide method [18]. But this route is definitely impractical from the synthetic viewpoint, since it requires the synthesis of an appropriate precursor containing an ester bond and loses the advantage of direct solid-phase derivatization. We here report a simple approach for the synthesis of oligodeoxynucleotides with a terminal lipophilic (palmitoyl) group attached through a biodegradable ester bond. This novel method employs ethanolamine for the selective deprotection of 5'-ester oligonucleotides with labile amino protecting groups.

RESULTS AND DISCUSSION

Acylation of 5'-OH

It is not useful to attempt 5'-esterification with a strong reagent such as acyl chloride, even in the case of protected oligonucleotides, due to possible base modifications. The use of acid anhydride is more appropriate, since 5'-acetylation with acetic anhydride is routinely used during oligonucleotide synthesis to cap unreacted 5'-OH groups. Nevertheless, the problem of cellular uptake does exist in the case of such polyanionic analogs as phosphorothioates and phosphorodithioates, as well as their co-polymers.

It has been shown that cellular uptake of native oligonucleotides and polyanionic analogs may be improved by the attachment to these molecules of terminal lipophilic groups, such as cholesterol, that are known to interact specifically with cell membranes [8—16]. But this approach may have a substantial drawback, in that the lipophilic groups of these conjugated oligomers may remain attached to cell membranes, and this anchoring may make the antisense molecules unavailable for binding to their mRNA targets.

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Synthesis of 5'-ester oligonucleotides

As a model compound, decathymidine (5'-(-Tp)9T-3') was synthesized by the β-cyanoethyl phosphoramidite method (4×1 μmol) on oxalyl-CPG (19) and, after removing the terminal DMT group, was 5'-esterized with palmitoyl tetrazole. But, in this case, apart from a great excess of acylating reagent, the reaction time was increased to 30 min to ensure complete derivatization. Efficiency of derivatization was checked by PAGE. A small portion of protected oligomer was treated with aqueous ammonia for 6 min at room temperature. This short treatment is enough to cleave the oxalyl anchor [19] and to almost completely remove β-cyanoethyl phosphate protecting groups, but is not enough to extensively hydrolyze the ester bond. As can be seen from Figure 1, lane 2, three distinct bands were observed on the electrophoresis gel. As we expected the main product migrates slower than authentic 5'-(-Tp)9T-3' (Fig. 1, compare lanes 1 and 2) that indicates the presence of the palmitoyl group. The upper minor band probably corresponds to the palmitoylated 10-mer with one unremoved β-cyanoethyl group that reduces the overall negative charge by one, accordingly decreasing mobility. In support of this assumption the upper band completely disappeared after prolonged ammonia treatment. The relatively small amount of underivatized 5'-(-Tp)9T-3' (lower minor band) indicates the high efficiency of derivatization.

Figure 1. PAGE analysis of crude 5'-palm-(Tp)9T-3' prepared on oxalyl-CPG and deprotected with (2) NH₃ for 6 min; (3) NH₃ for 2 h; (4) EA for 10 min; (5) the mixture N₂H₄/EA/MeOH (1:1:5, v/v/v) for 3 min. (1) is an authentic 5'-(-Tp)9T-3'; (6) represents HPLC-purified 5'-palm-(Tp)9T-3'.

Figure 2. HPLC analysis of crude 5'-palm-(Tp)9T-3' prepared on oxalyl-CPG and deprotected with (2) NH₃ for 2 h; (3) EA for 10 min; (4) the mixture N₂H₄/EA/MeOH (1:1:5, v/v/v) for 3 min. (1) is an authentic 5'-(-Tp)9T-3'; (5) represents HPLC-purified 5'-palm-(Tp)9T-3'.
Due to the great susceptibility of the ester bond toward base, incorporation of such a bond into a synthetic oligonucleotide with the classical amino protecting groups (benzoyl for A and C, and isobutyryl for G) is impossible, as they are routinely removed in strongly basic conditions (aqueous ammonia, overnight heating). However, protection of nucleoside heterocycles may be successfully accomplished by utilizing so-called base-labile amino protecting groups, such as phenoxyacetyl [20], isopropoxyacetyl (IPA) [21] and tert-butyl-phenoxyacetyl (t-BPA) [22]. For example, aqueous ammonia completely removes the t-BPA group from the least reactive guanine amino function in only 2 h at room temperature [22]. But these conditions are still too harsh for the ester bond. Therefore, to incorporate an ester bond in an oligonucleotide conjugates, another protecting or deprotecting strategy is necessary.

Recently we proposed two new techniques for accelerated oligo-nucleotide deprotection, based on the use of ethanolamine (EA) and a mixture of EA, hydrazine and methanol [23–26]. EA completely removes IPA and t-BPA amino protecting groups in 10 min at room temperature [25,26]. On the other hand, it takes almost 3 h to completely cleave the standard succinic ester anchor with this reagent (data not shown). This observation, encouraged us to investigate the use of EA deprotection for preparing 5′-esterified oligonucleotides.

We used a base-sensitive oxalyl anchor that is completely cleaved within 2 min by both EA and the mixture \( \text{N}_2\text{H}_4/\text{EA}/\text{MeOH} \) [25]. Initial experiments were performed with decathymidine. For the synthesis of 5′-esterized oligomers, incorporating all nucleosides, the t-BPA amino protecting group was chosen (appropriate monomers are commercially available from Millipore). Three small portions of CPG with immobilized protected 5′-palmitoyl decathymidine prepared as described above were treated at room temperature with: 1) aqueous ammonia for 2 h; 2) ethanolamine for 10 min; and 3) the mixture \( \text{N}_2\text{H}_4/\text{EA}/\text{MeOH} \) (1:1:5, v/v/v) for 3 min. In every case the selected time is the minimum necessary for the full deprotection of oligonucleotides synthesized with the use of t-BPA amino protected groups, β-cyanoethyl phosphate protecting group, and an oxalyl anchor [25,26].

To prevent prolonged exposure of the ester bond towards reactive EA and \( \text{N}_2\text{H}_4 \), they were immediately neutralized with acetic acid after deprotection. The reaction mixtures were desalted on Sephadex G25 and analyzed by PAGE and reverse phase HPLC (Figs. 1 and 2). As expected, the ester bond was almost completely hydrolyzed during ammonia treatment and, according to HPLC data, only 15% of the 5′-palmitoylated decathymidine was obtained (Fig. 1, lane 3; Fig. 2, profile 2). In contrast, EA deprotection gave the desirable conjugate with 68% yield (Fig. 1, lane 4; Fig. 2, profile 3). The mixture \( \text{N}_2\text{H}_4/\text{EA}/\text{MeOH} \) was more selective than aqueous ammonia but considerably less selective than EA (34% of derivatized oligomer, Fig. 1, lane 5 and Fig. 2, profile 4). EA deprotection was further used for the large-scale preparation of 5′-palmitoyl decathymidine. The conjugate was purified by preparative reverse phase HPLC with

![Figure 3. 1H-NMR spectrum of HPLC-purified 5′-palm-(Tp)_9T-3′. The ratio between the integrals of thymidine 1′-protons (m, 10H, 6.18–6.36 ppm) and CH₃ protons of the palmitoyl residue (t, 3H, 0.71–0.81 ppm) equals 3.1 that is in good agreement with theoretical value 3.3. Signal A (s, 4.75–4.90 ppm) corresponds to HDO. Signals B (q, 3.00–3.30 ppm, (−CH₂−)₉) and C (t, 1.15–1.40 ppm, (CH₃−)₃) corresponds to triethylammonium cation. Insert shows 31P-NMR spectrum of the same conjugate. The only signal at ~0.07 ppm corresponds to phosphodiester groups.](image-url)
50% isolated yield. The integrity of the product was checked by PAGE and reverse phase HPLC (Fig. 1, lane 6; Fig. 2, profile 5) and its structure was confirmed by $^1$H- and $^{31}$P-NMR (Fig. 3).

The above strategy was also successfully applied for the preparation of 5'-palmitoylated 17-mer (Palm-dCACCAACTTCTTCCACA) antisense to the coding region of the β-globin gene [27] and some other 5'-derivatized oligonucleotides containing G. The isolated yields were 40–50%. It is important to point out that phosphorothioate conjugates may also be prepared since it was shown that EA does not affect PS bonds [26].

CONCLUSION

We found that the combination of labile N-protecting groups and the easily cleavable oxalyl anchor, along with application of ethanolamine as a deprotecting agent, allows efficient incorporation of the ester bond into oligonucleotide conjugates. This should have the advantage of a pro-drug, releasing the intact antisense oligomer as a result of intracellular esterase activity after it permeates the cell membrane.

MATERIALS AND METHODS

The following reagents were purchased from commercial sources: t-BPA protected nucleoside N,N-diisopropyl-2-cyanoethylphosphoroamidites (Millipore); palmitoyl chloride; 1H-tetrazole; N,N-diisopropylphosphoramidite; 3'-O-acetylimidaine; ethanolamine; hydrazine (Aldrich), acetonitrile; dichloromethane; chloroform; phosphoramidites (Millipore); palmitoyl chloride; lH-tetrazole; N,N-diisopropyl-2-cyanoethylphosphoroamidites (Millipore) was used instead of acetic anhydride in the capping procedure. tert-Butylphenoxy-acetic anhydride was tried as an acylation agent.

Derivatization of 3'-O-acetylimidaine. 3'-O-Acetylimidaine (114 mg, 0.4 mmol) was co-evaporated with CH$_2$Cl$_2$ to remove traces of water. The acetylating mixture (10 ml, 2.0 mmol of palmitoyl tetrazole) and N,N-diisopropylethylamine (104 µl, 0.6 mmol) were added to the residue with stirring. The reaction was monitored by TLC. In 10 min the starting material (Rf = 0.33) was completely converted to the product (Rf = 0.57). The reaction mixture was diluted with CH$_2$Cl$_2$ and extracted with 10% aqueous NaHCO$_3$ (2×50 ml). The organic phase was dried over Na$_2$SO$_4$ and evaporated to dryness. The residue was chromatographed over silica gel using a gradient of 0–5% MeOH in CHCl$_3$ to yield 5'-O-Palmitoyl-3'-O-acetylimidaine as a white solid (194 mg, 0.37 mmol, 93%).

1H NMR (CD$_3$COCD$_3$) δ 7.31 (s, 1H, H6 (Thym)), 6.30–6.36 (m, 1H, H1'), 5.19–5.23 (m, 1H, H3'), 4.27–4.45 (m/d, 2H, CH$_2$:H2'), 2.42–2.52 (m, 1H, H2' (down)), 2.32–2.41 (t, 2H, CH$_2$:CO (Palm)), 2.09–2.19 (s+m, 4H, H2' (up), Ac), 1.93 (s, 3H, CH$_3$: (Thym)), 1.58–1.69 (m, 2H, CH$_2$:CH$_2$:CO (Palm)), 1.18–1.38 (m, 24H, (CH$_2$)$_{24}$. 0.84–0.93 (t, 3H, CH$_3$: (Palm)). FABMS m/z 521.4$^+$ (M + H$^+$), 255.3$^+$ (CH$_3$:CH$_2$)$_2$:CO$^+$. 

5'-Palmitoylation of oligonucleotides

The 5'-terminal DMT group was removed on the DNA synthesizer. The CPG support with immobilized oxalyl-bound protected oligonucleotide (1 µmol) was dried under vacuum and then treated with the acylating mixture (1 ml, 0.2 mmol of palmitoyl tetrazole, see above) and N,N-diisopropylethylamine (10 µl, 67 µmol) for 30 min at room temperature. The reaction vessel was shaken several times during this procedure. The liquid was filtered out, CPG was washed with CH$_3$CN and CH$_2$Cl$_2$ and dried under vacuum.

Deprotection of 5'-esterified oligonucleotides

With EA. 5'-Palmitoylated support-bound oligonucleotide (1–2 µmol) was treated with EA (200 µl) for 10 min at room temperature. The reaction mixture was diluted with EtOH (400 µl), cooled on dry ice and neutralized with acetic acid (190 µl). The pH was neutralized by adding 1M TEAB (1 ml) and the final solution was desalted on Sephadex G25 (Pharmacia NAP-25 column may also be used). All work up must be performed as quickly as possible to avoid considerable de-esterification. For 0.2 µmol synthesis the amount of reagents should be decreased times three.

With the mixture N$_2$H$_4$/EA/MeOH. Deprotection with the mixture N$_2$H$_4$/EA/MeOH (1:5:5, v/v/v) was performed as with EA, except the deprotection time was decreased to 3 min. With ammonia. The deprotection with aqueous ammonia (25%, w/w) was performed at room temperature. After a specific time the reaction mixture was diluted with water and the solution was directly desalted on Sephadex G25.
ACKNOWLEDGEMENTS

We thank Mridul Ghosh, Nasser Farschtschi, and Tilak Raj for helpful discussions in the early stages of this project. We are also very grateful to Alan Morocho for his technical assistance and to Pat Faustino for recording the NMR spectra. This work was supported by a research grant from the Cystic Fibrosis Foundation.

REFERENCES


