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Form 3

Abstract of Thesis

Name (Md Ariful Islam)	
Title	**Chemical and Biochemical Studies on Backbone-Modified Oligonucleotides** -Chiral Phosphorothioate and Achiral 5’-Phosphorothiolate Linkage. (骨格修飾型オリゴヌクレオチドに関する化学的・生化学的研究)

Chemically modified nucleic acid analogues that resemble naturally occurring DNA and RNA, are widely used in biochemical research and medicine. Among so many chemical modifications, in my current research I focused on two-backbone modified oligonucleotides (ONs) known as phosphorothioate (PS), where a non-bridging oxygen on phosphorus is replaced by a sulfur atom and 5’-phosphorothiolate (5’-thio) modification, where a sulfur atom replacing the 5’-bridged oxygen connected to the sugar moiety.

In the first part of this research, I investigated a hypothetical chemical property of phosphorothioate modified oligonucleotide (PS-ON) in duplex form. As we know PS-ONs contain chiral centers at the phosphorus atom, resulting in two stereoisomers *S*p and *R*p at phosphorous atom. These stereoisomers have distinct physical and biochemical properties. However, as we know that when a PS-ON forms double helix with its complementary strand, the sulfur substituents on the chiral phosphorus atoms are faced either inside (*S*p) or outside (*R*p) of the duplex depending on the absolute configuration of each phosphorus atom. In this research, I assumed that these type configurations have influence on their reactivity when they are in duplex structure with their complementary stands. So if the free thiol group of PS-ON reacts with an alkyl halide in duplex structure, sulfur atom of *S*p isomer should not participate in the alkylation reaction due to their steric hindrance. On the other hand, due to stereochemical configuration, reactivity of *R*p isomer should increase. In this study, I have been done a known type of alkylation reaction on two stereoisomerically pure *S*p and *R*p PS-ONs bearing a polypyrimidine tract, in duplex structure with their complementary strands. Reactions were performed in both HEPES buffer pH 6.5 and phosphate buffer pH 7.0 using different concentration of alkylating reagent. After several trials, I found reactivity of PS-ON largely depends on stability of duplex and stereochemistry of sulfur substituents. In HEPES buffer where the duplex stability low considering the melting temperature, I found, *S*p isomer reactivity did not have much significantly change due the absence or presence of complementary strands. But in case of *R*p isomer reactivity significantly changed in the presence of complementary strands. On the other hand, in phosphate buffer where the duplex stability much higher than in HEPES buffer showed very interesting results. In phosphate buffer reactivity of *S*p isomer decrease due to the presence of complementary strands where as reactivity of *R*p isomer increase when it participate in reaction in presence of complementary strand. To my best knowledge, this account represents the first demonstration of how a stereochemistry influences the reactivity profile of PS-ONs in duplex.

In the second part of my study, I have synthesized four 5’-phosphorothiolate or 5’-thio analogues and evaluate some biochemical properties of ONs containing 5’-thio modified analogues. Unlike PS-ON, 5’-thio modified ON do not have a chiral center at the phosphorous atom. The importance of the 5’-thio modification is reflected in the large number of synthetic chemical and enzymatic approaches reported to date, yet significant synthetic challenges remain for the fully automated solid-phase synthesis of 5’-thio modified ONs. Moreover, to my best knowledge, despite the large numbers of work conducted on the chemical synthesis of 5’-thio-modified ONs, there were no comprehensive studies on physical or biophysical properties of 5’-thio-modified ONs. Here, in first

step of this research, I have synthesized a 5'-thio thymidine analogue using a known protocol and by using one of the intermediate of that compound, I have synthesized a new 5'-thio-2',5'-dideoxy-5-metylcytidine analogue. These two 5'-thio DNA monomers were then incorporated into a series of ONs using a fully automated solid support DNA synthesizer in 8.5 to 20% yields. Those 5'-thio modified ONs were subjected to evaluated a numbers of physical and biological properties. Melting temperature (*Tm*) study showed that as like phosphorothioate modified ONs, single or multiple incorporation of the 5'-thio-modified monomer into different positions of the ONs showed acceptable differences in binding affinity following annealing to complementary DNA and RNA (-2 to 1 °C /modification). Again, the enzymatic stability of the 5'-thio modified ONs were evaluated using phosphodiesterase I enzyme. Results showed that the incorporation of 5'-thio analogues improved their 3'-exonuclease stability compare to their corresponding unmodified ONs. Then, the effects of 5'-thio modification *in vitro* gene silencing properties was evaluated in mice hepatic NMuLi cells treated with 0.25-200 nM of each antisense oligonucleotide (ASO) having 5'-thio-thymidine modification by using a lipofection method. From experimental results it was found that, 5'-thio-thymidine modified ASO at the fourth and eighth positions and 5'-thio-thymidine modified ASO at the fourth position from the 5'-end were showed very good *in vitro* gene silencing activity with a high IC_{50} of 13 nM and 7 nM respectively, which was similar to the positive control IC_{50} value (9 nM). In contrast, 5'-thio-thymidine modified ASO at the eighth position from 5'-end had a moderate IC_{50} 28 nM, compare to the positive control. This is the first demonstration, which showed that 5'-thio modified ASOs had very good *in vitro* gene silencing properties. The *in vitro* results inspired to evaluate the mRNA silencing activity of 5'-thio-modified ASO in an *in vivo* model. But introduction of 5'-thio reduced their antisense activity *in vivo* model compare to positive control. But in upside introduction of 5'-thio modification reduced the hepatotoxicity. Initially, I thought that 5'-thio-modified ASOs were not facilitated the RNase H mediated scission or were not stable at blood serum, but 5'-phosphorothiolate-modified ASO showed slow but significant RNase H activation as well as good stability in blood serum for its action in liver.

Again, later part of this study I have synthesized two new 5'-thio analogue, which contain a 2'-*O*,4'-*C*-methylene bridge. By using those 5'-thio-modified 2',4'-BNA/LNA analogues, a numbers of ONs have been synthesized in a fully automated solid support DNA synthesizer. Introduction of 2'-*O*,4'-*C*-methylene bridge improved the hybridizing ability as expected. As like hybridizing ability, those 5'-thio 2',4'-BNA/LNA modified ONs also showed very good nuclease stability compare to corresponding ONs. The *in vitro*, *in vivo*, RNase H activity and serum stability assay data of 5'-thio modified ONs showed discrepancies: the *in vitro* data showed that 5'-thio-modified ASO looked promising whereas the *in vivo* data did not. On other the hand, introduction of a 2'-*O*,4'-*C* methylene-bridge into 5'-thio analogues and there corresponding ONs showed significantly improved duplex stability and nuclease resistance. I believe in future, fine-tuned ASOs having 5'-thio 2',4'-BNA/LNA analogues may solve the inconsistencies between the *in vitro* and *in vivo* data.

様式 7

論文審査の結果の要旨及び担当者

氏　名　（ ISLAM MD ARIFUL ）	
論文審査担当者	（職）　　氏　名 主 査　教授　小比賀 聡 副 査　教授　藤岡 弘道 副 査　教授　赤井 周司

論文審査の結果の要旨

現在研究開発が進められている核酸医薬品の多くは、その化学構造に含まれるリン酸ジエステル結合部の一つの酸素原子を硫黄原子に置換しホスホロチオアート結合とすることで生体内での安定性向上を実現している。一方、このホスホロチオアート結合を含むオリゴヌクレオチド（S-オリゴ）は、リン原子上にキラリティを有するため数多くのジアステレオマー混合物として得られてくることが一つの課題であった。

こうした背景のもとISLAM MD ARIFUL君は、リン原子上のキラリティの違いがS-オリゴの反応性（硫黄原子のアルキル化反応）に与える影響を精査した。その結果、S-オリゴが適切な条件下において相補鎖と二重鎖を形成した場合に、その反応性がリン原子上のキラリティの違いによって大きく異なるという興味深い知見を得た。この結果は、今後の核酸医薬品の研究開発において非常に重要な意義を持つものであると言える。

さらに、ISLAM MD ARIFUL君は、ホスホロチオアート結合と同じくリン酸ジエステル結合部の一つの酸素原子を硫黄原子に置換した結合であるもののリン原子上のキラリティを生じさせないホスホロチオラート結合に着目した。彼は5’位の酸素原子を硫黄原子に置換した各種ヌクレオシド誘導体を合成することに成功し、さらにそれらを導入したオリゴヌクレオチドの効率的な合成を達成した。このようにして得られたホスホロチオラート結合を含む各種オリゴヌクレオチドの機能評価を行った結果、in vivoでの活性確認には至らなかったものの、培養細胞系において十分な有効性を見出した。この結果は、今後の核酸医薬品開発における化学修飾の重要性を示すもので、核酸化学、生物有機化学という学問領域においても重要な意義を持つものと判断できる。

これらの点から、本研究成果は、博士（薬科学）の学位論文に値するものと認める。