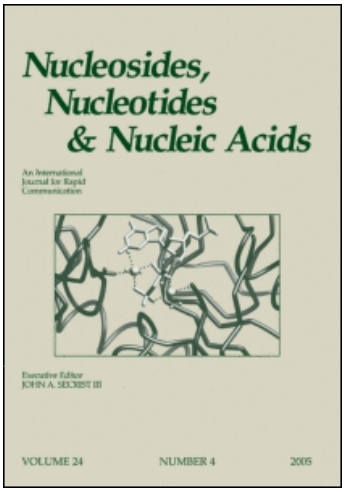


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# ANTISENSE OLIGONUCLEOSIDE METHYLPHOSPHONATES AND THEIR DERIVATIVES

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**Abstract:** The effects of psoralen derivatized oligo-2'-deoxyribonucleoside methylphosphonates on VSV-infected cells and the interaction of a oligo-2'-O-methylribonucleoside methylphosphonate with a complementary oligoribonucleotide are described.

Oligo-2'-deoxyribonucleoside methylphosphonates serve as antisense inhibitors of mRNA translation and processing both *in vitro* and in cell culture systems[1,2]. The inhibitory effects of these nonionic, nuclease resistant nucleic acid analogs most likely result from formation of hydrogen-bonded duplexes with their targets. Such duplex formation could prevent proteins which are responsible for the expression or processing of the mRNA from binding to the mRNA in a productive manner. Efforts have been underway for some time to improve the efficacy of the methylphosphonate oligomers through derivatization with various functional groups or through further modification of the sugar phosphate backbone of the oligomer. In this report we describe recent studies on the effect of antisense psoralen-derivatized oligo-2'-deoxyribonucleoside methylphosphonates on vesicular stomatitis virus infected cells. We also present preliminary studies on a new type of nonionic oligonucleotide analog, the oligo-2'-O-methylribonucleoside methylphosphonates.

Psoralen Derivatized Oligo-2'-deoxyribonucleoside Methylphosphonates We have previously reported the synthesis of psoralen derivatized oligonucleoside methylphosphonates and their interactions with complementary single-stranded oligo-DNA and with rabbit globin mRNA[3,4,5,6]. Methylphosphonate oligomers have been derivatized with either

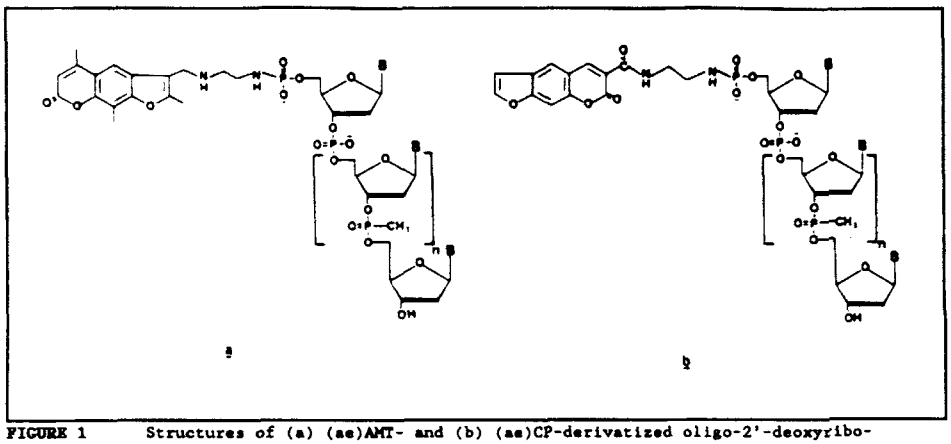

FIGURE 1 Structures of (a) (ae)AMT- and (b) (ae)CP-derivatized oligo-2'-deoxyribonucleoside methylphosphonates.



4'-[[*N*-(2-aminoethyl)amino]methyl]-4,5',8-trimethylpsoralen, (ae)AMT, or with 3-[(2-aminoethyl)carbamoyl]psoralen, (ae)CP. In each case the psoralen group carries a linker arm which is attached to a 5'-terminal phosphate group of the oligomer *via* a nuclease resistant phosphoramidate linkage (see FIGURE 1). When the oligomer binds to its complementary target sequence, the psoralen group is positioned such that it can cross-link with a pyrimidine residue in the target. Cross-linking is initiated by irradiation with long wavelength (365 nm) ultraviolet light. This wavelength is above the absorption range of the nucleic acid bases and therefore irradiation does not affect the integrity of non-targeted nucleic acids. In the case of (ae)AMT-oligomers, cross-linking occurs through photocycloaddition of the psoralen pyrone ring whereas in the case of (ae)CP-oligomers, cross-linking occurs through the psoralen furan ring.

The interaction and photochemistry of psoralen-derivatized oligonucleoside methylphosphonates have been studied most extensively with single-stranded DNA targets[3,5,6]. The extent of cross-linking depends upon the length of the oligomer, the type of psoralen derivative [(ae)AMT or (ae)CP] the temperature at which irradiation is carried out, and the nucleotide sequence at the psoralen cross-linking site. The psoralen group can bind to the target strand by two different modes. The psoralen ring can stack on the base-pair formed between the 5'-

terminal nucleoside of the oligomer and its complementary base in the target and partially intercalate with the next 3'-base in the target. This binding mode appears to be favored by (ae)AMT-oligomers. Alternatively the psoralen ring can fold back and intercalate between the last two base pairs formed between the oligomer and the target, a mode favored by the (ae)CP-oligomers[6].

Cross-linking of (ae)AMT-oligonucleoside methylphosphonates with random coil, single-stranded oligo RNA targets proceeds with the same kinetics as are observed with single-stranded DNA targets. The rate of crosslinking is essentially linear during the first 5 min of irradiation, begins to level off after 10 min and remains constant upon further irradiation. Failure to achieve further cross-linking after 10 min is apparently due to photodegradation of the psoralen. Cross-linking of (ae)CP-oligomers with oligo-RNA has not yet been studied nor has cross-linking with oligo-RNA targets which contain stem-loop secondary structure been examined. The latter RNA targets would be expected to have structural features similar to those which exist at oligomer binding sites in biological RNAs.

(ae)AMT-derivatized methylphosphonate oligomers specifically crosslink with rabbit α- or β-globin mRNA *in vitro* and as a result, specifically inhibit translation of the globin mRNA in a cell-free reticulocyte lysate at low (5 $\mu M$) oligomer concentrations[4]. The phosphoramidate linkage of (ae)AMT-methylphosphonate oligomers is resistant to hydrolysis by both endo- and exonucleases, whereas the single phosphodiester linkage is slowly hydrolyzed after prolonged incubation with $S_1$ nuclease. An (ae)AMT-derivatized octamer was shown to be taken up intact by mammalian cells in culture. These properties suggest that (ae)AMT-oligomers could be used as antisense inhibitors in living cells. To test this, we have prepared (ae)AMT-derivatized oligonucleoside methylphosphonates with sequences complementary to the coding regions near the initiation codon of vesicular stomatitis virus (VSV) M-protein mRNA. The sequence of one of these oligomers, M-2, is shown below, where the box represents the psoralen group and the methylphosphonate linkages are underlined.

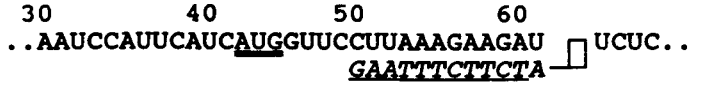

TABLE 1. Crosslinking of M-2 with nucleic acids in VSV-infected cells.

| Series[a] | Virus | UV | Nuclease | fmoles Oligomer in Cell Residue Expt 1 | Expt 2 |
|---|---|---|---|---|---|
| 1 | - | - | none | 27 | -- |
| | - | + | " | 25 | 18 |
| | + | - | " | 17 | 16 |
| | + | + | " | 171 | 133 |
| 2 | + | + | none | 352 | 281 |
| | + | + | DNase | 310 | 270 |
| | + | + | RNase | 85 | 81 |

a. $6X10^4$ and $1X10^5$ cells were used in series 1 and 2 respectively



The ability of M-2 to cross-link to nucleic acids within VSV-infected cells was investigated. [$^{32}$P]-Labeled M-2 at a concentration of 1 $\mu$M was added to VSV-infected mouse L-cells at the time of infection or to uninfected mouse L-cells and the cells were incubated at 37°C for 6 hrs. The cells were then irradiated for 5 min at 365 nm. Control cells remained in the dark. The cells were then fixed with methanol and the fixed cells were extensively washed with 50% acetonitrile/water until radioactivity was no longer removed from the plate. The cell residue was solubilized with phosphate buffered saline containing 1% sodium dodecylsulfate and counted (see TABLE 1 Series 1). In a separate series of experiments (Series 2) the fixed cells were incubated with DNase or RNase after the aqueous acetonitrile wash and were then washed again with aqueous acetonitrile after the nuclease treatment. The results of these experiments are shown in TABLE 1.

Crosslinking occurs only in virus-infected cells which have been irradiated. The sensitivity of the crosslinked material to RNase, suggests that the oligomer is cross-linked to VSV RNA. Further experiments will be required to determine if the oligomer is in fact cross-linked to VSV M-protein mRNA. However, it appears that psoralen cross-linking will provide a unique way to study the interaction and mechanism of action of antisense oligomers in living cells.

TABLE 2. Effect of d-(ae)AMTpApTCTTCTTTAAG on VSV protein synthesis in VSV-infected mouse L-cells[a]

| VSV Protein | % Inhibition[b] |
|---|---|
| L | - 14 |
| G | - 10 |
| NS | 9 |
| N | 14 |
| M | 37 |

a. VSV-infected mouse L-cells were irradiated in the presence of 5 μM oligomer for 5 min at 22°C.
b. "-" indicates stimulation



The effects of M-2 on VSV protein synthesis were also investigated. Mouse L-cells were infected with VSV at a multiplicity of 5 and oligomer M-2 was added to the culture medium at a concentration of 5 μM. The infected cells were incubated in the presence of the oligomer for 6 hrs. The cell culture medium was then removed and replaced with methionine-free medium. After further incubation for 15 min, [$S^{35}$]-methionine was added and the cells were immediately irradiated at 365 nm for 5 min at room temperature. The cells were then incubated for an additional 5 min and lysed. The 5 VSV proteins in the lysate were separated by polyacrylamide gel electrophoresis and quantitated by scanning densitometry. The amounts of each of the proteins were compared with proteins from VSV-infected cells which had been similarly irradiated in the absence of the oligomer. Control experiments showed that irradiation for 5 min did not affect virus protein synthesis to a significant extent.

The effect of M-2 on VSV protein synthesis is shown in Table 2. It appears that M-2 inhibits synthesis of M-protein in a specific manner and has little or no inhibitory effect on synthesis of the other four VSV proteins. The inhibitory effect is produced by irradiation of the cells. Thus control experiments showed that incubation of VSV-infected cells with oligomer in unirradiated cells produced no inhibitory effects on synthesis of VSV proteins. Previous experiments have shown that oligonucleoside methylphosphonates complementary to the initiation codon

regions of either N-, NS- or G-protein mRNA inhibit syntheses of all five VSV proteins in VSV-infected mouse L-cells 10%-45% at 50 μM concentration[7]. Psoralen derivatization appears to increase the efficiency of inhibition by antisense oligonucleoside methylphosphonates approximately 10 fold. As described above, similar increases were observed for inhibition of *in vitro* translation of globin mRNA. Thus derivatization by psoralen or other functional groups which are capable of cross-linking oligonucleoside methylphosphonates to their targeted nucleic acids, would appear to be a valuable approach to enhancing the efficacy of these antisense molecules.

Oligo-2'-O-methylribonucleoside Methylphosphonates Previous studies have shown that oligonucleoside ethylphosphotriesters which contain 2'-O-methylribonucleosides form more stable complexes with transfer RNA than do those which contain 2'-deoxyribonucleosides[8]. Similarly, oligo-2'-O-methylribonucleotides form duplexes with complementary oligoribonucleotides which have higher melting temperatures (Tms) than duplexes formed by oligo-2'-deoxyribonucleotides of the same sequence[9]. These observations suggested that antisense oligonucleoside methylphosphonates in which 2'-O-methylribonucleosides replaced 2'-deoxyribonucleosides might show enhanced stability when interacting with their target nucleic acids.

To test this possibility, we have prepared r-U$^{m}$pI$^{m}$A$^{m}$U$^{m}$C, where N$^{m}$ represents a 2'-O-methylribonucleoside and the underline shows the position of the methylphosphonate linkages. The internucleotide linkage at the 5'-terminus of the oligomer is a phosphodiester group. The required 2'-O-methylnucleosides were prepared by literature procedures[10,11]. These were converted to 5'-O-dimethoxytrityl-3'-O-methyl-N,N-diisopropylaminophosphonamidites by reaction of the 5'-O-dimethoxytrityl-2'-O-methylribonucleoside with methyl dichlorophosphine followed by reaction with diisopropylamine in the presence of ethyldiisopropylamine. The overall yield starting from the 2'-O-methylribonucleoside was 60% to 65%. The 6-amino group of A$^{m}$ was protected with a phenoxyacetyl group.

The phosphonamidite synthons were used to prepare r-U$^{m}$pI$^{m}$A$^{m}$U$^{m}$C on a Biosearch Model 8700 DNA synthesizer. Controlled pore glass derivatized with N-benzoyl-5'-dimethoxytrityl-3'-O-*t*-butyldimethylsilylcytidine was used as the support. The phosphonamidite synthons were used at a

concentration of 0.065 M and the coupling time was 15 min for each synthetic cycle. The last (5'-) nucleotide unit was added as the 5'-O-dimethoxytrityl-2'-O-methyluridine-3'-O-N,N-diisopropylamino-ß-cyanoethyl phosphoramidite. The average coupling yield was 96%, which is comparable to the average coupling yields obtained in syntheses of oligo-2'-deoxyribonucleoside methylphosphonates.

The oligomer was deprotected and simultaneously removed from the support by sequential treatment with hydrazine hydrate in pyridine-acetic acid buffer followed by treatment with ethylenediamine in 95% ethanol (1:1 v/v). The oligomer with the 2'-O-t-butyldimethylsilyl group still attached, was purified by DEAE cellulose chromatography followed by reversed phase HPLC and was obtained in 46% overall yield. The silyl protecting group was removed quantitatively by treating the oligomer with 0.05 N HCl in acetonitrile/water (2:1) at 37°C for 20 hrs.

Like oligo-2'-deoxyribonucleoside methylphosphonates, r-U$^{m}$pI$^{m}$A$^{m}$U$^{m}$C could be phosphorylated by polynucleotide kinase in the presence of ATP. The phosphorylated oligomer migrated as a single band when subjected to polyacrylamide gel electrophoresis under denaturing conditions. Treatment of the phosphorylated oligomer with 1 M aqueous piperidine resulted in cleavage of the methylphosphonate linkages and this reaction coupled with polyacrylamide gel electrophoresis[12] was used to confirm the chainlength of the oligomer.

The presence of the 2'-O-methyl group stabilizes the phosphodiester linkage toward hydrolysis by the exonuclease, spleen phosphodiesterase. As shown in FIGURE 2, the diester linkage of r-U$^{m}$pI$^{m}$A$^{m}$U$^{m}$C is partially hydrolyzed by 0.69 units of enzyme in 45 µL with a half life of approximately 15 min whereas the diester

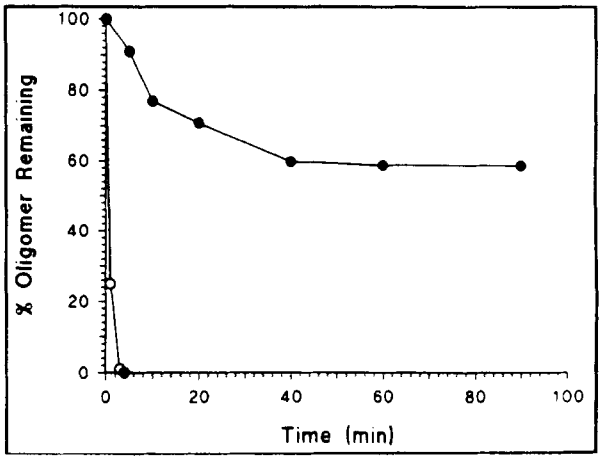


FIGURE 2. Hydrolysis of r-U$^{m}$pI$^{m}$A$^{m}$U$^{m}$C (open circles) and d-TpGATC (closed circles) by spleen phosphodiesterase at 37°C.

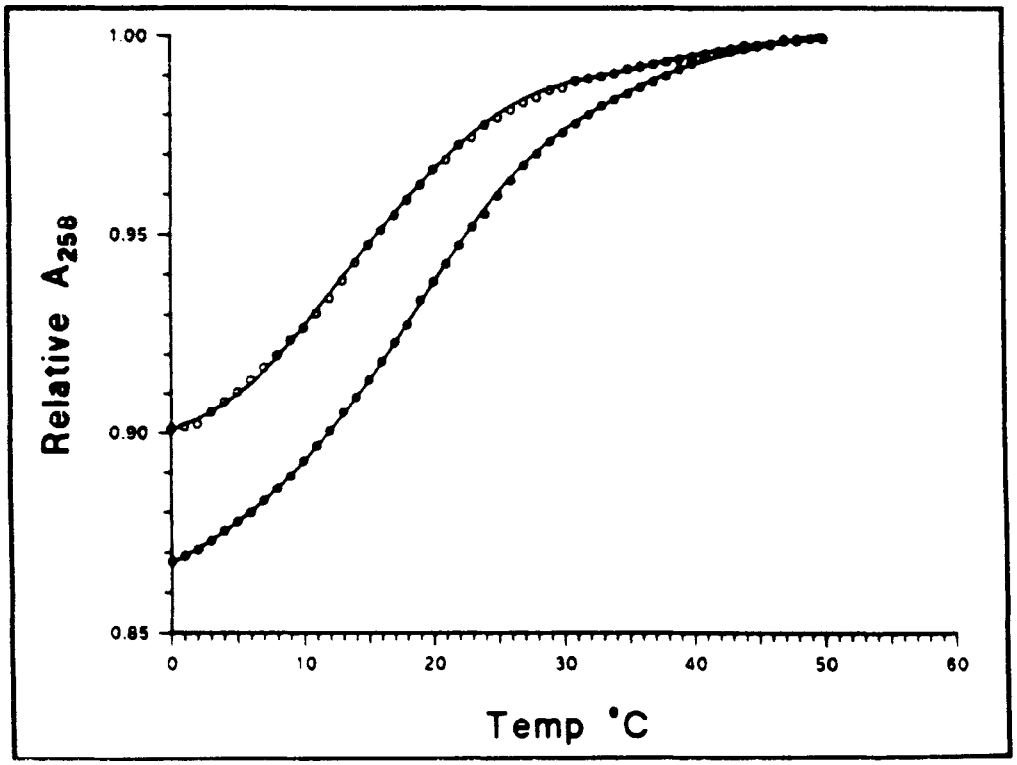


**FIGURE 3** . Melting transitions of the r-U$^m$pI$^m$A$^m$U$^m$C/r-GAUCA duplex in 50 mM Tris (open circle) and 50 mM Tris-0.1 M NaCl (closed circle), pH 7.5.



linkage in d-TpGATC is completely hydrolyzed within 4 min at this enzyme concentration. Addition of fresh enzyme to the r-U$^m$pI$^m$A$^m$U$^m$C reaction mixture did not result in further hydrolysis. This result suggests the rate of hydrolysis of the phosphodiester linkage may be influenced both by the presence of the 2'-O-methyl group and by the configuration of the neighboring methylphosphonate linkage(s).

The stability of the duplex formed between r-U$^m$pI$^m$A$^m$U$^m$C and an oligo-RNA target, r-GAUCA, was compared with those of similar duplexes formed by d-TGATC and with d-TpGATC. The experiments were carried out in 50 mM Tris or 50 mM Tris containing 0.1 M NaCl at a total oligomer strand concentration of 20 $\mu$M. As shown in FIGURE 3, r-U$^m$pI$^m$A$^m$U$^m$C forms a stable duplex with r-GAUCA under these conditions. The melting

temperature of the duplex increases with increasing ionic strength. This effect most likely results form the reduced charge repulsion between the 5'-phosphodiester linkage of r-U$^{m}$pI$^{m}$A$^{m}$U$^{m}$C and the negatively charged backbone of the target. The rather broad transition curve is reflective of the short length of the oligomer and is also observed with the d-TGATC/r-GAUCA duplex.

TABLE 3. Melting temperatures of r-GAUCA duplexes.

| | $T_m$°C | |
|---|---|---|
| OLIGOMER | 50 mM Tris | 50 mM Tris/ 0.1 M NaCl |
| d-TGATC | 15 | 22 |
| d-TpGATC | < 9 | 18 |
| r-U$^{m}$pI$^{m}$A$^{m}$U$^{m}$C | 15 | 21 |

The melting temperatures of the various duplexes are shown in TABLE 3. The d-TpGATC/ r-GAUCA duplex melted over a broader range than did r-U$^{m}$pI$^{m}$A$^{m}$U$^{m}$C /r-GAUCA and had a significantly lower melting temperature in 50 mM Tris. The melting temperature of r-U$^{m}$pI$^{m}$A$^{m}$U$^{m}$C/r-GAUCA was similar to that of d-TGATC/r-GAUCA under both salt conditions, although the transition curves of the r-U$^{m}$pI$^{m}$A$^{m}$U$^{m}$C-duplex were somewhat sharper. The I$^{m}$ residue of r-U$^{m}$pI$^{m}$A$^{m}$U$^{m}$C forms only two hydrogen bonds with C whereas the G residues of d-TGATC and d-TpGATC each form three hydrogen bonds. Thus one would expect a further increase in the stability of the oligo-2'-O-methylribonucleoside methylphosphonate if the more difficult to synthesize G$^{m}$ is substituted for I$^{m}$.

The increased stability of r-U$^{m}$pI$^{m}$A$^{m}$U$^{m}$C/r-GAUCA may be due to the ability of r-U$^{m}$pI$^{m}$A$^{m}$U$^{m}$C to more readily assume a A-type conformation. Coupled with psoralen derivatization, oligo-2'-O-methylribonucleoside methylphosphonates could prove to be effective antisense inhibitors in cell culture at low oligomer concentrations.

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