Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 15, pp. 1735-1740, 1995

0960-894X/95 $9.50+0.00

0960-894X(95)00292-8

SYNTHESES AND PROPERTIES OF NOVEL THIONO TRIESTER MODIFIED ANTISENSE OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES

Zhaoda Zhang, Jimmy X. Tang and Jin Yan Tang*

Hybridon, Inc., One Innovation Drive, Worcester, MA 01605, USA

ABSTRACT. A new class of the thiono triester containing oligodeoxynucleotide phosphorothioates, an antisense inhibitor against HIV, has been synthesized, which shows improved nuclease stability, cellular association and binding affinity.

Antisense oligonucleotides have been shown to regulate the expression of genes and to be effective inhibitors against HIV, influenza virus, human papilloma virus and herpes simplex virus in tissue culture studies.[1] In order to use antisense oligonucleotides as therapeutic agents, it is necessary to develop compounds that are stable under biological and physiological conditions, that are capable of binding to a complementary nucleic acid target effectively, and can be readily taken up by cells. To meet these criteria, considerable efforts have recently been made in the structure modification of oligonucleotides.[2]

We report herein synthesis and properties of a new class of the thiono triester modified oligodeoxynucleotide phosphorothioates (S-triester-phosphorothioates) that contain a non-ionic internucleotide linkage and a lipophilic group. A number of small alkyl (e.g., methyl, ethyl, isopropyl, trifluoroethyl and neopentyl) triester linkages have been incorporated into oligonucleotides.[3] Several large lipophilic groups (e.g., Cholesterol and adamantane moieties) were connected to the 3' end via a short linker on the CPG, and attached directly to the 5' end by post-synthesis method, H-phosphonate chemistry and phosphoramidite chemistry.[2b] However, the most commonly used linkers (the corresponding phosphoramidites) do not contain nucleosides, which restricts their use as terminal modifiers. Since the nature and position of the groups incorporated may influence the ability of the modified oligonucleotide to transverse cell membranes or to hybridize effectively to mRNA, it is desired to be able to introduce a non-ionic internucleotide linkage and a lipophilic group into oligonucleotides at any preselected positions (terminal and/or internal). Therefore, we have synthesized a series of 5'-DMT-nucleoside-*O*-alkyl-phosphoramidites and used them in the syntheses of S-triester-phosphorothioates. The sequence of the oligonucleotide phosphorothioate modified here, 5'-dCTCTCGCACCCATCTCTCTCCTTCT-3' (**GEM 91**), has been identified as an antisense inhibitor against HIV.[4] Five alkyl groups (i.e., *ethyl, isopropyl, 1- adamantyl-2-ethyl, Cholesteryl-3-carboxyamino-6-*

hexyl and *1-hexadecyl*) have been incorporated into the oligonucleotide phosphorothioates to form a thiono triester internucleotide linkage (Figure 1). To minimize changes in the geometry of the internucleotide bond and maintain the stability and fidelity of oligomer binding, only one or two triester linkages having a bulky group (1-adamantyl-2-ethyl, Cholesteryl-3-carboxyamino-6-hexyl and 1-hexadecyl) were incorporated into the oligonucleotides.

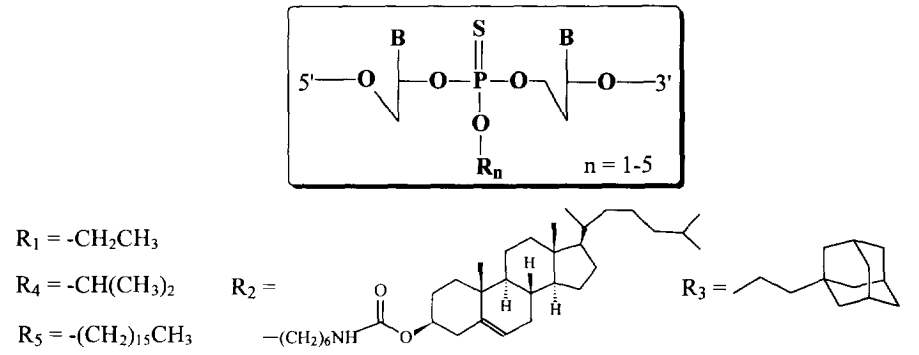
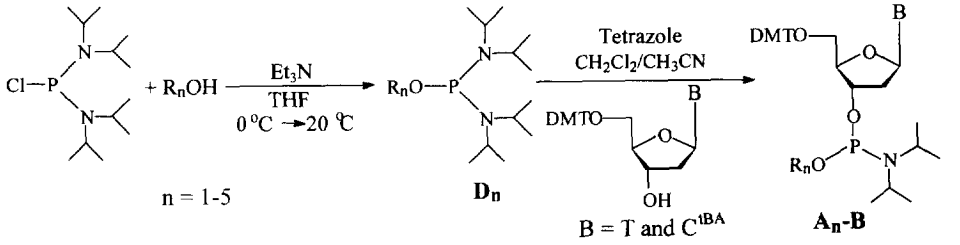


Figure 1. The Thiono Triester Internucleotide Linkages.

The 5'-DMT-nucleoside-*O*-alkyl-phosphoramidites (**A_n-B**) were synthesized in two steps as shown in Scheme 1: (1) bis(diisopropylamino)chlorophosphine was converted to the *O*-alkyl-phosphordiamidites (**D_n**) in the reaction with the corresponding alcohols and triethylamine in dichloromethane; (2) phosphitylation of 5'-DMT-2'-deoxynucleosides, dC^{tBA} and T, by the resultant *O*-alkyl-phosphordiamidites (**D_n**) plus tetrazole gave the 5'-DMT-nucleoside-*O*-alkyl-phosphoramidites (**A_n-B**) in good to excellent yield.[5] All the phosphoramidites were purified by silica-gel column chromatography (eluent: CH_2Cl_2/AcOEt/Et_3N) and some (**A_1-B** and **A_4-B**) were also precipitated from hexane at -78 °C.



Scheme 1. Synthesis of 5'-DMT-Nucleoside-*O*-Alkyl-Phosphoramidites.

The S-triester-phosphorothioate oligonucleotides were synthesized using the phosphoramidites protected by the base labile *tert*-butylphenoxyacetyl (tBA)[6a] group on the exocyclic amine (dA, dC, and dG). The increased lability of the tBA group over the standard benzoyl and isobutyryl protection permitted

the use of milder conditions for the deprotection of the bases and the release from solid support with ammonium hydroxide (room temperature, 2 h), which was crucial in the syntheses of the *O*-ethyl containing oligonucleotides.[6b] All of the triester containing oligonucleotides were synthesized on a 1 μmol scale using an automated synthesizer (Millipore 8909 Expedite™, Bedford, MA). The oligonucleotides were purified and analyzed by reverse-phase HPLC and/or PAGE, and the PAGE purified samples were desalted by using SEP-PAK cartridges.

A critical step in the action of antisense oligonucleotides involves hybridization to their complementary nucleic acid targets. The melting temperatures[7] were measured for the modified oligonucleotides as well as the unmodified analog to understand and predict the duplex stability (Table 1). Modification with triester linkage(s) reduces the total negative charge of the oligonucleotide. It would therefore be expected that these modifications would enhance hybridization. As Table 1 shows, the melting temperatures of duplexes formed between S-triester-phosphorothioates (**1-11**) and complementary DNA strands are higher than that formed by the equivalent unmodified phosphorothioate (**GEM 91**).

Table 1. The Melting Temperature (Tm) and Cellular Uptake of Oligonucleotides.

Number	Sequence[a,b]	Modification	Tm (°C)	Cell association[c]
GEM 91	5'-dCTC TCG CAC CCA TCT CTC TCC TTC T-3'	P=S	52.2	7.4
1	5'-dCTC TCG CAC CCA TCT CTC TC$\underline{C_1}$ $\underline{T_1T_1C_1}$ T-3'	P=S	56.8	11.4
1-O	5'-dCTC TCG CAC CCA TCT CTC TC$\underline{C_1}$ $\underline{T_1T_1C_1}$ T-3'	P=O	65.8	--
2	5'-dCTC TCG CAC CCA TCT CTC TCC TT$\underline{C_2}$ T-3'	P=S	57.5	19.9
3	5'-dCTC TCG CAC CCA TCT CTC TCC TT$\underline{C_3}$ T-3'	P=S	56.8	11.6
4	5'-dCTC TCG CAC CCA TCT CTC TCC $\underline{T_3}$T$\underline{C_3}$ T-3'	P=S	55.9	--
5	5'-dCTC TCG CAC CCA TCT CTC TCC T$\underline{T_3C_3}$ T-3'	P=S	55.2	14.1
6	5'-dCTC TCG CAC CCA TCT CTC TC$\underline{C_4}$ $\underline{T_4T_4C_4}$ T-3'	P=S	55.5	--
7	5'-dCTC TCG CAC CCA TCT CTC TCC TT$\underline{C_5}$ T-3'	P=S	56.1	19.8
8	5'-d$\underline{C_3}$TC TCG CAC CCA TCT CTC TCC TT$\underline{C_3}$ T-3'	P=S	55.8	--
9	5'-d$\underline{C_1}$TC TCG CAC CCA TCT CTC $\underline{T_1}$CC TT$\underline{C_2}$ T-3'	P=S	55.2	--
10	5'-d$\underline{C_1}$TC TCG CAC CCA TCT CTC TCC TT$\underline{C_2}$ T-3'	P=S	57.1	--
11	5'-d$\underline{C_5}$TC TCG CAC CCA TCT CTC TCC TT$\underline{C_5}$ T-3'	P=S	54.5	--

[a] $\underline{N}$ indicates the triester linkage at 3' side and subscripts correspond to R_1-R_5. [b] R_1= ethyl; R_2= Cholesteryl-3-carboxyamino-6-hexyl; R_3=1-adamantyl-2-ethyl; R_4= isopropyl; R_5=1-hexadecyl. [c] Mean FITC fluorescence.

These results indicate that the modified oligonucleotides would be better candidates than the

chimeric oligodeoxynucleotides containing the methyl phosphonate and phosphonothioate because the latter showed lower melting temperatures and poor hybridization.[8] It also shows that the melting temperature of O-triester-phosphodiester (**1-O**) is about 10 degrees higher than that of the corresponding S-triester-phosphorothioates, which is also consistent with the results obtained from the unmodified oligonucleotides.[2a]

The effect of the S-triester-phosphorothioate modification on cellular association was studied by flow cytometry using the fluorescein (FITC) conjugated-oligonucleotides (Table 1).[9] For these S-triester-phosphorothioates (**2, 5,** 7) which were modified with a large lipophilic group (i.e., 1-adamantyl-2-ethyl, Cholesteryl-3-carboxyamino-6-hexyl and 1-hexadecyl), cellular association was significantly enhanced.

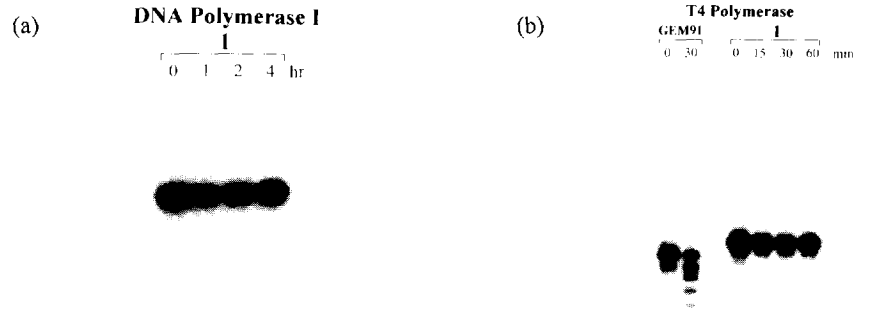


Figure 2. Digestion of the Modified *O*-Ethyl Triester Phosphorothioate Oliginucleotide (**1**) by 3'-Exonuclease Activity: (a) DNA Polymerase I, (b) T4 Polymerase.

The nuclease sensitivity of the S-phosphorothioate (**GEM 91**), S-triester-phosphorothioate and O-triester-phosphodiester oligonucleotides were studied. Since degradation of phosphorothioates oligonucleotides occurs with enzymes primarily on the 3' end,[1d,10] two exonucleases, T4 polymerase and DNA polymerase I, were chosen for comparative digestion studies.[11] The modified oligonucleotide (**1**) was examined for its sensitivity to digestion by 3' exonuclease using DNA polymerase I (Figure 2a). The S-phosphorothioate (**GEM 91**) and S-triester-phosphorothioate (**1**) were compared for their sensitivities to digestion using T4 polymerase (Figure 2b). Since no significant degradation of **1** was observed in either experiment, it is clear that

the modified S-triester-phosphorothioate is much more stable than the unmodified S-phosphorothioate.

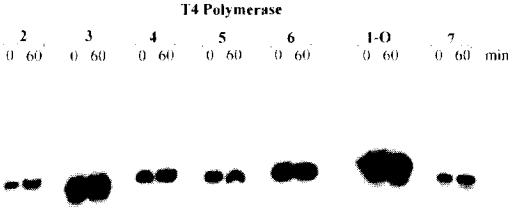


Figure 3. Digestion of the *O*-alkyl triester modified oligonucleotides by T4 polymerase.

The exonuclease resistance of the other modified S-triester-phosphorothioates (**2**-**7**) and O-triester-phosphodiester (**1-O**) oligonucleotides were also studied using T4 polymerase (Figure 3). It is shown that not only the modified S-triester-phosphorothioate, but also the O-triester-phosphodiester oligonucleotides, have increased exonuclease resistance. This is important because the S-triester-phosphorothioate may be converted to O-triester-phosphodiester in cells,[12] and the increased persistence of O-triester-phosphodiester would provide a longer duration of action *in vivo*.

In summary, this research demonstrates that several biologically important properties of oligonucleotide phosphorothioates can be improved by introducing triester linkage(s). These thiono triester modified oligonucleotide phosphorothioates exhibit enhanced nuclease stability, cellular association and binding affinity. In addition, the S-triester-phosphorothioate might be slowly hydrolized *in vivo* to phosphorothioate, and would function as a prodrug for the corresponding phosphorothioates. Consequently, it appears that these thiono triester modified oligonucleotide phosphorothioates have opened a new avenue for the development of newer and more potent antisense drugs. These modified oligonucleotides are currently being studied for their bio-reversibility and gene regulation activity. The approach to S-triester-phosphorothioates described here is also applicable to other antisense oligonucleotides.

Acknowledgement. We thank Ms. B. Cauley, Dr. Y. Li and Dr. J. Zhao for their expert technical assistance.

References and Notes

1. (a) Zamecnik, P .C. In *Prospects for Antisense Nucleic Acid Therapy for Cancer and AIDS*; Wickstrom, E., Ed.; Wiley Liss: New York, 1991; pp 1-6. (b) Agrawal, S. *Trends Biotechnol.* **1992**, *10*, 152. (c) Wickstom, E. *Trends Biotechnol.* **1992**, *10*, 281. (d) Mirabelli, C. K.; Crooke, S. T. In *Antisense Research and Applications*; Crook, S. T.; Lebleu, B., Eds.; CRC: Ann Arbor, 1993; pp 7-35, and the

literatures cited in.

2. (a) Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 543. (b) Manoharan, M. In *Antisense Research and Applications*; Crook, S. T.; Lebleu, B., Eds.; CRC: Ann Arbor, 1993; pp 304-340, and the literatures cited in. (c) Cook, P. D. In *Antisense Research and Applications*; Crook, S. T.; Lebleu, B., Eds.; CRC: Ann Arbor, 1993; pp 149-187. (d) Goodchild, J. *Bioconjugate Chem.* **1990**, *1*, 165.
3. Koziolkiewicz, M.; Wilk, A. In *Protocols for Oligonucleotides and Analogs*; Agrawal, S., Ed.; Humana: New Jersey, 1993; pp 207-224, and the literatures cited in. (b) Guga, P.; Koziokiewicz, M.; Okruszek, A.; Uznanski, B.; Stec, W. J. *Nucleosides Nucleotides* **1987**, *6*, 111.
4. Agrawal, S.; Tang, J. Y. *Antisense Res. Dev.* **1992**, *2*, 261.
5. The yields and ^{31}P NMR ($CDCl_3$) data are given below: **D_1** (93.3%, δ 137.8); **D_2** (73.4%, δ 127.0); **D_3** (86.3%, δ 136.7); **D_4** (98.4%, δ 136.2); **D_5** (88.1%, δ 136.3); **A_1-T** (47.0%, δ 157.7, 158.5); **A_1-C** (47.0%, δ 157.7, 158.5); **A_2-T** (48.7%, δ 156.6, 157.1); **A_2-C** (61.1%, δ 157.2, 157.6); **A_3-T** (54.7%, δ 157.9, 158.3); **A_3-C** (58.6%, δ 158.4, 158.7); **A_4-T** (95.2%, δ 158.6, 158.3); **A_4-C** (90.4%, δ 158.9, 159.3); **A_5-T** (98.0%, δ 159.0, 158.3); **A_5-C** (93.5%, δ 158.7, 159.5).
6. (a) Sinha, N. D.; Michaud, D. P.; Roy, S. K.; Casale, R. A. *Nucleic Acids Res.* **1994**, *22*, 3119. (b) The ^{31}P NMR spectrum of the ethyl triester containing oligonucleotide phosphorothioate (**1**) in D_2O shows that the corresponding peaks for S-triester-phosphorothioate and phosphorothioate internucleotide linkages appear at about 62.9 and 51.6 ppm, respectively (the chemical shift was correlated to 85% H_3PO_4). The ratio between S-triester and phosphorothioate is equal to the calculated value (4:20).
7. Thermal melting data were collected from GBC 920 UV-Vis spectrophotometer (Dandenong, Victoria, Australia). Melting temperatures were determined for the duplexes of unmodified or modified oligonucleotides with the complementary DNA. Each oligonucleotide (0.2 A_{260} units) and its complementary DNA was annealed in 1 mL buffer (10 mM Na_2HPO_4, pH 7.4, 0.1 M NaCl) by heating to 80 °C and then cooling down to 40 °C at a rate of 2 °C/minute. The mixture was then reheated to 80 °C at a rate of 1 °C/minute and the A_{260} was continuously recorded.
8. Cook, P. D. *Anti-Cancer Drug Des.* **1991**, *6*, 585.
9. (a) Fluorescein-ON phosphoramidite was purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA). Fluorescein was conjugated to the 5' end of the oligonucleotides by either an automated DNA synthesizer or by a manual procedure using a fluorescein-ON™ phosphoramidite as described in (b). The efficiency of fluorescein labeling was determined by using a spectrofluorometer (excitation 488 nm, emission 520 nm). (b) Technical bulletin for Fluorescein-ON™ Phosphoramidite (TB62036, CLONTECH). (c) Human T cell and leukemia cell line H9 were used in the study. The methods for cell culture and measurement of cell uptake are described in (d). Flow cytometric data on 5,000 viable cell was acquired in listmode on Epics XL (Coulter, Hialeah, FL), and data were analyzed by Epics XL (version 1.5 software) after gating on living cells by forward scatter versus side scatter and propidium iodide staining. (d) Zhao. Q.; Matson, S.; Herrera, C. J.; Fisher, E.; Yu, H.; Krieg, A. M. *Antisense Res. Dev.* **1993**, *3*, 53.
10. Tang, J. Y.; Temsamani, J.; Agrawal, S. *Nucleic Acids Res.* **1993**, *21*, 2729.
11. (a) To study the resistance against DNA polymerase I, 5'-^{32}P labeled oligonucleotides (30 pmole) were dissolved in 20 μL of buffer (50 mM Tris, pH 8.0, $MgCl_2$, 5 mM DTT, 0.05% BSA) and incubated with DNA polymerase I (5.0 units) at 37 °C. Aliquots (5 μL), inhibited by 6 μL of the stop solution (95% formamide, 10 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), were removed at 0, 60, 120, 180 and 240 min, analyzed by PAGE (20% polyacrylamide containing 8.3 M urea), and followed by autoradiography. (b) To study the resistance against T4 DNA polymerase, 5'-^{32}P labeled oligonucleotides (45 pmole) were dissolved in 30 μL of buffer (50 mM Tris, pH 8.0, $MgCl_2$, 5 mM DTT, 0.05% BSA) and incubated with T4 DNA polymerase (7.5 units) at 37 °C. Aliquots (5 μL), inhibited by 6 μL of the stop solution (95% formamide, 10 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), were removed at 0, 60 min, analyzed by PAGE (20% polyacrylamide containing 8.3 M urea), and followed by autoradiography.
12. (a) Wyrzykiewicz, T. K.; Cole, D. L. *Nucleic Acids Res.* **1994**, *22*, 2667. (b) Chae, M. Y.; Postula, J. F.; Raushel, F. M. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1473.

(*Received in USA* 7 *March* 1995; *accepted* 29 *June* 1995)