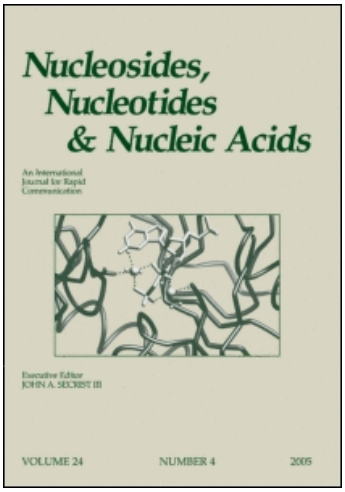

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Mori, Kenya , Subasinghe, Chris , Stein, C. A. and Cohen, Jack S.(1989) 'Synthesis and Properties of Novel 5'-Linked Oligos', Nucleosides, Nucleotides and Nucleic Acids, 8: 5, 649 — 657
To link to this Article: DOI: 10.1080/07328318908054205
URL: http://dx.doi.org/10.1080/07328318908054205

NUCLEOSIDES & NUCLEOTIDES, 8(5&6), 649-657 (1989)

SYNTHESIS AND PROPERTIES OF NOVEL 5'-LINKED OLIGOS

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Abstract: We have synthesized normal and phosphorothioate oligos with 5'-linked groups, using either a phosphoramidite with the linked group attached or a mercaptopropanol linker. These linked oligos have been studied for cellular uptake as fluorescent labels, and for inhibition of gene expression in a cell free expression system, and in several other biological systems.

INTRODUCTION

The ability to covalently attach a chemical group onto an oligodeoxynucleotide may provide compounds with interesting biological properties. These include the use of intercalation to improve the hybridization of an oligo[1-3], or a reactive group to cause selective DNA damage[4-6]. In order to improve the facility with which such compounds can be synthesized, we have investigated the synthesis of several 5'-linked oligos[7]. We chose the 5' rather than the 3' end of the oligo on which to link the group, because this is much easier in the automated synthesis, which proceeds from the 5' end with the 3' end attached to the solid support (usually controlled pore glass beads).

Asseline et al.[8], compared the results of thermal melting studies on duplexes with both 3' and 5' linked acridines, and found that the greatest increase in Tm was associated with the 3' linked acridine oligo. Consequently, in their subsequent studies they have consistently used 3'-linked oligos. However, for our applications,

where duplex stabilization was not the major criterion, but rather ease and scale of synthesis, we have focused on the preparation of 5'-linked oligos by the automated method via intermediate 5'-linked phosphoramidites.

With 6-chloro-2-methoxyacridine linked via the 9-amino position to a methylene chain[8], the conditions of demethylation surprisingly resulted in facile substitution by thiophenol at the 6-position[7]. Also, use of blocked A,G, and C monomers resulted in base-catalyzed cleavage of the linker at the 9-amino acridine position, so that only dT oligomers with 5'-linked 6-thiophenoxy-2-methoxyacridine could be prepared in good yield by this method. These substituted dT-oligos exhibited only small increases in Tm[7].

In order to overcome these problems in the present work we have used an alternative synthesis with a maleimide linked acridine,[9] which was attached to a mercaptopropanol linker.[10] In this way we were able to prepare 5'-linked acridine with any sequence oligo. We have also prepared a phosphoramidite with an unsubstituted acridine and from it prepared 5'-linked dT oligomers.

These compounds have been made both as normal phosphodiester oligos and as phosphorothioates (S-oligos), since these have been found to exhibit selective inhibition *in vitro* of HIV[11-13] and of mammalian oncogene expression.[14] The combination of the 5'-linked group with the S-oligos is a novel approach to attempt to increase the effectiveness (potency) of these potential drugs.

MATERIALS AND METHODS

Synthesis of Mercapto-Linker: Following the method of Connolly and Rider[10] S-trityl-3-mercaptoethanol (334 µg, 1 mmol) was dissolved in methylene chloride (2 ml), and N-ethyl-di-isopropylamine (760 µl, 4 mmol) was added followed by slow addition of methoxy-di-isopropychlorophosphite (180 µl, 1.2 mmol) at 0°C. The mixtures were left for a further 15 min at 0°, and then poured into ethylacetate (5 ml) containing triethylamine (0.5 ml), and then washed twice with 5% sodium bicarbonate (5 ml) and saturated sodium chloride (5 ml). The organic phase was dried over sodium

sulfate and evaporated to an oil. The ^{31}P NMR spectrum showed a single peak (δ=146.408 ppm) and tlc on silica gel gave a single spot (petroleum ether 9:triethylamine 1; Rf of starting material 0.3; Rf of product 0.45). The clear oil was dissolved in dry acetonitrile without further purification to make an approximately 100 mM solution. Yields of about 60% were typical.

Synthesis of 5'-Acridine-Maleimide Linked Oligo: Oligodeoxynucleotide synthesis was carried out using the solid-phase phosphoramidite method on an Applied Biosystems 380B synthesizer. The S-trityl group was removed as described by Connolly and Rider[10]. The mercapto-linked oligo (1 μmol scale synthesis) was in triethylammonium acetate buffer was adjusted to pH 8-8.5 with 5% $NaHCO_3$, and N-9-acridinyl maleimide (4 mg in 4 ml acetone) was added following the procedure of Nara and Tuzimura[9]. The mixture was kept at 4°C for 16 hr, and then extracted with ethylacetate (3x2 ml). The aqueous phase was concentrated by blowing nitrogen gas, and the products were purified by hplc. Yields of 35-40% were typical (from a 1 μmol scale synthesis).

Synthesis of 5'-Acridine-Maleimide Linked Phosphorothioate Oligo: The S-oligos were synthesized as described previously[16], and the 5' linkage of acridine-maleimide was carried out as described above except for the removal of S-trityl groups. The S-trityl containing S-oligo was treated with a 30-fold excess of 10 mM $AgNO_3$ in deionized water solution. After several hours a 35-fold excess of 10 mM dithiothreitol (DTT) was added, and the precipitated silver salt of DTT was removed. However, the yields in these cases were very low (ca. 3-7%), because of the general difficulty of removing the silver salt from the S-oligo.

Melting studies: Melting curves were carried out and fitted and melting temperatures (Tm) were obtained as described previously[15]. All values were comparative and determined at 260 nm under the same experimental conditions, including 140 mM NaCl in 20 mM cacodylate buffer.

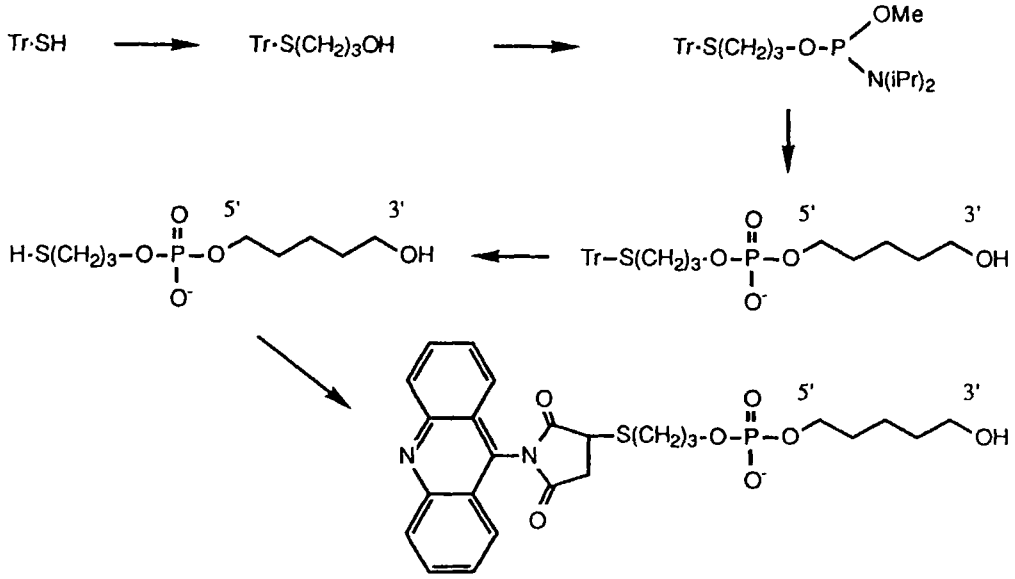
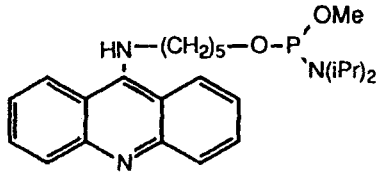


FIG. 1. Reaction scheme for the synthesis of an acridine linked through a mercapto-linker and a maleimide to an oligodeoxynucleotide (represented as a wavy line).

RESULTS

Synthesis: The synthesis of 5'-acridine normal and S-oligos via a 5'-linked phosphoramidite has been described elsewhere[7]. For this work we synthesized an unsubstituted acridine phosphoramidite and used it to make several 5'-acr-linked oligos by the same method.



The synthesis of the maleimide-linked acridine oligo is shown schematically in Figure 1. By this approach it was possible to link an acridine in high yield to an oligo of any sequence, as opposed to the

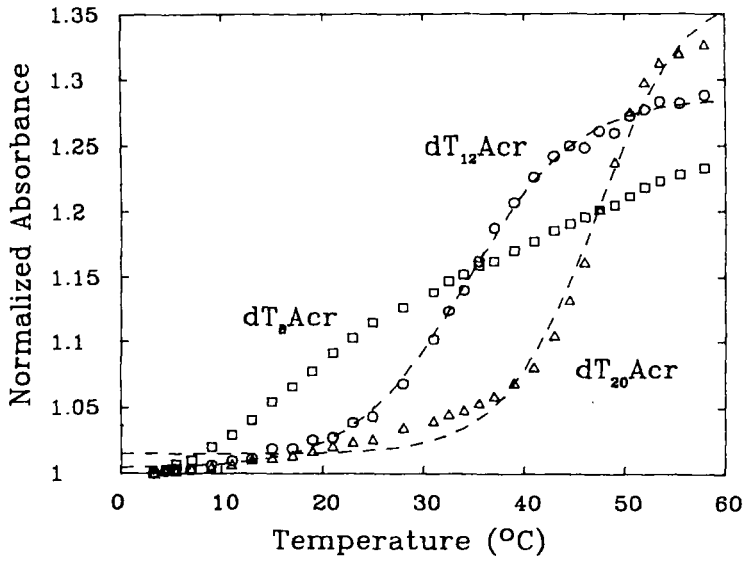


FIG. 2. Melting curves of Acr-dT_n (n=8,12,20) with poly-rA. Absorbance at 260 nm was normalized and dashed lines are fits to a sigmoidal curve. For details see ref. 16.

previous synthesis[7] in which the acridine was found to be cleaved on treatment with the ammonia reagent used to de-block the bases A,G, and C. The synthesis described here has also been accomplished with S-oligo using the stepwise method described previously,[15] although the yields were less than for the normal oligo due to the de-tritylation process requiring a silver salt.

Melting temperatures: The melting curves of the 5'-acridine-maleimide linked oligo dT's with poly-rA are shown in Figure 2. It was not possible to fit the data or obtain a meaningful Tm value for the Acr-dT_8, however the data for the Acr-dT_{12} and dT_{20} with poly-rA gave good fits. The results were: Acr-dT_{12}, Tm = 34.2°C, ΔH = 34.3 kcal/mole; Acr-dT_{20}, Tm = 47.0°C, ΔH = 44.9 kcal/mole. These values compare very well to the values of the controls without the linked groups attached, namely dT_{12} (32°, 42), dT_{20} (45°, 65). The absence of a large increase in Tm indicates that the acridine group in this case cannot effectively intercalate.

By contrast, the unsubstituted acridine with a five-methylene chain did show a significant increase in Tm, presumably due to intercalation. The results were (the numbers in parenthesis were determined for the visible band at 415 nm): Acr-dT_8, Tm = 32.3° (31.6°), ΔH = 63.2 kcal/mole (75.1); Acr-dT_{12}, Tm = 41.7°, ΔH = 60.5 kcal/mole.

Cell-free Expression: A wheat germ cell free expression system was used with globin mRNA, and will be described in detail elsewhere.[16] The globin antisense sequence:

5'-GGG AGA CAG CAC CAT-3'

was used, and the oligo with the 5'-linked maleimide acridine was compared. The cut-off of antisense effect was ca. 500 nM with the normal oligo and ca. 1μM with the acridine linked oligo, so that the acridine in this case made little difference to the antisense effect.

DISCUSSION

In principle there are three basic ways that one could synthesize an oligo with a covalently attached chemical group. These are shown schematically in Figure 3. The first approach is that used by most previous workers in which the oligo is synthesized in the automatic synthesizer and then purified, followed by attachment of the linker and/or the linked group. Asseline et al.[8] found that the 3'-linked acridine oligo hybridized best with its complement, and consequently preferred this linkage in their further studies. Since the oligo is attached to the solid support at the 3' end in the phosphoramidite synthesis, this mandated their synthetic approach (although it is possible to interpolate a linker between the oligo and the support).

We preferred to prepare 5'-linked oligos because of our need for large quantities of fluorescently labelled material that could be used to monitor uptake into cells by fluorescence activated cell sorting (FACS)[7,14] as well as to monitor the biological properties of these linked oligos as phosphorothioates. In order to prepare 5'-linked oligos by the automated method it was necessary to

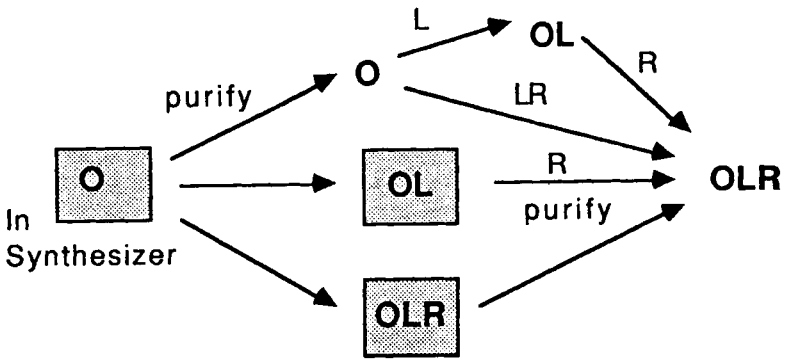


FIG. 3. Methods of synthesis of an oligo (O) linked (L) to a 5' group (R). *Upper*, the oligo is synthesized first, and then purified, and the group is added; or *Middle*, a 5'-linker is attached to the oligo and then the group is attached; or *Bottom*, a 5'-linked phosphoramidite is used to synthesize the 5'-linked oligo in the synthesizer.

synthesize the phosphoramidite of either the linker and/or the linked chemical group, such as acridine. These alternative approaches are also shown in Figure 3. Initially we synthesized the 6-chloro-2-methoxy acridine derivative via the acridine linked phosphoramidite in order to compare our results with those of Asseline et al.[8]. In principle this is the most efficient method, since most of the synthesis occurs in the automatic synthesizer. However, surprisingly in this case we found that the 6-chloro group was substituted under the conditions of the thiophenol treatment in the synthesizer. The instability of the acridine linkage to base treatment also reduced the range of compounds we could prepare by this method to oligo-dT's[7]. In order to overcome these difficulties we reverted to a synthesis as described here, in which the oligo was 5'-terminated by reaction with a mercapto-linked phosphoramidite[10], and following purification, the acridine group was attached via maleimide[9].

The Tm's determined for the maleimide linked 5'-Acr-dT_n's in this case (with n=12, 20) were very similar to those found previously for the controls with poly-rA[7]. This indicates that intercalation is not

occurring efficiently for the acridine in this case, and this may be attributed to the presence of the sterically hindering maleimide group, that reduces the flexibility of the linker and prevents intercalation. We observed a similar effect previously with the 6-thiophenoxy-acridine deriviative[7]. The use of an unsubstituted acridine with a flexible methylene linker to dT gave a comparable increase in Tm as expected from the previous work of Asseline et al.[8], and this confirms the steric hinderance from the thiophenoxy substituent and the maleimide linker.

The cell free expression inhibition observed with the 5'-Acr maleimide linked antisense oligo for (α,β)globin expression were very similar to those found previously for the unlinked oligo[16]. This indicates not surprisingly that the 5'-linked group plays little role in modulating the hybridization of an oligo of this length (16-mer), although this result is different from that observed in a T4 phage system[3]. On the other hand, to obtain gene inhibition one would prefer not to use a much shorter oligo due to loss of selectivity.

While this cell-free inhibition result was not surprising, it is a useful control in view of other results that we have obtained. We have found that the use of a 5'-Acr maleimide group linked to the antisense α-rev S-oligo 28-mer sequence,[13] gave increased inhibition of HIV in a T-cell assay (Mori et al., unpublished results). In view of the absence on any increased Tm with these linked compounds found here, this cannot be explained by any intercalative effect of the 5'-linked group. It is possible that the 5'-linked group increases cellular uptake of the oligo. However, such an effect may vary from cell to cell, and may be accentuated by the use of S-oligos, which we have found tend to enter cells much more slowly than the normal congeners.[7,17]

This effect of 5'-linked groups has been confirmed by comparing a 5'-anthroquinone linked α-rev S-oligo, (Mori et al., unpublished results) that gave even higher inhibitory effects than the 5'-Acr-maleimide group. We are attempting to discover the origin of this effect, which may also depend on the chemical reactivity of the 5'-linked group attached to the oligo. If this is the case then the combination of a 5-linked reactive group with an S-oligo may represent a new class of drugs[18] in which the nuclease

resistant S-oligo acts as a long-lived "magic bullet" containing the antisense base sequence for the mRNA "target", and the 5'-linked group is delivered to produce irreversible chemical damage to the target. We are attempting to improve this strategy by modulating the activity of the 5'-linked group to avoid unnecessary toxicity to the cell.

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