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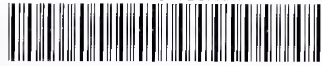
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ANTISENSE OLIGONUCLEOTIDES: PSORALEN PHOTOREACTIVITY AND ENZYMATIC RESISTANCE

Steven Jay Ugent

Yale University

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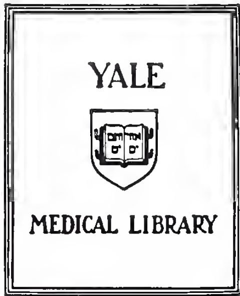
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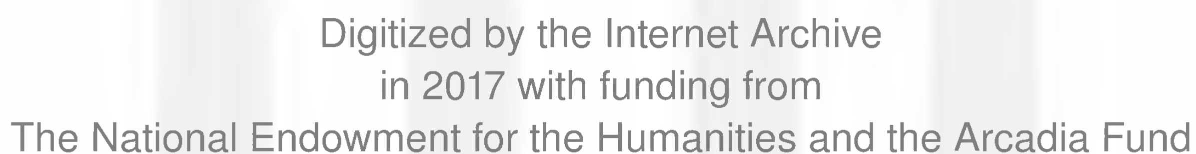
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ANTISENSE OLIGONUCLEOTIDES:

PSORALEN PHOTOREACTIVITY AND ENZYMATIC RESISTANCE

A Thesis Submitted to the Yale University School of Medicine

in Partial Fulfillment of the Requirements for the

Degree of Doctor of Medicine

by

Steven Jay Ugent

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Finally, I am grateful to my parents for their emotional and financial support of my medical education. I consider myself extremely fortunate to have parents and a sister who have encouraged me and have always been there to listen.

ABSTRACT

ANTISENSE OLIGONUCLEOTIDES: PSORALEN PHOTOREACTIVITY AND ENZYMATIC RESISTANCE. Steven J. Ugent and Francis P. Gasparro. Department of Dermatology, Yale University School of Medicine, New Haven, CT.

Psoralen-modified antisense oligodeoxyribonucleotides previously have been used to prolong sequence-specific inhibition of gene expression. The efficacy of oligonucleotides has been limited, in part, by their degradation by cellular nucleases. Modifications of the sugar-phosphodiester backbone with methylphosphonates in order to increase resistance to enzymatic degradation have produced several disadvantages. In order to determine whether limited substitutions of methylphosphonates confers ezymatic resistance, three different oligonucleotides each with selected bases having this modification were reacted with a 3'-exonuclease, a 5'-exonuclease, and a phosphohydrolase. A psoralen-modified oligonucleotide was also tested. In addition, the ability of methylphosphonated and phosphorothioated oligonucleotides to undergo psoralen modification was examined. It was found that selective methylphosphonate substitution does increase ezymatic resistance, that the psoralen monoadduct, itself is somewhat protective, and that psoralen photochemistry with either methylphosphonates or phosphorothioates is comparable in efficiency to that of unmodified oligonucleotides.

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INTRODUCTION

The use of psoralens as chemotherapeutic agents dates back thousands of years, having been used in ancient Egypt and India since 1200-2000 B.C.E. as a remedy for vitiligo. Preparations from the boiled extracts of certain umbelliferous plants either were applied to the skin or were ingested. Patients then exposed their skin to the intense Egyptian or Indian sunlight.[1] That psoralens, per se, were photoactive agents was not known until 1938, when Kuske identified natural furocoumarins in plants as photosensitizers and isolated bergapten (5-MOP) from the oil of bergamot.[1] Modern PUVA (psoralens plus longwave UV radiation (320-400 nm) or UVA) photochemotherapy traces its origins to the isolation of 8-methoxypsoralen (8-MOP) from Ammi majus L. in 1947 by Fahmy and Abu-Shady[2] and the subsequent clinical use of crystalline 8-MOP and sunlight to treat vitiligo by El Mofty,[3] a leading Egyptian dermatologist. The development of high intensity UVA radiation sources by Levin and Parrish in 1975 made PUVA practical for large scale treatment of skin disorders throughout the entire year.[4,5]

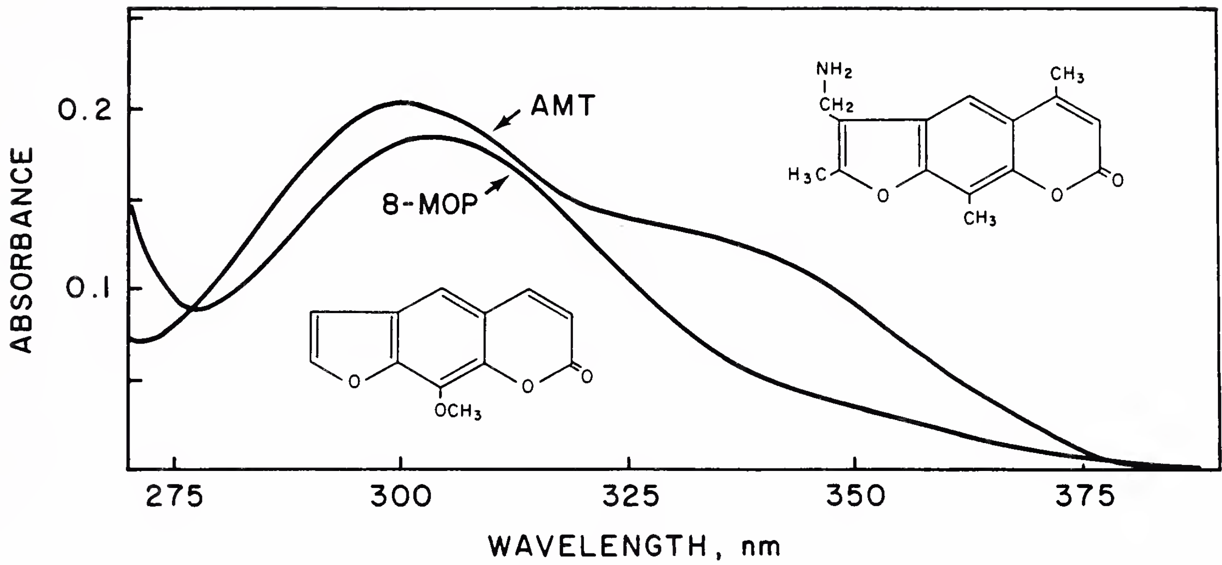
Currently, PUVA is used to treat several dermatologic disorders, including all forms of psoriasis, early stages of cutaneous T-cell lymphoma (CTCL), and vitiligo.[6] Another therapeutic modality employing 8-MOP is extracorporeal photopheresis. Unlike PUVA, this phototherapy, developed by Edelson et al.,[7] does not involve irradiation of the skin but irradiation of the blood extracorporeally.

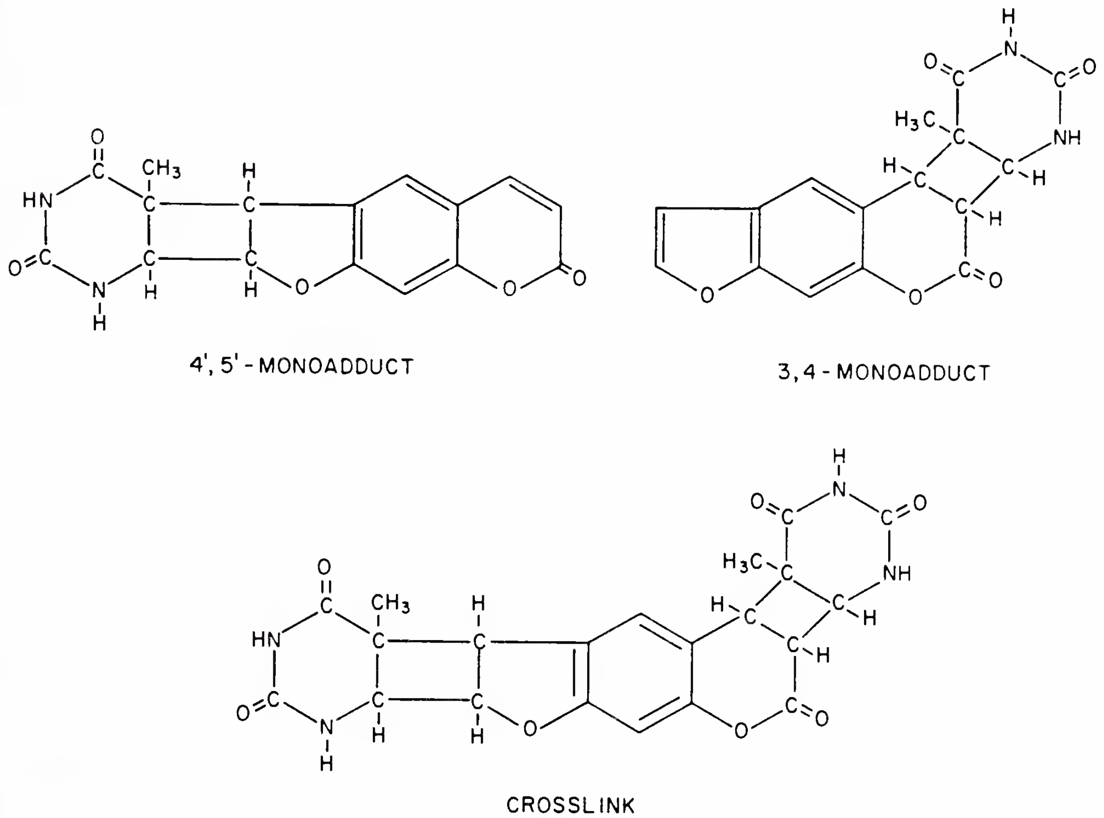
This approach has been used successfully to treat some patients with CTCL. During the last two years, trials have begun to test the efficacy of photopheresis in the treatment of progressive systemic sclerosis,[8] rheumatoid arthritis,[9] and rejection in cardiac transplant.[10]

Experiments to study the immunological effects of PUVA have recently begun. Preliminary evidence indicates that PUVA is able to suppress skin allograft rejection in mice. Pretreatment with PUVA and reinfusion of photodamaged cells into the host attenuated the in vivo and in vitro response to alloantigen.[11] Although the precise mechanism of this effect has yet to be elucidated, this result along with the preliminary data from cardiac transplants and the successes with CTCL seem to indicate that PUVA and photopheresis may be affecting T-cells.

PHOTOBIOLOGY OF 8-MOP

8-MOP is a planar tricyclic aromatic compound that can intercalate between nucleic acid base pairs. Diagram 1 A shows the structure and UV absorbance spectra for 8-MOP and another psoralen compound, 4'-aminomethyl-4,5', 8-trimethylpsoralen (AMT). The peak near 300 nm is typical of psoralen regardless of the nature of the substituents. In the absence of UV radiation, a molecular complex involving non-covalent linkages is formed. Activation of the intercalated complex with UVA light leads to a photochemical reaction which results in a covalent C_4*-cycloaddition with the pyrimidines, primarily thymines, in DNA. Two types of monoadducts (see diagram 1 B) can be formed, depending on which double bond is involved.*





A: Structure and UV absorbance spectra of 8-MOP and AMT.
B: Structure of the psoralen monoadducts and crosslink.

Diagram 1

Sage and Moustacchi have shown that the sequence 5'- TpApT -3' is a preferential site for 8-MOP photoadduct formation.[12] *The 4',5'-monoadduct can absorb a second UVA photon and undergo another C_4-cycloaddition with a pyrimidine on another strand of DNA resulting in an interstrand crosslink (see diagram 1 B).*

DNA is not the only target for psoralens. Covalent addition of psoralens to unsaturated fatty acids[13] *and proteins*[14] *as well as oxidation of lipids*[15] *has been reported. Several groups have demonstrated that 8-MOP illuminated with UVA produces singlet oxygen which in turn may cause DNA damage as well as oxidation of amino acids.*[16,17] *Which of these effects is responsible for the clinical efficacy of PUVA and photopheresis is not precisely known.*

OLIGONUCLEOTIDES AS INHIBITORS OF GENE EXPRESSION

One method by which 8-MOP could be made to specifically target DNA is by employing antisense oligodeoxynucleotides, short segments of DNA that have a complementary base sequence to a particular gene. The use of antisense RNA and DNA both naturally and synthetically has proven to be a powerful means of modulating gene expression. This regulation was first recognized as a naturally occurring mechanism in prokaryotes. It has been shown that processes such as plasmid replication,[18] *osmoregulation,*[19] *transposition,*[20] *and phage reproduction*[21] *all involve an interaction of a "sense" and a short "antisense" transcript. Several eukaryotic RNA species have been identified containing complementary sequences to known genes.*[22] *Although many have been isolated and shown to regulate translation in vitro, their regulatory role, in vivo, remains undefined.*[21]

Control of gene expression by synthetic oligonucleotides was first demonstrated in vitro by two different groups.[23,24] *Soon thereafter, in 1978, Zamecnik and Stephenson used a synthetic oligomer complementary to terminal repeats of Rous sarcoma virus (RSV) RNA to inhibit RSV replication and cell transformation in cultured cells.*[25] *Control of cellular gene expression by antisense oligonucleotides to mRNA was accomplished eight years later by both Zamecnik et. al.*[26] *and Wickstrom et. al.*[27] *These initial experiments have lead to the widespread use of this technique to inhibit gene expression. Yet, this inhibition was only transient because of the oligomers' susceptibility to nuclease digestion. One way to increase delivery of antisense oligomers to their targets is to integrate an antisense gene construct in the genome of a cell. Izant and Weintraub, in 1984, were able to suppress thymidine kinase expression by inserting an artificial antisense gene in the herpes simplex virus.*[28] *Since then, numerous artificial antisense genes have been described, with plants being the first multicellular eukaryotic organism in which this artificial regulation was reported.*[29]

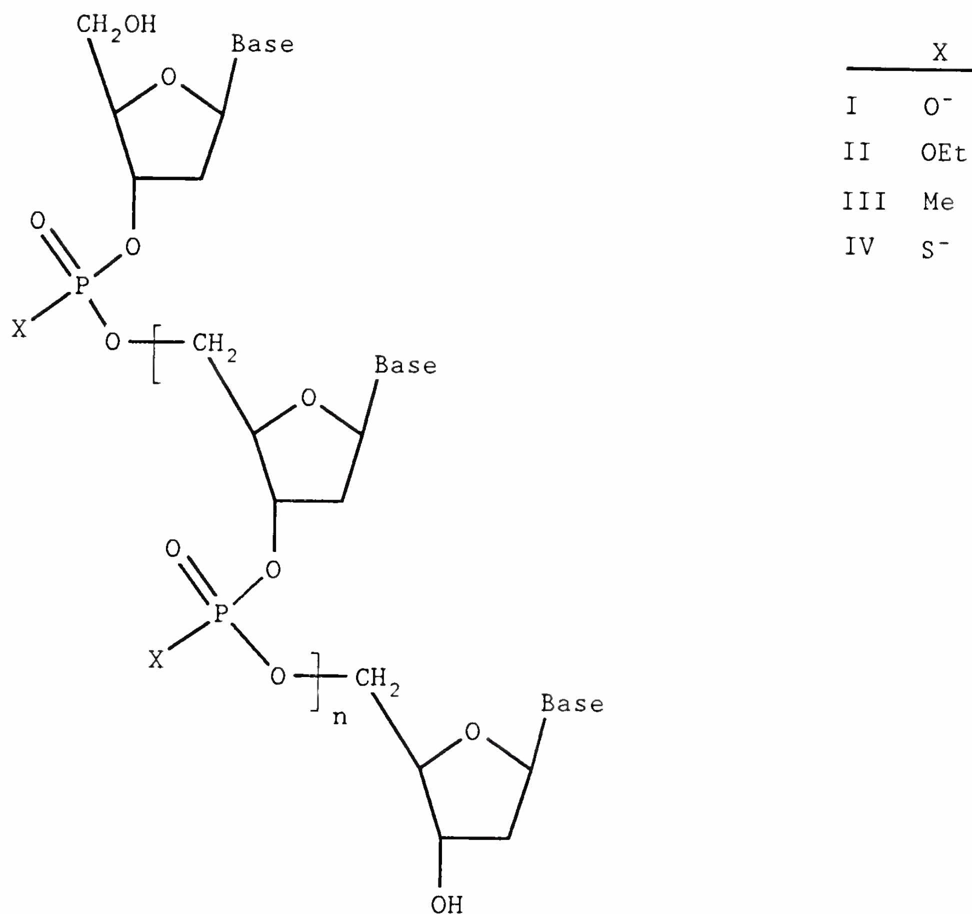
Although this is a powerful tool for elucidating the function of various genes and gene products, a simpler, more direct, and more applicable approach for higher eukaryotes is the use of extrinsic oligodeoxynucleotides, which can be prepared by automated chemical synthesis. Ideally, the oligomer must be able to reach its target, remain intact, bind selectively, and retain its effects over a long period of time.

UPTAKE OF OLIGONUCLEOTIDES

Two groups have shown that oligomers are actively transported across the plasma membrane via an endocytic process.[30,31] One of the groups, Loke et. al., suggested that this transport may involve the binding of the oligomer to a plasma membrane protein.[30] Using oligo(dT)-cellulose beads, they were able to affinity purify an 80-kDa protein that may be associated with cellular uptake of the oligos. They also showed that this uptake is temperature dependent and is saturable. Yakubov et. al. demonstrated that undegraded oligonucleotides may be found in cellular cytoplasm and nuclei. Recently, it has been shown that an antisense oligonucleotide phosphorothioate (see diagram 2) specific for the rev gene-encoded RNA of HIV-1 is concentrated in the nucleus and achieves a steady state concentration within 0.5 to 5 hours depending on the cell line.[32] This is especially important if the target of the antisense is not RNA, on which most of the antisense work focuses, but the genomic DNA, itself. Consideration of this endocytic mechanism and the role of the oligomer binding protein may allow the construction of oligomers that more efficiently reach their targets. This was the rationale behind the work of Leonetti et. al. who attached poly(L-lysine), a polycationic drug carrier, to an oligonucleotide to enhance its uptake by endocytosis.[33] In their experiments, antisense oligomers to a particular mRNA of the vesicular stomatitis virus (VSV) exhibited antiviral properties at lower concentrations than unmodified oligomers.

STABILITY

Even if the oligomer can get into the cytoplasm or into the nucleus, its susceptibility to nucleases may prevent it from reaching its target intact in sufficient concentrations. Because of their greater stability, oligodeoxynucleotides, as opposed to oligoribonucleotides, have been used.[34] Yet, oligodeoxynucleotides, themselves, are sensitive to nuclease digestion. Several laboratories thus have developed oligomers with modifications of the sugar-phosphodiester backbone. Much of the early work on oligonucleotide analogs was performed by Miller et. al., who characterized antisense compounds modified with ethyl phophotriester or methylphosphonate linkages[35] (see diagram 2). These constructs have increased hydrophobicity and lowered water solubility because they lack ionizable groups and are uncharged. Tidd and Warenius[36] tested an N-ras antisense sequence with terminal methylphosphonate diester linkages for their ability to resist enzymatic degradation. They found that an 18-mer with two methylphosphonate diester linkages at the 3'-terminus was relatively resistant to enzymes found in fetal calf serum. These modified oligomers, however, have several drawbacks. Methylphosphonates have low solubility in aqueous media.[34] In addition, phosphorus atoms with four different substitutes exhibit diastereoisomerism. Since the synthesis of methylphosphonates is not stereospecific, an oligo of length n has $2^{(n-1)}$ *stereoisomers, some of which may have poor affinity for the target sequence. Higher concentrations are also required to achieve inhibition. Presumedly, this is because unlike unmodified oligos, methylphosphonates do not function by causing*



Structure of normal and modified oligonucleotides. I, normal phosphate linkage; II, ethyl phosphotriester; III, a methylphosphonate; IV, a phosphorothioate.

Diagram 2

RNase H catalyzed cleavage of mRNA but by physically blocking ribosome binding during protein synthesis.

Another analogue that has been extensively studied is the phosphorothioate oligonucleotide, in which one of the nonbridging oxygen atoms is replaced by a sulfur atom (see diagram 2). This oligomer has good water solubility, unlike the methylphosphonates. It is also protected against nucleases,[37] *and has been shown to be stable for several hours (between 7 and 19 hours depending on the particular cellular system) in adult human serum, rat cerebrospinal fluid, RPMI with 10% fetal bovine serum, undiluted fetal bovine serum, and HeLa cell extract.*[38] *Matsukura et. al.*[39] *synthesized oligonucleotides complementary to regions of the rev (art/trs) gene of HIV-1 that are essential for efficient viral replication. These antisense oligos showed potent antiviral activity; yet, the control phosphorothioate oligomers which were not sequence specific also exhibited equally potent antiviral properties. In fact, the best inhibition for de novo infection was obtained with a homo-oligomer,* $S\text{-}dC_{28}$*. No effect, however, was observed when the homo-oligomer was applied to chronically affected cells. Phosphorothioates have been shown to inhibit reverse transcriptase in vitro,*[40] *and thus this may be one explanation of this non-specific effect in this system. This group has more recently demonstrated that there is sequence-specific antiviral activity associated with the phosphorothioates.*[41] *Antisense oligos complementary to rev mRNA caused a translational arrest, as measured by Northern blots, whereas control sequences had no such effects. In addition, as mentioned earlier, these oligomers preferentially target the nucleus, and*

therefore may affect translation and transcription at a different stage than other oligomer analogs.

A third class of oligomer that is resistant to nucleases is the α-analogs, in which the naturally occurring β linkage formed by the deoxyribose moiety with the purine or pyrimidine base is replaced by an α-linkage. As compared to β oligomers, they bind more strongly to ribonucleotides rather than deoxyribonucleotides and are much more resistant to enzymatic degradation.[42] Another advantage is that the synthesis of these α-analogs yields only a single stereoisomer.[43] An α-18-mer complementary to VSV mRNA coding for the nucleocapsid protein, however, did not inhibit in vitro translation.[44] This result was ascribed to the failure to induce degradation of the target RNA because the α-oligo - RNA complex did not activate RNase H.

SPECIFICITY

Oligomers specifically bind to complementary sequences of DNA or RNA through hydrogen bonding. Statistically, a sequence of 12 nucleotides should be found only once in the E. coli genome. Yet, in human cells where the (A+T)/(G+C) ratio is 0.6, the minimum length should range from 15 (if there are only G's and C's in the oligonucleotide) to 19 (if there are only A's and T's) when a DNA sequence is the target.[45] When mRNA is the target, assuming that about 0.5% of the genome is transcribed into mRNA, the minimum length for uniqueness should be 11 to 15. The actual calculation depends on the sequence, especially for (G+C)-rich oligonucleotides, because one

dinucleotide sequence, CpG, is under-represented in eukaryotic genomes as compared to a random distribution.[45] The evaluation of specificity must also take into account the formation of stable "mismatched" non-Watson-Crick base pairs, especially G-T.[35] This could lead to an increase of non-specific binding. It also has been shown statistically that increasing the length of the oligonucleotide beyond 25 bases confers no additional specificity to it. At this length, a single mismatch would represent only a small percentage of the total oligonucleotide and thus would be "tolerated" quite well.

CONJUGATED OLIGONUCLEOTIDES

In order to increase the affinity of the oligomers for their target sequence and prolong their inhibitory effects, several groups have covalently linked the oligomers to intercalating agents. Intercalating agents are polycyclic aromatic compounds which insert their planar aromatic ring between two consecutive base pairs of double-stranded DNA. They increase the affinity of the oligo by providing additional binding energy which stabilizes the complex. Asseline et. al.[46] constructed oligos with a acridine covalently linked to the 3′ terminus. Subsequently, oligos with an attached acridine derivative were reported to specifically inhibit translation of mRNA for phage T4[47] and of rabbit β-globin[48] more efficiently than unmodified oligos. In order to increase the affinity and the stability of the oligomers, Thuong et. al.[42] linked an acridine derivative to an α-oligo. In addition, a number of reagents have been linked to oligos to induce irreversible modifications in the target nucleic acids. Knorre and Vlassov

covalently attached an alkylating group to 3' terminus.[49] *Iron EDTA has also been conjugated to antisense oligos. This modification can give rise to reactive oxygen-containing radicals in the vicinity of the DNA; however, Boutorin et. al.*[50] *reported that there was some nonspecificity and autodegradation with time. Other metal complexes that have been used include o-phenantroline-Cu(I)*[51] *and porphyrin-Fe(II).*[52]

PHOTOACTIVE INTERCALATING AGENTS

The synthesis of oligomers containing a photoinducible crosslinking group was originated to overcome some of the above mentioned problems with oligos in vivo. Ts'o and Miller et. al. reasoned that methylphosphonated oligos could be more effective if they could bind covalently to their target.[53] *To this end, they linked a psoralen molecule (AMT) to an oligodeoxyribonucleoside via an amino-alkyl tether. Lee et. al. in the same laboratory were able to demonstrate that these psoralen-modified oligomers were able to crosslink single-stranded DNA in vitro in the presence of UVA.*[54] *Using a different psoralen derivative, 4,5',8-trimethylpsoralen, Pieles and Englisch showed that the intercalated psoralen group contributed to the stability of the duplex formed between the psoralen-modified oligodeoxyribonucleotide and a synthetic 21-mer.*[55] *They reported that the* T_m *of this complex increased by about 8°C. In addition, their group confirmed that a psoralen-modified antisense oligo can specifically bind and "crosslink" single-stranded DNA in vitro. Kean et. al.*[56] *used the psoralen-derivatized oligonucleoside methylphosphonates mentioned*

above to target rabbit globin mRNA in vitro. Crosslinking was detected in the single-stranded regions of the mRNA. Further work by Lee et. al.[57] *suggested that in vitro this psoralen-modified oligo did not crosslink double-stranded DNA. Even when the duplex was in an open form during transcription, it was not accessible to oligomer binding. Other photocrosslinking agents that have been tethered to oligonucleotides include azidophenacyl,*[58] *proflavine,*[58] *and porphyrins.*[59]

DNA AS A TARGET

Synthetic oligonucleotides and oligonucleosides have been used to inhibit DNA replication, retroviral replication, pre-mRNA processing and protein synthesis. The target of these oligos is mRNA. Very little work has been done on the use of antisense directed against DNA. One approach to specifically target DNA has involved the formation of a triple helix. A homopyrimidine oligonucleotide with EDTA-Fe attached at a single position bound the corresponding homopyrimidine-homopurine tracts within double-standed DNA by triple helix formation and cleaved the DNA at that sight.[60] *The oligo was shown to be bound by Hoogsteen hydrogen bonds in the major groove. Unfortunately, this recognition has only been possible with homopyrimidine-homopurine tracts. This technique, therefore, does not have the same specificity as oligos that are directed to the linear DNA or RNA sequence.*

Recently, Gasparro et. al. have synthesized antisense oligos containing psoralen monoadducts.[61] *This strategy avoids using the chemically reactive site on the psoralen for the attachment of the*

hydrocarbon tether, as is the case in the experiments described above. In a series of experiments, they targeted the β-lactamase gene in E. coli and caused otherwise resistant strains to be susceptible to ampicillin. An effect was seen at a concentration about 100 times lower than would be needed to target mRNA. This is the first report of antisense being directed against DNA in cells in culture.

Described in this paper is a series of experiments designed to test the susceptibility of modified oligonucleotides to enzymatic degradation. Previous work cited above tested oligos where every base was modified with a methylphosphonate. Here, only selected bases have the methylphosphonate substitution. Several combinations of the substitutions are compared to each other. Gasparro et. al. synthesized a 9-mer complementary to a sequence within the initiation region of the β-lactamase gene of E. coli. It is this sequence that is used for these experiments. Modified and unmodified oligos containing psoralen monoadducts were synthesized and also analyzed for resistance to enzymatic degradation. Finally, the synthesis and purification of phosphorothioate oligos containing psoralen monoadducts is described.

MATERIALS AND METHODS

OLIGONUCLEOTIDES

A 9-mer (5'-GAGTATGAG-3') was synthesized on a multicolumn solid phase synthesizer (Applied Biosystems, Foster City, CA). 9-mers with methylphosphonates on the middle three bases, TAT, (9-mer-TAT), on the second, third, eighth, and ninth bases (9-mer-AG), and on every other base- the second, fourth, sixth and eighth bases- (9-mer-alt) were also synthesized by the same method (see table 1). These oligos were 5'-end labelled using T4 kinase (Boehringer-Mannheim, Indianapolis, IN) and $[^{32}P]$-γ-ATP (Amersham, Arlington Heights, IL) according to the procedure of Maniatis, et. al.[62] *Salts and enzymes were removed prior to the reactions using Nensorb ion exchange columns (Dupont, Wilmington, DE). A 15-mer phosphorothioated oligo antisense to part of a sequence encoding for ICAM-1 in human keratinocytes was purchased from Synthecell (Gaithersburg, MD).*

DIGESTION WITH T4 POLYMERASE

To test for 3' exonuclease sensitivity the oligonucleotides (100 μg/mL) were dissolved in 67 mM Tris HCl, 6.7 mM $MgCl_2$, 16.7 mM $(NH_4)_2SO_4$ and 10 mM β-mercaptoethanol (pH 8.8). T4 polymerase (Boehringer-Mannheim, Indianapolis, IN) was added (3.3 units/μg oligo) and incubated at room temperature. The polymerase was inactivated at 0 time, 1 min., and 10 min. after the start of the reaction with 0.5 M EDTA and by heating the reaction

TABLE 1: OLIGONUCLEOTIDES USED

Abbreviation	*Sequence*	*Description*
9-mer	*pGpApGpTpApTpGpApG*	*unmodified 9-mer*
9-mer-alt	*pGmApGmTpAmTpGmApG*	*9-mer with every other base a methylphosphonate*
9-mer-TAT	*pGpApGmTmAmTpGpApG*	*9-mer with the three middle bases methylphosphonated*
9-mer-AG	*pGmAmGpTpApTpGmAmG*	*9-mer with second, third, eighth, and ninth bases methylphosphonated*
9-mer-MA	*pGpApGpT*pApTpGpApG*	*monoadducted 9-mer*
5-mer	*pCpApTpApC*	*complementary strand to middle 5 bases of the 9-mer*
15-mer	*sGsGsTsCsTsCsTsAsT sGsCsCsCsAsA*	*phosphorothioated 15-mer*
9-mer-comp	*pGpGpCpApTpApGpApG*	*complementary strand to middle 9 bases of 15-mer*
15-mer-MA	*sGsGsTsCsTsCsT*sAsT sGsCsCsCsAsA*	*monoadducted 15-mer*

* *location of 4',5'-monoadduct*

mixture to 75°C for 3 min. Each of the above 5'-end labelled oligos were tested in this manner.

DIGESTION WITH ACID PHOSPHATASE

To test for phosphohydrolase sensitivities, the oligonucleotides (200 μg/mL) were dissolved in 10 mM Na acetate (pH 5.0). Acid phosphatase (Sigma, St. Louis, MO) was added (0.12 units/μg oligo) and incubated at 37°C. The phosphatase was inactivated at 0 time, 1 min., 10 min., and 30 min. after the start of the reaction with 1 M Na phosphate (pH 6.8) and by heating to 80°C for 3 min.

DIGESTION WITH PHOSPHODIESTERASE II

Because DNA is resistant to phosphodiesterase II degradation if a 5' terminal phosphate exists, prior treatment with acid phosphatase, which removes this phosphate, was necessary. In order to follow the reaction, the oligos were 3'- end labelled, as the 5' label will be cleaved by the acid phosphatase. This labelling used terminal deoxynucleotidyl transferase (IBI, New Haven, CT) and [^{32}P]-α-CTP (Amersham, Arlington Heights, IL) according to the procedure by Maniatis et. al.[62] Acid phosphatase (0.12 units/μg oligo) was allowed to react with the oligos for 20 min. The reaction was stopped as described above. Phosphodiesterase II (Sigma, St. Louis, MO) in 125 mM succinate HCl (pH 6.5) was added (0.01 units/μg oligo and 0.001 units/oligo). This enzyme was stopped at 0 time, 1 min., 10 min., and 30 min. by heating to 75°C for 5 min.

GEL ELECTROPHORESIS

Results of the above reactions were analyzed using 20% polyacrylamide gels under denaturing conditions (8.3 M urea in Tris-borate-EDTA). Autoradiograms were taken using diagnostic XR film (Kodak, Rochester, NY).

PHOTOCHEMISTRY WITH METHYLPHOSPHONATES

The psoralen monoadducts of the 9-mer, 9-mer-TAT, 9-mer-alt, and 9-mer-AG were prepared. Equimolar amounts (40 µM) of the 9-mer and a 5-mer (5'CATAC-3') complementary to the middle five bases of the 9-mer were prepared in irradiation buffer (10 mM Tris, 1 mM EDTA, 0.5 M NaCl, pH 7.8). The minihelix formed between these two oligomers provided intercalation sites for the psoralen (AMT or 8-MOP, 80 µM). The chilled solution (4°C) were exposed to 400 nm light emanating from a monochromator (Photon Technologies International Inc., Princeton, NJ). Samples were withdrawn from the photoreaction chamber at 0 time, 1 hour, and 3 hours and then analyzed on gel electrophoresis as described above.

PHOTOCHEMISTRY WITH PHOSPHOROTHIOATES

The psoralen monoadduct of the 15-mer phosphorothioated oligo was also prepared. Equimolar amounts (80 µM) of the 15-mer and a 9-mer complementary to its middle 9 bases were prepared in irradiation buffer (composition described above). 8-methoxypsoralen (8-MOP) was added to yield a final concentration of 640 µM. The chilled solution (4°C) was exposed to 400 nm light as described above for a total of 4 hours.

PURIFICATION OF 9-MER MONOADDUCT

The 9-mer monoadduct was purified on HPLC. Reversed phase HPLC facilitated the separation and purification of psoralen-modified oligos because volatile organic solvents (acetonitrile) and salts (triethylammonium acetate) were used. 50 ul aliquots were applied to a C8 column (Vydac, Hesperia, CA). A mobile phase consisting of acetonitrile and 0.1 M triethylammonium acetate (TEA) was used with the following gradient to elute the oligonucleotides: 0 min: 2.5% TEA; 2.5 min: 2.5% TEA; 22.5 min: 12.5% TEA; 27.5 min: 12.5% TEA; 35 min: 25% TEA; 35 min: 50% TEA; 40 min: 50% TEA; 45 min: 50% TEA. A scanning detector (Spectra-Physics, San Jose, CA) was used to determine the ultraviolet absorption spectra of the eluting compounds. Absorbance data monitored from 200-370 nm was accumulated by a direct memory access board (IBM AT). Fractions were collected off the column and concentrated by lyophilization (Savant, Farmingdale, NY). Purity was confirmed on gel electrophoresis.

PURIFICATION OF 15-MER MONOADDUCT

The 15-mer phosphorothioated monoadduct was also purified on reversed phase HPLC. 500 ul aliquots were applied to a C4 column as above with the mobile phase being acetonitrile and TEA. The following gradient was used to elute the oligonucleotides from the column: 0 min: 12.5% TEA; 5 min: 12.5% TEA; 30 min: 20% TEA; 35 min: 20% TEA; 40 min: 25% TEA; 50 min: 25% TEA. Fractions were collected, pooled, and concentrated as above. Purity was checked by applying a small portion of the concentrated sample on a C8 column (Regus, Morton, IL) and by running gel electrophoresis.

DIGESTION OF THE 9-MER MONOADDUCT

A psoralen modified oligonucleotide (9-mer-MA) was also tested for its sensitivity to T4 polymerase. T4 polymerase (15 units/μg oligo) was added to 9-mer-MA (30 μg/mL) purified by HPLC. The reaction proceeded as described above and was stopped at 0, 1, 3, 10, and 30 min. The results were analyzed by gel electrophoresis as described above.

FATE OF 9-MER IN VIVO

The stability of the oligonucleotides when incorporated into cells was determined by adding the 9-mer and 9-mer-MA to E. Coli (XL1-Blue cells). 40 uL aliquots of competent XL1-Blue cells were exposed to either 9-mer or 9-mer-MA at a 2.5:1 oligo:plasmid ratio. The oligos were introduced into the bacteria by the following heat shock method. After a 30 min. incubation on ice, the cells were placed in a 42°C water bath for 45 sec. The cells were then placed on ice for 2 min. and then incubated at 37°C with shaking for 1 hour. Aliquots were taken at 0, 10 20, and 30 min. during the initial 30 min. incubation as well as following the 42°C heat shock and the one hour incubation at 37°C. Cells and media were separated by centrifugation, the oligonucleotides were isolated from these fractions, and then the oligos were analyzed on polyacrylamide gels. (Work done by Mr. Mark O'Malley)

RESULTS

DIGESTION WITH T4 POLYMERASE

Denaturing polyacrylamide gel electrophoresis was used to analyze the results of the enzymatic degradation experiments. Figure 1 shows the results of the reactions of the 9-mer and the three methylphosphonated 9-mers with T4 polymerase. Lanes 4-6 correspond to the unmodified 9-mer at 0, 1 min., and 10 min. after the start of the ezymatic reaction. The single band corresponding the 9-mer in lane 4 is completely degraded in one minute to at least three shorter oligonucleotides. After 10 min., only a single band (lane 6) corresponding to an oligo about 3 bases long remains.

The results of the same experiment using 9-mer-alt can be seen in lanes 1-3. In lane 1, the starting material is shown to be far from pure with evidence of 8-mer, 7-mer, 6-mer, and 5-mer contamination. Yet, at 1 and 10 min. the band corresponding to the 9-mer (the top band) is decreased in intensity and another band (about 3 bases long) not present in the original material has appeared. Although some degradation has taken place, the 9-mer-alt has not been completely degraded as was the unmodified 9-mer.

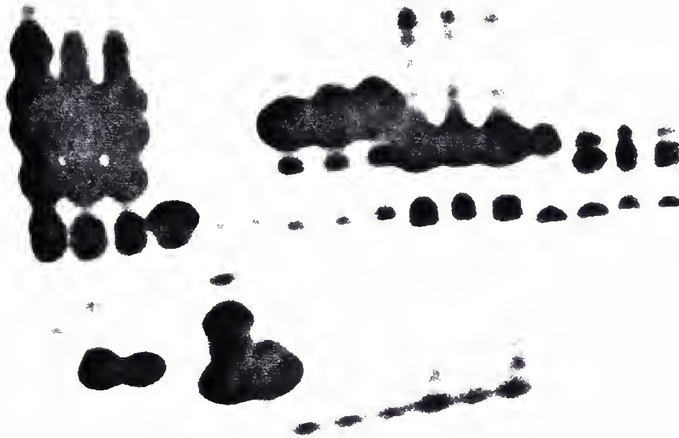
Lanes 7-9 show the results of the experiment with 9-mer-TAT. The starting material in lane 7 is fairly pure. After 1 min. of digestion, the band pattern appears virtually identical to the starting material. Even at 10 min., only a faint increase in the intensity of the bands corresponding to smaller oligos can be detected. Similar results were observed for 9-mer-AG. In lanes

10-12, it can be seen that the oligomer at 0 time is essentially identical to the oligomer at 1 min. and 10 min. These two methylphosphonated oligos appear to be more resistant to T4 polymerase than the unmodified 9-mer or the 9-mer-alt.

DIGESTION WITH ACID PHOSPHATASE

Figure 2 shows the reaction of unmodified 9-mer with acid phosphatase. Lane 1 corresponds to the starting material. Three different concentrations of acid phosphatase, 0.0012, 0.012, 0.12 units/μg oligo, were used, and the reaction was stopped at 1, 3, 10, and 30 min. An effect was seen only at the highest concentration. Since the acid phosphatase removes the 5′-terminal phosphate and the oligo is 5′-end labeled, it is the disappearance and the decrease in intensity of the bands that is observed. Lanes 10-13, corresponding to the concentration of 0.12 units/μg oligo and the four time periods mentioned above, reveal this decrease in intensity. By 30 min. (lane 13) the original band has virtually disappeared.

Figure 3 shows the results of the reaction using this highest concentration of acid phosphatase with the three methylphosphonated oligos. Each oligo is shown at 0, 1, 10, and 30 min. Lanes 1-4, detailing the reaction with 9-mer-AG, reveal the susceptibility of this oligo to acid phosphatase. By 10 min. (lane 3), the band is decreased in intensity and by 30 min. (lane 4), the band has disappeared. The results are similar for the experiment with 9-mer-alt (lanes 9-12). At 30 min. the band has disappeared. 9-mer-TAT (lanes 5-8), however, appears to be more resistant to the



Polyacrylamide gel analysis of the digestion of the 9-mer, 9-mer-MA, and the three methylphosphonated oligonucleotides with T4 polymerase. Lanes 1-3: 9-mer-alt at 0, 1, and 10 min. of digestion. Lanes 4-6: 9-mer at 0, 1, and 10 min. of digestion. Lanes 7-9: 9-mer-TAT at 0, 1, and 10 min. of digestion. Lanes 10-12: 9-mer-AG at 0, 1, and 10 min. of digestion. Lanes 13-16: 9-mer-MA at 0, 1, 10, and 30 min. of digestion.

Figure 1

Digestion of 9-mer with acid phosphatase. Lane 1: starting material. Lanes 2-5, 6-9, and 10-13: digestion times of 1, 3, 10 and 30 min. for acid phosphatase concentrations of 0.0012, 0.012, and 0.12 units/μg oligonucleotide, respectively.

Figure 2

acid phosphatase. Although it is decreased in intensity, the band corresponding to 30 min. of reaction time (lane 8) is still visible.

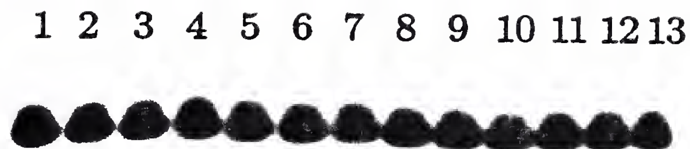
DIGESTION WITH PHOSPHODIESTERASE II

Phosphodiesterase II requires a 5'-hydroxyl terminus in its substrate in order to be active. Thus, oligonucleotides, which have a 5' phosphate group, are resistant to this enzyme. This is shown in figure 4. No digestion is detected at any of the concentrations or reaction times tested.

Acid phosphatase, as shown above, cleaves the 5'-phosphate group, exposing the 5'-hydroxyl terminus. Figure 5 shows the results of pre-treating the oligos with acid phosphatase and then exposing them to the phosphodiesterase Because the acid phosphatase cleaves the 5'-phosphate group, the oligos were 3'-end labeled so that results could be followed on gel electrophoresis. Lane 1 corresponds to unmodified 9-mer before the 20 min. reaction with acid phosphatase (0.20 units/μg oligo). Lanes 2-4 reveal the results of adding 0.001 units/μg oligo of phosphodiesterase with reaction times of 1 min., 10 min., and 30 min, respectively. Lanes 5-7 correspond to the same time points with ten times the amount of enzyme (0.01 units/μg oligo). It can be seen that the unmodified oligo is quite susceptible to the phosphodiesterase. As the reaction time is lengthened and the enzyme concentration increased, the original band decreases in intensity and numerous bands representing smaller oligos appear. After 30 min. at the higher concentration of enzyme, only a single band corresponding to an oligo about three or four bases long remains.

Digestion of methylphosphonated oligonucleotides with acid phosphatase. Each oligonucleotide is shown at 0, 1 min., 10 min., and 30 min. of digestion with acid phosphatase (0.12 units/μg oligonucleotide). Lanes 1-4: 9-mer-AG. Lanes 5-8: 9-mer-TAT. Lanes 9-12: 9-mer-alt.

Figure 3



Digestion of 9-mer with phosphodiesterase II. Lane 1: starting material. Lanes 2-5, 6-9, and 10-13 : digestion times of 30 sec., 3 min., 10 min., and 30 min. for phosphodiesterase concentrations of 0.001, 0.01, and 0.1 units/μg oligonucleotide, respectively.

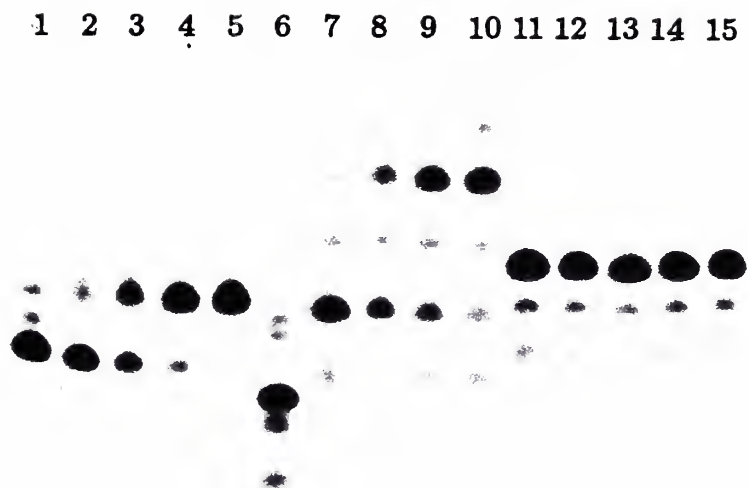
Figure 4

This same reaction was performed with the methylphosphonated oligonucleotides. In figure 6, lanes 1-5, 6-10, and 11-15 reveal the results of the reactions with 9-mer-alt, 9-mer-TAT, and 9-mer-AG, respectively. Unlike the unmodified oligos, the smaller fragments of the methylphosphonated oligos actually run slower on the gel than the larger fragments. This is because when the charge to mass ratio of the oligo is constant, the distance oligos migrate on a gel is proportional to their mass. This ratio, however, is not constant for the methylphosphonated oligos because while the phosphate groups are negatively charged, the methylphosphonated groups are not. A more negatively charged species will tend to migrate further than a less negatively charged one of equal mass. Thus, for example, the 9-mer-TAT has six negatively charged groups out of its nine bases (charge to mass ratio = -0.67-assuming for the example that a base has mass of one) while this oligo with its 5' base cleaved has 5 charged groups out of eight bases (charge to mass ratio = -0.63). Thus, although the 8-mer has less mass than the 9-mer, it is also has less charge density. It is this latter force that predominates in this case.

As above, two concentrations of the phosphodiesterase were tested, although at the higher concentration only a 10 min. time point was examined. The results show that the 9-mer-AG (lanes 11-15) is resistant to this enzymatic degradation; whereas, the 9-mer-alt and 9-mer-TAT are both degraded. It appears that the reaction removes one base from the 9-mer-alt and about three bases from the 9-mer-TAT. After 30 min., the bands representing the original material for these two oligos have almost completely disappeared.

Digestion with acid phosphatase and phosphodiesterase II. Lane 1: 9-mer. 9-mer was treated with acid phosphatase (0.2 units/μg oligonucleotide) and then digested with phosphodiesterase. Lanes 2-4: 0.001 units/μg oligonucleotide phosphodiesterase at 1, 10, and 30 min. of digestion. Lanes 5-7: 0.01 units/μg oligonucleotide at 1, 10, and 30 min. of digestion.

Figure 5



Digestion of methylphosphonates with acid phosphatase and phosphodiesterase II. As in figure 5, oligonucleotides were pretreated with acid phosphatase. Each oligo is shown before reaction with acid phosphatase, at 1, 10 and 30 min. following addition of phosphodiesterase (0.001 units/μg oligo), and 10 min. following addition of phosphodiesterase (0.01 units /μg oligo). Lanes 1-5: 9-mer-alt. Lanes 6-10: 9-mer-TAT. Lanes 11-15: 9-mer-AG.

Figure 6

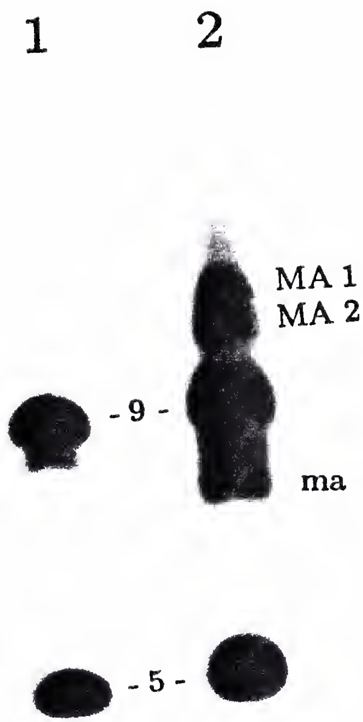
PREPARATION OF 9-MER MONOADDUCT

The monoadduct of the unmodified 9-mer was prepared with AMT. A representative autoradiograph of the gel used to analyze the reaction mixture is shown in figure 7. The reaction between the 9-mer:5-mer minidouble helix complex and AMT following exposure to 400 nm radiation for 4 hours is shown. The primary photoreaction was the formation of 9-mer-monoadducts (labeled MA 1 and 2). Earlier studies showed that MA-1 and MA-2 correspond to two forms of the 4',5'-monoadduct (G. Miolo, unpublished results). The 5-mer photoproduct (ma) also was formed.

Each of the methylphosphonated oligos were tested to see if this modification would interfere with the photochemistry. Figure 8 shows the results of the photoreactions at time 0, 1 hour, and 3 hours of 9-mer-TAT, 9-mer-AG, and 9-mer-alt, respectively. As with the unmodified 9-mer, the principal reaction is formation of 9-mer monoadducts (labeled MA). Thus, this shows that it is possible to create monoadducts with the modified oligos. Although only one of the monoadducts can be seen at the three hour time point, at longer reaction times both of these bands can be seen (data not shown). Also, it appears that the yield of monoadduct is lower for the 9-mer-alt than for the other two 9-mers at the three hour time point; yet, by six hours no differences among the three can be detected (data not shown).

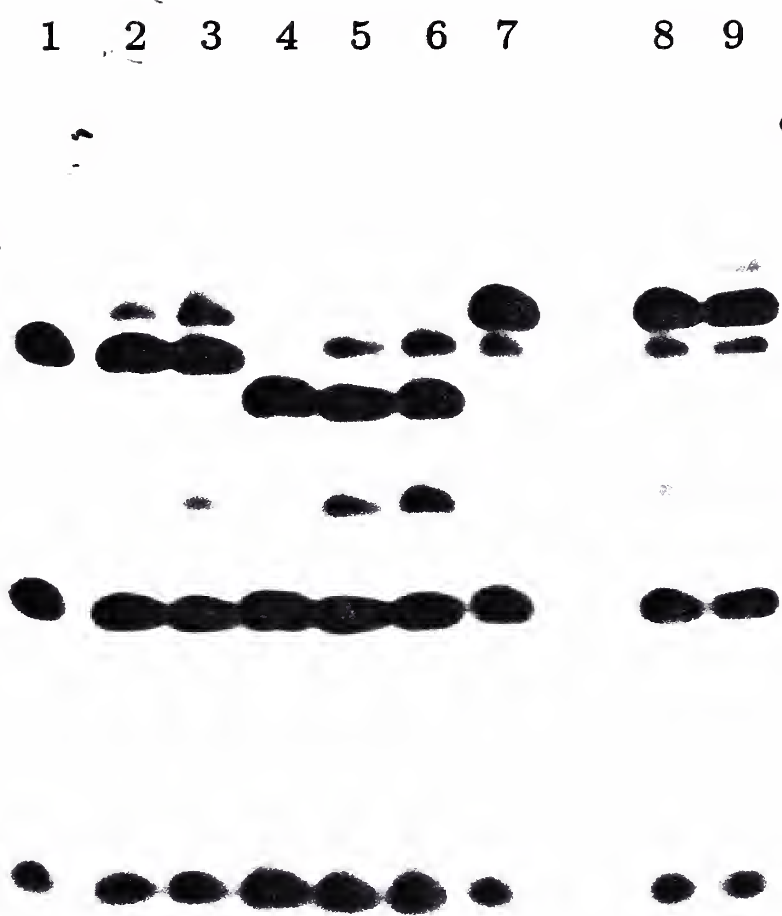
PURIFICATION AND ANALYSIS OF THE 9-MER MONOADDUCT

The monoadduct of the unmodified 9-mer was isolated and purified by reversed phase HPLC. The photoreaction mixture was applied to the



9-mer:5-mer photoreactions with AMT. Equimolar amounts (40 μM) of the 9-mer and 5-mer were equilibrated with AMT (80 μM) and irradiated with 400 nm radiation at 4°C. Lane 1: 0 time. Lane 2: 4 hours of reaction time.

Figure 7



Preparation of monoadduct with methylphosphonated oligonucleotides. Procedure as above in figure 7. Each oligonucleotide is shown at 0, 1, and 3 hours. Lanes 1-3: 9-mer-TAT. Lanes 4-6: 9-mer-AG. Lanes 7-9: 9-mer-alt.

Figure 8

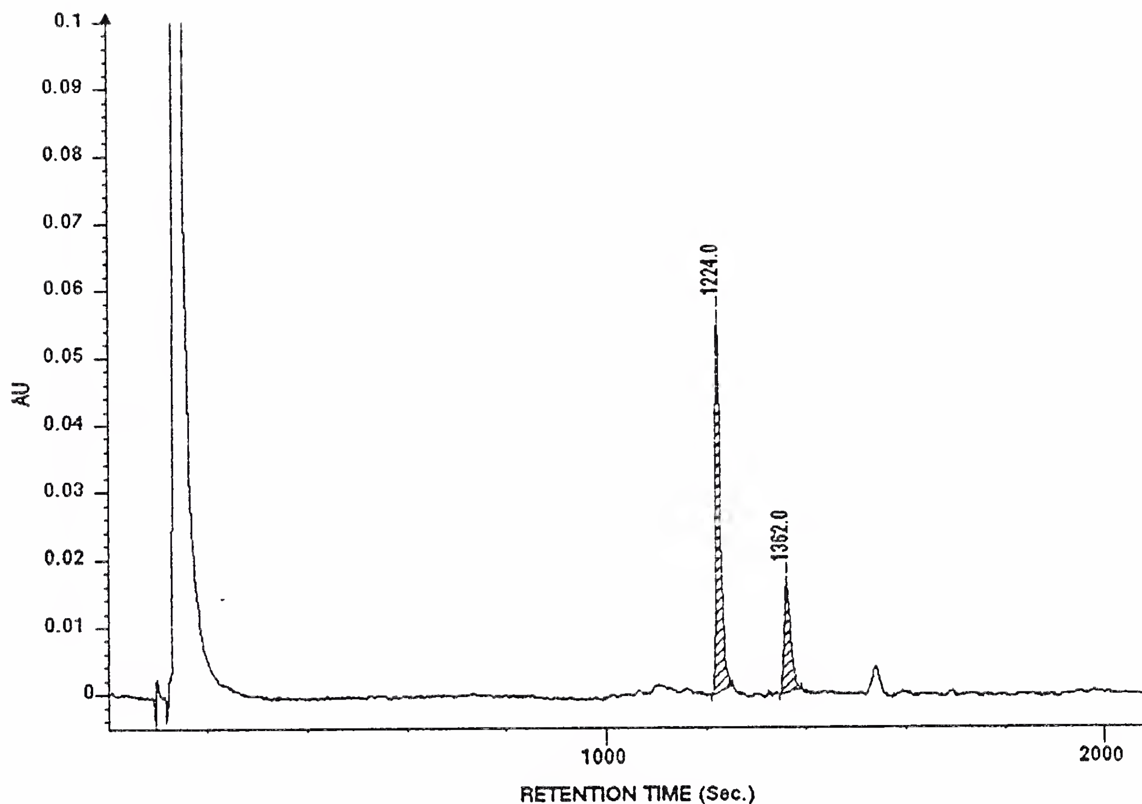
HPLC column as described above. Chromatographic analysis of the peaks eluted off the column is shown is figure 9. The peaks eluting at 1224 s and 1362 s were characterized by recording their UV spectra (figures 10 and 11) using a scanning detector as they eluted from the HPLC column. The spectrum of the peak at 1224 s seen in figure 10 is characteristic of unmodified DNA and has been shown to be the unreacted 9-mer (Gasparro <u>et</u>. <u>al</u>., unpublished results). Figure 11 shows the UV spectrum of the species eluting at 1362 s. The shoulder at 327 nm is characteristic of 4',5'-monoadducts. Using the integrated areas of these two peaks, it was calculated that 27.6% of the original 9-mer was converted to monoadduct. (For the several runs done, the yields ranged from about 20% - 30%). The fractions containing the monoadduct were pooled and concentrated.

DIGESTION OF THE 9-MER MONOADDUCT

The 9-mer monoadduct was tested for its susceptibility to T4 polymerase. Figure 1 (lanes 13-16) reveals of the results of this ezymatic digestion after 0, 1 min., 10 min., and 30 min. By 30 min., the reaction mixture appears to contain 8-mer, 7-mer, and a small amount of 6-mer as well as some of the original 9-mer. Although some digestion has taken place, the monoadduct is far more stable than the unmodified 9-mer, which, as discussed above, is completely degraded by 10 min. (lane 6).

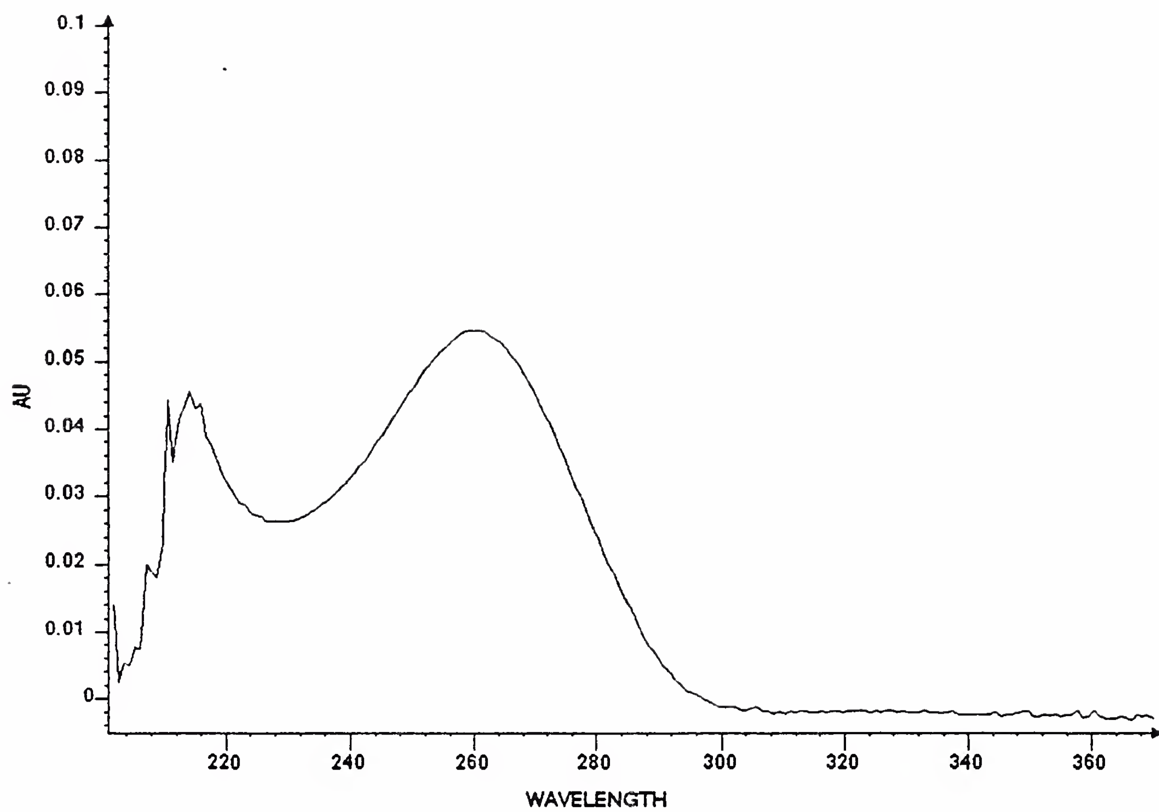
FATE OF THE 9-MER MONOADDUCT <u>IN</u> <u>VIVO</u>

The stability of the 9-mer monoadduct after incorporation into <u>E</u>. <u>coli</u> cells was also determined. Fractions of cells were collected at



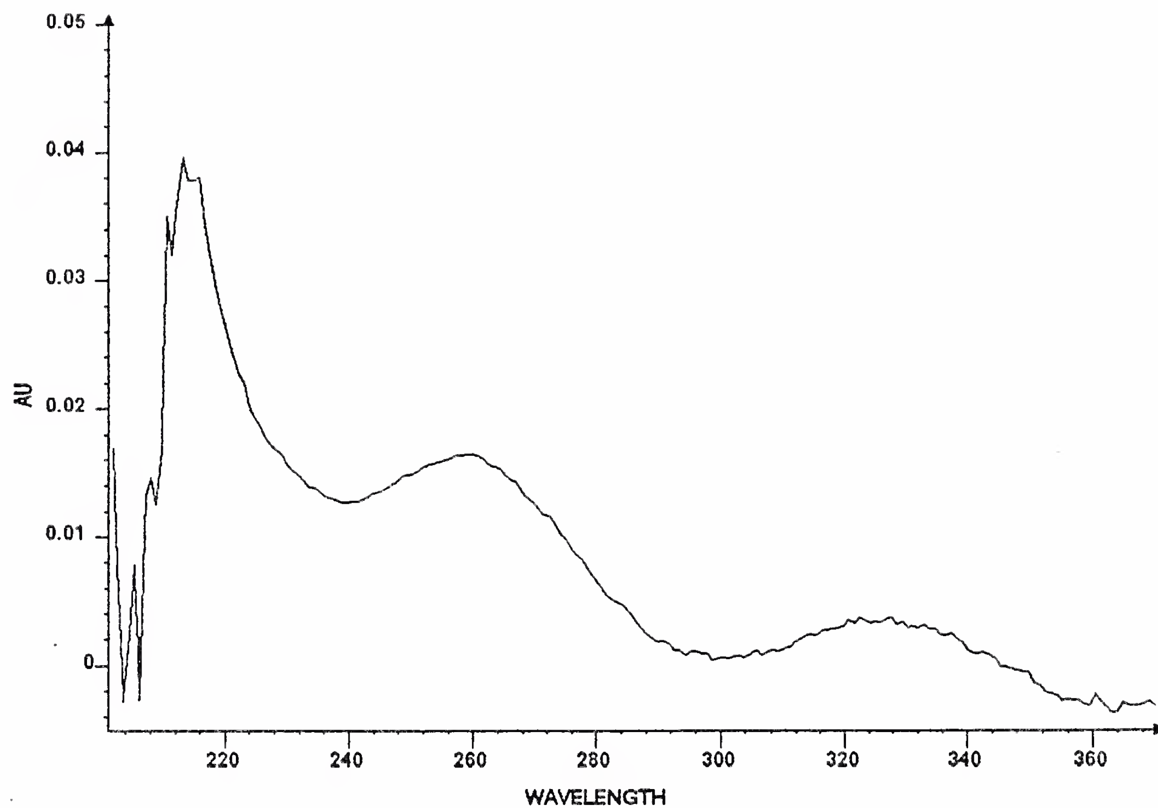
HPLC analysis of 9-mer:5-mer photoreaction with AMT. A mobile phase consisting of acetonitrile and 0.1 M triethylammonium acetate was used with the gradient as described above to elute the oligonucleotides from the C8 column. Absorbance at 260 nm is recorded. The AMT came off the column after 2000 sec. and therefore is not shown on this graph.

Figure 9



UV absorbance spectrum of the peak eluting at 1224 sec.

Figure 10



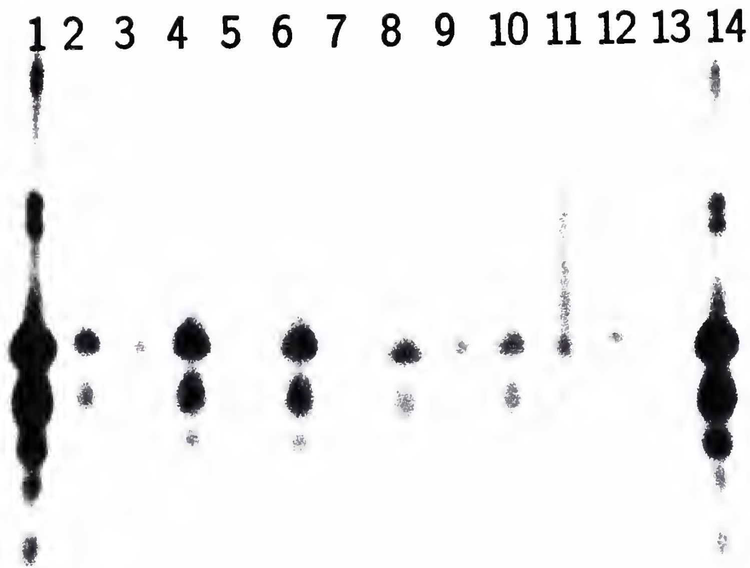
UV absorbance spectrum of peak eluting at 1361 sec.

Figure 11

0, 10, 20, and 30 min. during the initial incubation of the cells with the 9-mer monoadduct as well as following the heat shock and the one hour incubation at 37°C. The results of the 20% polyacylamide gel are shown in figure 12. Lanes 1 and 14 correspond to the end labeled 9-mer monoadduct reaction mixture that has not been incubated with the cells. The even numbered lanes correspond to the supernatant from the cells after exposure to the 9-mer monoadduct at each of the above points during the protocol. The odd numbered lanes represent the cellular fractions in the same sequence. The results indicate that the monoadduct remains intact throughout, even after heat shock and one hour incubation. Although the band in lane 13, representing the cellular fraction following the one hour incubation, is quite faint, the cpm for this sample (5.18×10^2 cpm) compared with the total cpm added (6.89×10^3 cpm) indicates that 7.5% of the monoadduct was incorporated into the cells. This is in agreement with previous data from Gasparro et. al. In contrast, samples incubated with unmodified 9-mer in which the oligos undergo degradation after heat shock and incubation (data not shown). These results support the <u>in vitro</u> data and indicate that the presence of the monoadduct imparts some degree of nuclease resistance upon the oligonucleotide.

PREPARATION OF A PHOSPHOROTHIOATED OLIGO MONOADDUCT

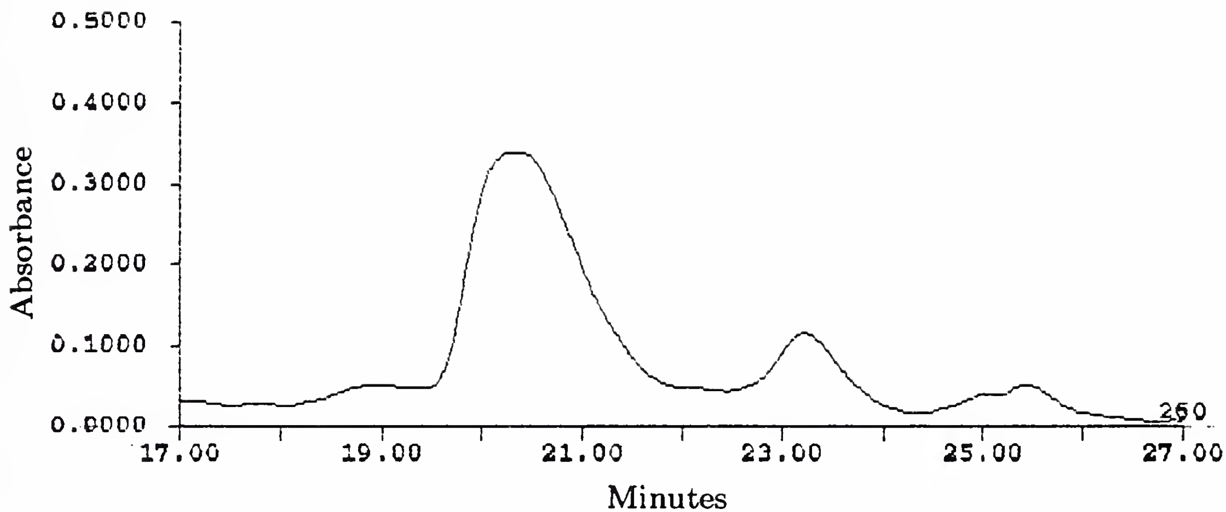
A phosphorothioated 15-mer was also used in the photoreaction experiments. A reaction mixture consisting of the 15-mer:9-mer minihelix and 8-MOP was exposed to 400 nm radiation for 4 hours and



Stability of 9-mer-MA in E. coli. Lanes 1 and 14: 9-mer-MA marker. Lanes 2-13: even numbered lanes - supernatant from cells after exposure to 9-mer-MA for 0 min (lane 2), 10 min. (lane 4), 20 min. (lane 6), and 30 min. (lane 8) incubations on ice, 42°C heat shock (lane 10), and 37°C incubation (lane 12); odd numbered lanes - cellular fractions in the same sequence as the samples above.

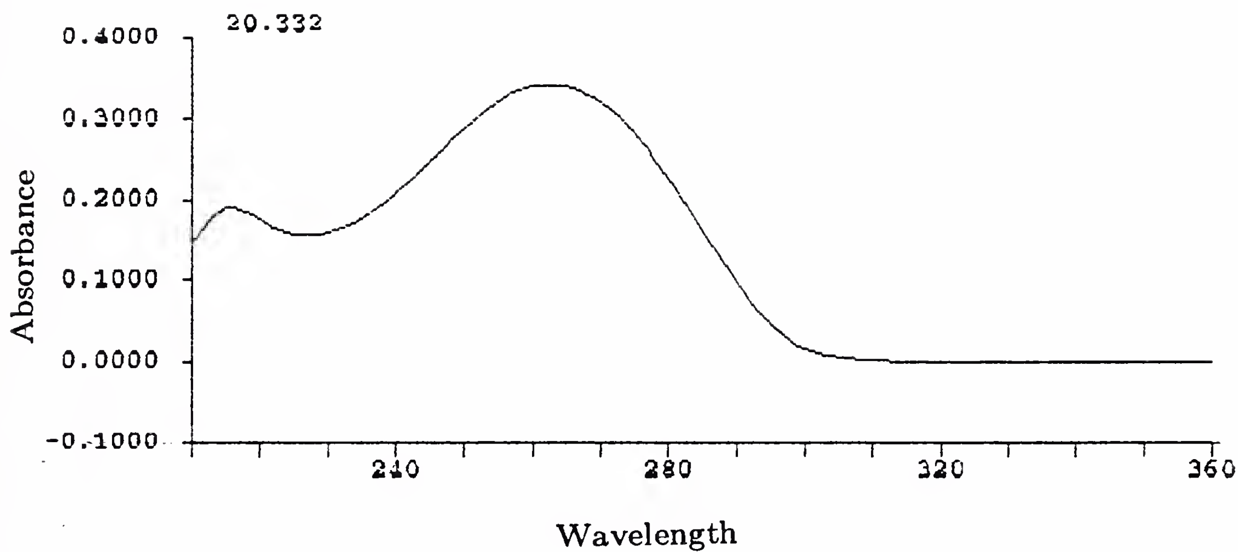
Figure 12

then applied to reversed phase HPLC. Figure 13 shows the chromatographic analysis of this reaction mixture. As above, the peaks were characterized by recording their spectra using a scanning detector as they eluted from the HPLC column. The spectrum of the first peak (figure 14) is classic for DNA. The shoulder at 330 nm in the spectrum of the second peak is characteristic of 4′,5′-monoadducts (figure 15). The percentage of starting material converted into monoadduct ranged from 18-28. The fractions corresponding to the monoadduct were pooled and concentrated. A UV spectrum of the purified monoadduct is found in figure 16. Again, the expected shoulder at 330 nm is observed.



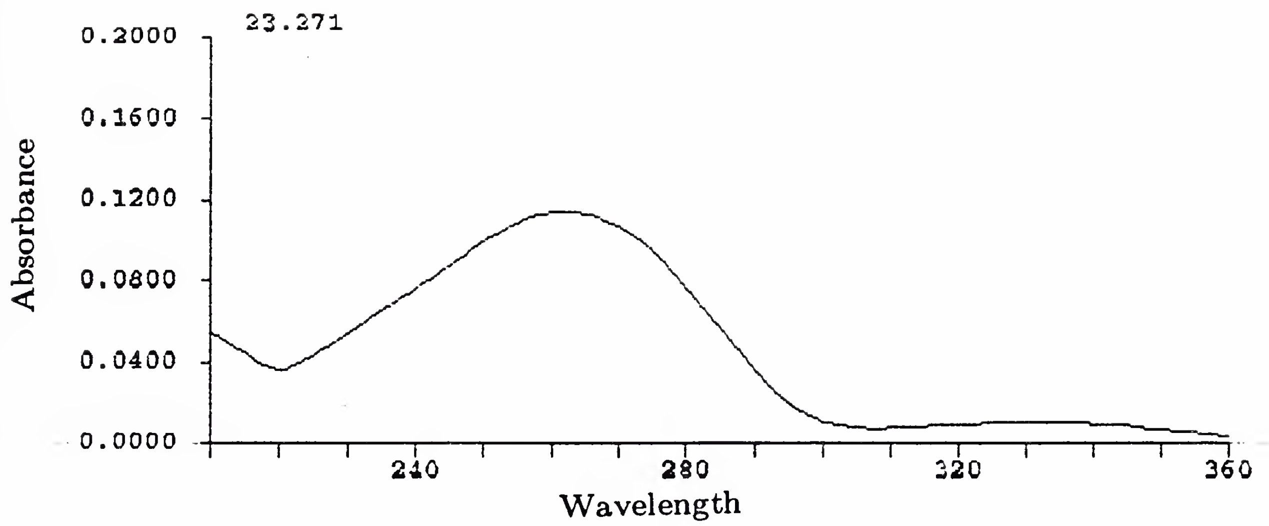
HPLC analysis of 15-mer:9-mer photoreactions. Conditions and gradient of this C4 column are found in the text. Absorbance at 260 nm is shown. Note: scaffolding 9-mer elutes from the column within the first 4 min. of the gradient and is not shown on this figure.

Figure 13



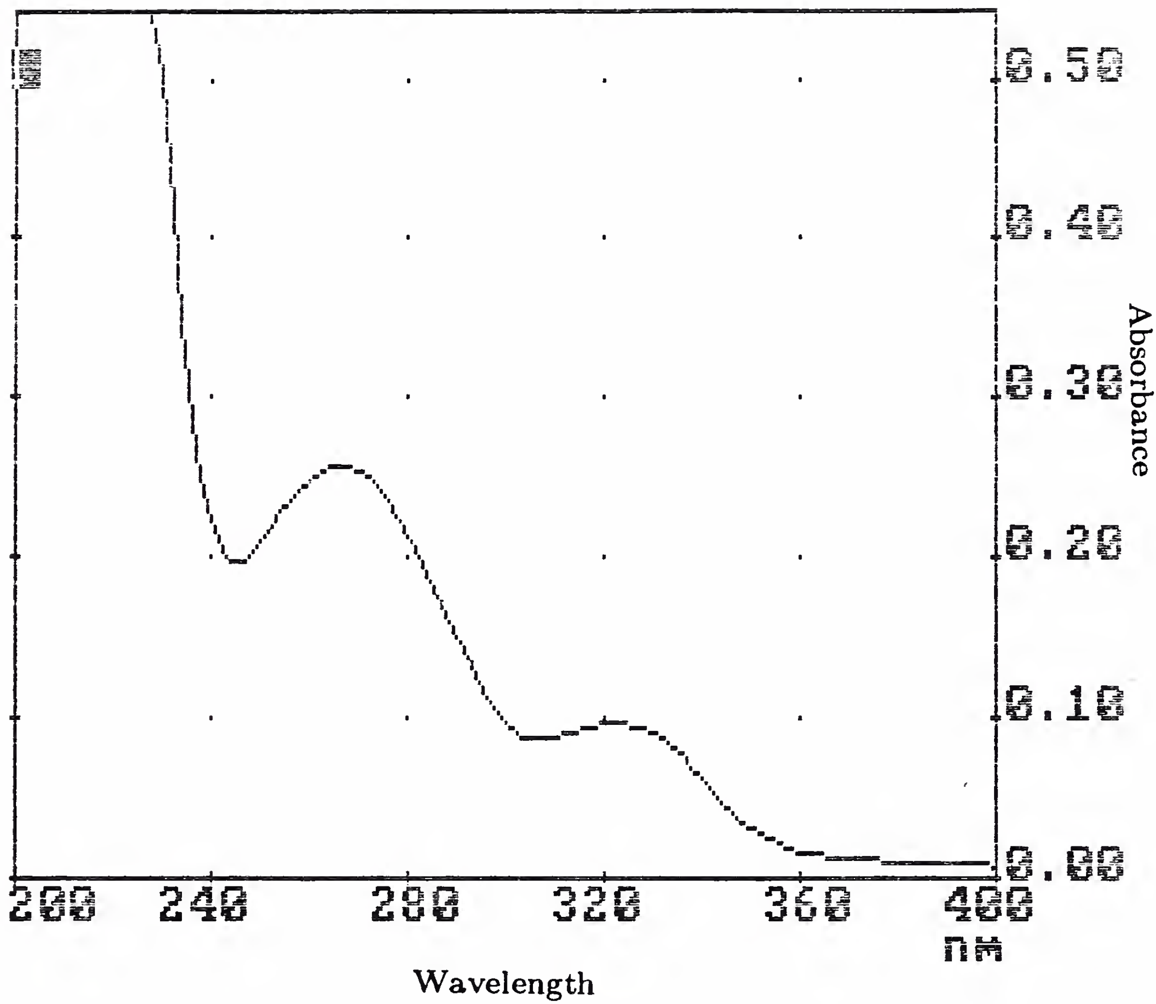
UV absorbance of peak eluting at 20.3 min.

Figure 14



UV absorbance of peak eluting at 23.3 min.

Figure 15



UV absorbance of pooled 15-mer-MA

Figure 16

DISCUSSION

This study was designed to determine whether oligonucleotides could be modified to protect them from enzymatic degradation without destroying their ability to participate in photochemical reactions with psoralens. The particular oligonucleotides studied, a 9-mer and a 15-mer, were chosen because of their use in ongoing antisense experiments. In these investigations, the antisense oligos are psoralen-photomodified and directed against cellular genomic DNA. In the presence of UVA, the psoralen will covalently crosslink the DNA thereby potentially influencing gene expression.[61]

It has been well established that unmodified oligos are very susceptible to degradation by enzymes found in the cytoplasm of cells. This has been confirmed in the present study. Unmodified oligonucleotides were shown to be susceptible to degradation by a 5'-exonuclease (phosphodiesterase II), a 3'-exonuclease (T4 polymerase), and acid phosphatase. They were also digested rapidly when introduced into an in vivo system (E. coli).

As discussed above, several groups have modified the sugar-phosphodiester backbone with methylphosphonate linkages in order to retard digestion by enzymes.[35,36] *Although this modification has successfully increased the stability of the oligos, it has produced several disadvantages. These modified oligos have low solubility in aqueous media,*[34] *involve a synthesis that is not stereospecific thereby increasing the number of stereoisomers produced, and when directed against mRNA do not cause RNase catalyzed*

cleavage of the target. One way to limit these drawbacks is to modify only a subset of the bases of a particular oligo. Tidd and Warenius[36] employed this strategy of partial modification in order to isolate the relative contributions to the degradation of oligonucleotides of several enzymes in fetal calf serum. They modified the 3'-end, the 5'-end, or both and concluded that blockage of the 3'-end was sufficient to enhance the lifetime of the oligo in the presence of fetal calf serum.

In this present work, three different methylphosphonated oligos, each nine bases long, were used: one with methylphosphonates on the three middle bases (9-mer-TAT), one with methylphosphonates on every other base (9-mer-alt), and one with methylphosphonates on the second, third, eighth, and ninth bases (9-mer-AG) (see table 1). When reacted with the 5'-exonuclease, it was the 9-mer-AG that was most resistant to degradation. This oligo is the only one of the three that has two methylphosphonate groups next to each other close to the 5'-terminus.

Similarly, the 9-mer-AG is also most resistant to the 3'-exonuclease. Surprising, however, is the result that 9-mer-TAT, which only has methylphosphonates on the three middle bases, is also quite resistant to this enzyme. One might have expected that the first three unmodified bases of 9-mer-TAT would be cleaved by the exonuclease. This may suggest that the methylphosphonates in the middle alter the conformation of the oligo, thereby decreasing the likelihood of contact with the enzyme or the binding affinity. The results of the experiments with acid phosphatase support this hypothesis. 9-mer-TAT is the only one of the three oligos in which

any of the starting material remains after the 30 min. digestion. Since the 5'-terminus is unmodified in all three oligos, there must be another factor allowing 9-mer-TAT to be more resistant. A conformational change could explain this difference.

In addition, 9-mer-MA also conferred increased stability to the oligo. This was seen both in the in vitro and the in vivo settings. The in vivo data shows that the 9-mer-MA introduced into E. coli persists even after one hour incubation at 37°C. As in the above example, this oligo has unmodified ends potentially at risk for degradation. Yet, the psoralen attached in the middle of the piece of DNA makes it less susceptible to degradation as compared to the unmodified oligonucleotide. Several explanations are possible to account for this result. It is possible that the enzyme is blocked by steric hinderance - that is that the somewhat "bulky" psoralen physically impedes the contact of the enzyme with the oligo. The above hypothesis of a conformational change is also conceivable.

These findings demonstrate an increased utility for the psoralen-modified oligos. The monoadduct in the presence of UVA forms a covalent crosslink with the target DNA thereby increasing the length of time the target is inactivated. In addition, it now appears that the psoralen also allows more oligos to reach their targets intact.

One concern of using modified oligonucleotides, either methylphosphonates or phosphorothioates, is that these changes in the backbone would interfere with the photochemistry necessary to create the psoralen monoadducts. The photochemical experiments done, however, demonstrate that both methylphosphonates and

phosphorothioates can be photomodified with psoralen with approximately the same yields as with unmodified 9-mer. Even the 9-mer-TAT and the 9-mer-alt, in which the particular thymidine that is photomodified has the methylphosphonate substitution, are able to react with psoralen.

As Gasparro et. al.[61] *have stressed, this method of photomodification offers several advantages over other strategies used to attach psoralens to oligos. Other groups*[53,54,55] *(see above) have employed a tether to link the psoralen to the oligo. The strategy used by Gasparro et. al. and used in this paper is relatively straightforward, involving a four hour irradiation and HPLC purification. Second, in order to attach the tether to the oligo, the above groups have had to use the 4′ position in the furan ring. These papers have noted that use of this sight has disrupted effective photochemical reactions and led to increased photodegradation of the product.*

Thus, these experiments have shown several ways to improve upon the antisense technology. Several additional studies are being planned to further characterize the phosphorothioates and the monoadducts. The in vivo study should be repeated in a eukaryotic system. Previous work (see above) has shown that phosphorothioates preferentially target the nucleus. Experiments to localize phosphorothioate monoadducts should also be performed.

The problem of stability of the oligonucleotides is of great concern when trying to apply this technology to the clinical setting. The ability to control gene expression at the level of the DNA has great therapeutic potential; however, in order for the

oligonucleotide to reach the DNA it must cross two hostile compartments- the cytoplasm and the nucleus. It is imperative, therefore, that the oligonucleotides are protected, but protected without destroying their usefulness.

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