Phosphorothioate oligonucleotides

Literature Seminar

B4 Miura Kensuke

Outline

- **Antisense oligonucleotide (ASOs)**

- **Phosphorothioate linkage**

- **Main topic:**
  “Control of phosphorothioate stereochemistry substantially increase the efficacy of antisense oligonucleotides”
  (Iwamoto, N.; Butler C. D. D.; Svrzikapa, N.; Mohapatra, S.; Zlatev, I.; Sah, W. Y. D.; Meena,; Standley, M. S.; Lu, G.; Frank-Kamenetsky, M.; Zhang, J. J.; Vargeese, C.; Verdine, L. G. *Nat. Biotech.* 2017, *35,* 845-851.)

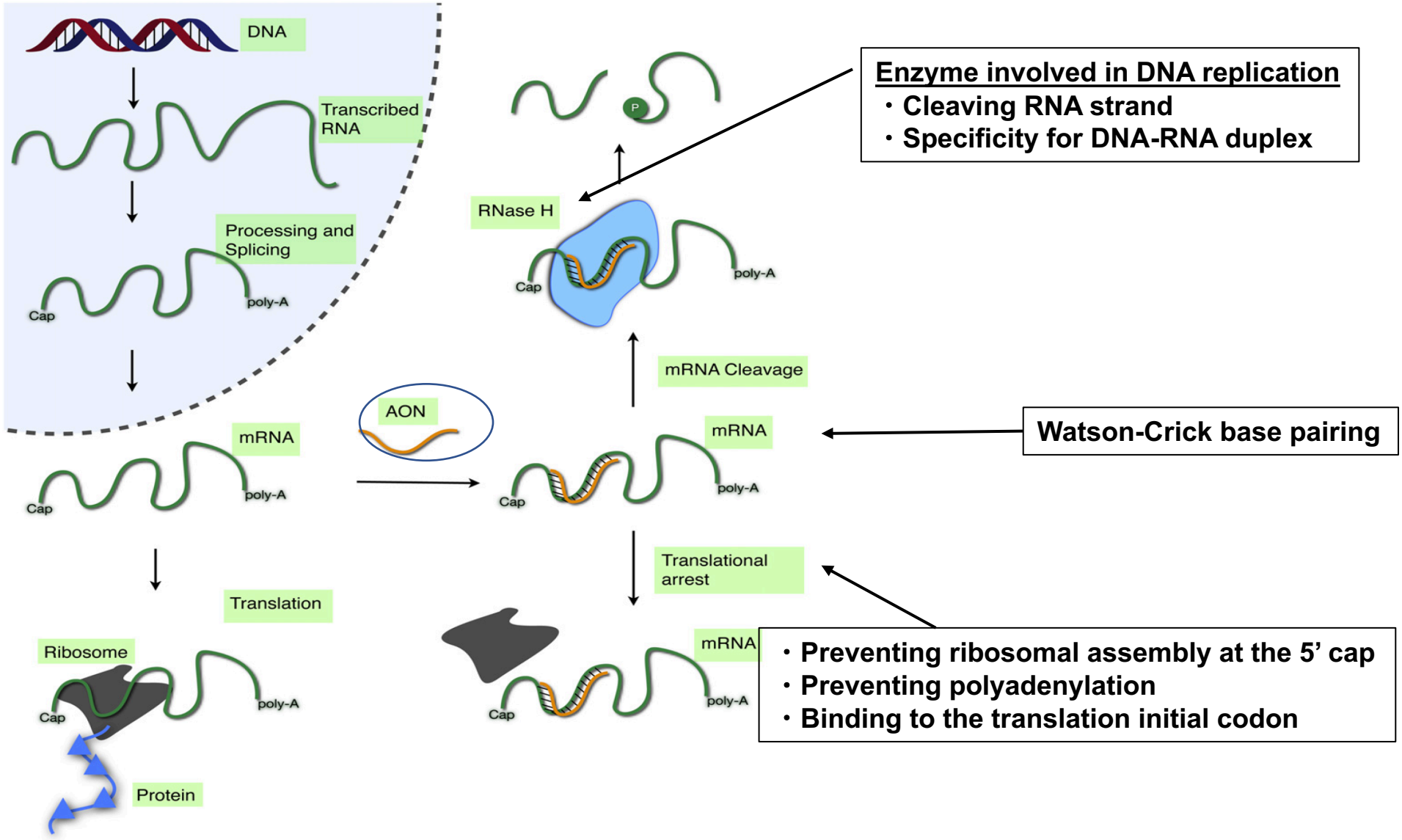
Gene Silencing

- **RNA interference (RNAi)[1)]**
  - **Discovery of RNAi pathway in 1998 (Fire, A. *et al*.)[2)]**
  - **micro RNA (miRNA)**
  - **Mimic of miRNA (siRNA)**
  - **RNA-induced silencing complex (RISC)**

- **Antisense[1)]**
  - **First demonstratation in 1978 (Zamecnic, P. C.; Stephenson, M. L)[3)]**
  - **Antisense oligonucleotides (ASOs)**
  - **RNase H**

1) Deleavey, G. F.; Damha, M. J. *Chem. Biol*. **2012**, *19*, 937-954.
2) Fire, A.; Xu, S.; Montgoormery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C. *Nature* **1998**, *391*, 806-811.
3) Zamecnic, P. C.; Stephenson, M. L. *Proc Natl Acad Sci*. **1978**, *75*, 280-284.

Gene Silencing Mediated by ASOs



1) Deleavey, G. F.; Damha, M. J. *Chem. Biol*. **2012**, *19*, 937-954.

ASOs-Based Therapeutics

- **Low molecular-weight drug**

  - **Targeting for protein**
  - **Synthesized chemically**
  - **Exerting a side effect**

- **Antibody drug[1)]**

  - **Targeting for protein**
  - **Synthesized by cells**
  - **Increasing specificity and reducing normal tissue toxicity**

- **Antisense drug[2)]**

  - **Targeting for mRNA**
  - **Increasing specificity**
  - **Synthesized chemically**

1) Bagshawe, K. D. *Br. J. Cancer*. **1987**, *56*, 531-532.
2) Deleavey, G. F.; Damha, M. J. *Chem. Biol*. **2012**, *19*, 937-954.

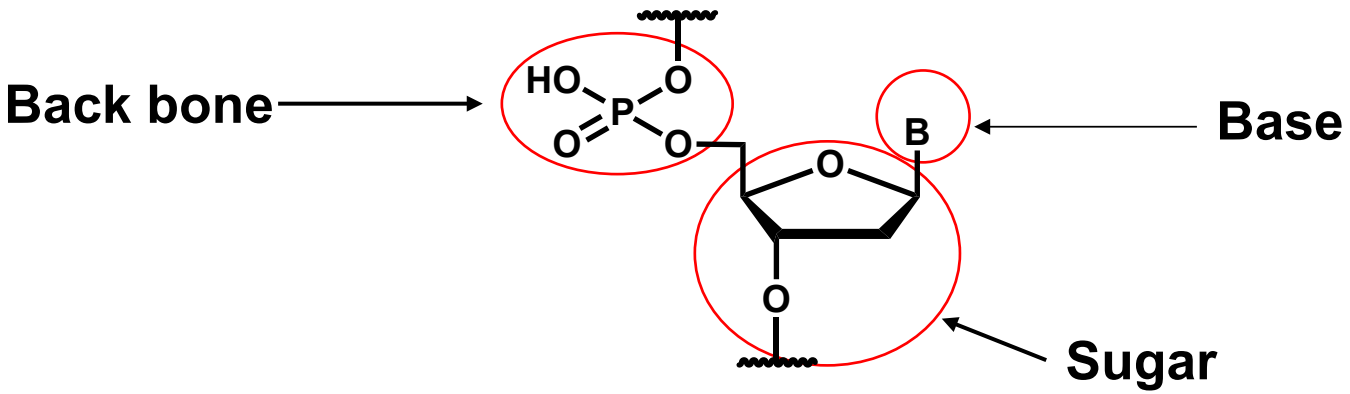
Obstacles of Oligonucleotides Therapeutics[1)]

- **Poor extracellular and intracellular stability**
- **Low efficiency of intracellular delivery to target cells**
- **The potential for 'off-target' gene silencing**
- **Immunostimulation**

- **Natural oligonucleotide (DNA/RNA) is not useful to therapeutics**
- **Chemical modification is needed**

1) Deleavey, G. F.; Damha, M. J. *Chem. Biol*. **2012**, *19*, 937-954.

Chemical Modifications of ASOs

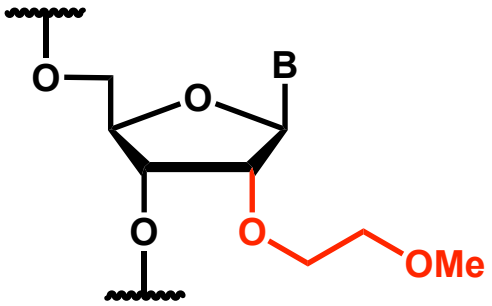


Aims of chemical modification[1,2)]

- **Increasing nuclease resistance**
- **Good binding affinity for target sequence**
- **Increasing potency of RNase H**

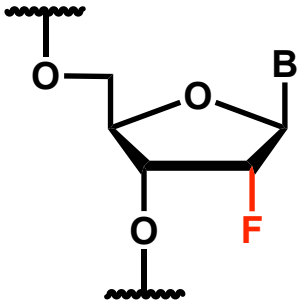
1) Deleavey, G. F.; Damha, M. J. *Chem. Biol*. **2012**, *19*, 937-954.
2) Sharma, V. K.; Sharma, R. K.; Singh, S. K. *Med. Chem. Commun.* **2014**, *5*, 1454-1471.

Sugar Modified ASOs



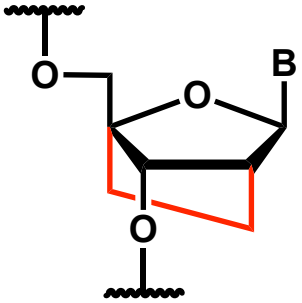
2' methoxyethyl (MOE) RNA[2)]

- Improving nuclease resistance
- Activating RNase H
- Used in Mipomersen



2'F-RNA[3)]

- Increasing binding affinity
- Enhancing stacking interactions



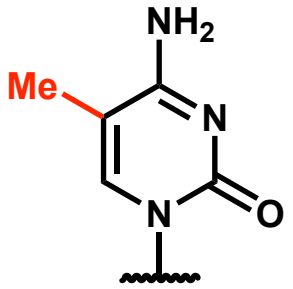
Locked nucleic acid (LNA)[4)]

- Improving nuclease resistance
- Forming a restricted conformation
- Enhancing duplex stability

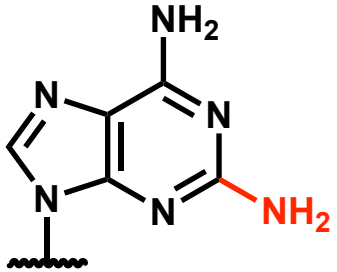
- **Improving binding affinity for target RNA**
- **Improving stability of ASOs-RNA duplex**

1) Deleavey, G. F.; Damha, M. J. *Chem. Biol*. **2012**, *19*, 937-954.
2) Rettig, G. R.; Behlke, M. A. *Mol. Ther*. **2012**, *20*, 483-512.
3) Manoharan, M. *Nucleic Acid Drug Dev.* **2002**, *12*, 103-128.
4) Wahlestedt, C.; Salmi, P.; Good, L.; Kela, J.; Johnsson, T.; Hokfelt, T.; Broberger, C.; Porreca, F.; Lai, J.; Ren, K. *Proc. Natl. Acad. Sci.* **2000**, *97*, 5633-5638.

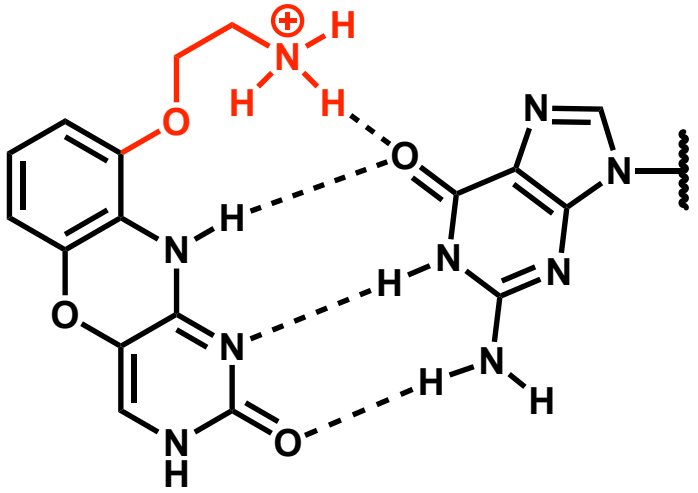
Nucleobase Modified ASOs



5-Me cytosine[3)]



2,6-diamiinopurine
(replacement for adenin)[4)]



Cytosine modified with G-clamp (red)[5)]

- Reducing immunostimulation response
- Used in Mipomersen

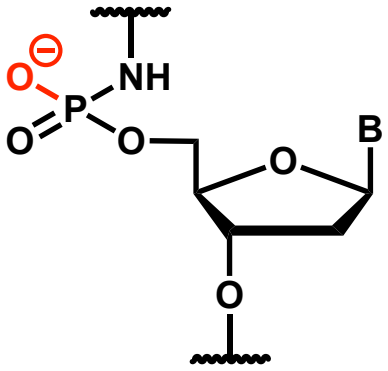
- Stabilizing A-T(U) pairs

- Dramatically enhancing stability
- Introducing hydrogen-bond donor

- **For increased duplex stability**
- **Less common than sugar and back bone modification**

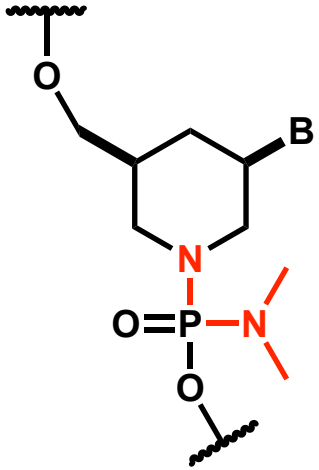
1) Deleavey, G. F.; Damha, M. J. *Chem. Biol*. **2012**, *19*, 937-954.
2) Sharma, V. K.; Sharma, R. K.; Singh, S. K. *Med. Chem. Commun.* **2014**, *5*, 1454-1471.
3) Krieg, A. M. *Nucleic Acid Ther*. **2012**, *22*, 77-89.
4) Deleavey, G. F.; Watts, J. K.; Alain, T.; Robert, F.; Kalota, A.; Aishwarya, V.; Pelletier, J.; Gewirtz, A. M.; Sonenberg, N.; Damha, M. J. *Nucleic Acid Res.* **2010**, *38*, 4547-4557.
5) Lin, Y. K.; Matteucci, D. M. *J. Am. Chem. Soc*. **1995**, *117*, 3373-3386.

Backbone Modified ASOs



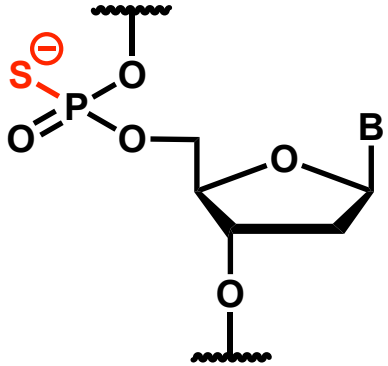
N3' Phosphoramidate (NP)[1)]

- **Improving binding affinity**
- **Improving nuclease resistance**



Morpholino[2)]

- **Containing uncharged substitute of the linkage**
- **Containing franose sugars**
- **Improving nuclease resistance**



Phosphorothioate (PS)[3)]

- **Used in Formivirsen**
- **Used in Mipomersen**

- **Often combined with sugar modification**
- **Improving nuclease resistance**

1) Heidenreich, O.; Gryaznov, S.; Nerenberg, M. *Nucleic Acids Res*. **1997**, *25*, 776-780.
2) Summerton, J. *Biochim. Biophys*. **1999**, *1489*,141-158.
3) Deleavey, G. F.; Damha, M. J. *Chem. Biol*. **2012**, *19*, 937-954.

Outline

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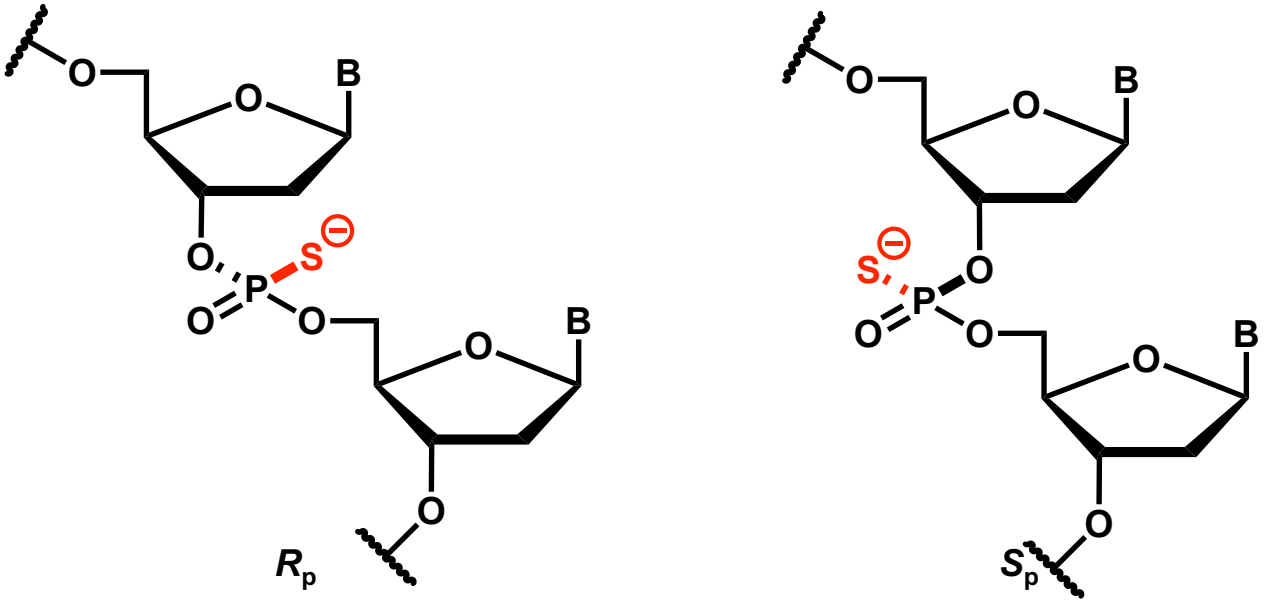
PS-Modified ASOs



- **First internucleotide linkage modifications[1)]**
- **Existence of S_p/R_p isomer**
- **Compatibility with solid-phase synthetic method[2)]**
- **Less binding affinity than phosphodiester[3)]**
- **Compatibility with RNase H-mediated cleavage[4)]**

1) Eckstein, F. *Tetrahedron Lett*. **1967**, *8*, 1157-1160.
2) Sharma, V. K.; Sharma, R. K.; Singh, S. K. *Med. Chem. Commun.* **2014**, *5*, 1454-1471.
3) Kilver-Herzog, L.; Zon, G.; Uznanski, B.; Whittier, G.; Wilson, W. D. *Nucleic Acid Res*. **1991**, *19*, 2979-2986.
4) Agrawal, S.; Kandimalla, E. R. *Mol. Med*. **2000**, *6*, 72-81.

ASOs Drugs Containing PS -Mipomersen-

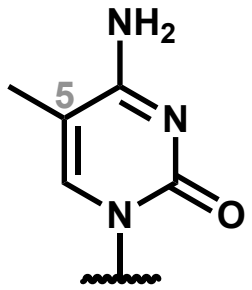
1)

Structual features

5'-wing gap 3'-wing

GCCTCAGTCTGCTTCGCACC

- 20 nucleotides long
- All 5-Me cytosine residue
- 'Gapmer' design
- MOE modification at ends (wings)
- DNA in middle (gap)



5-Me cytosine

Introduction

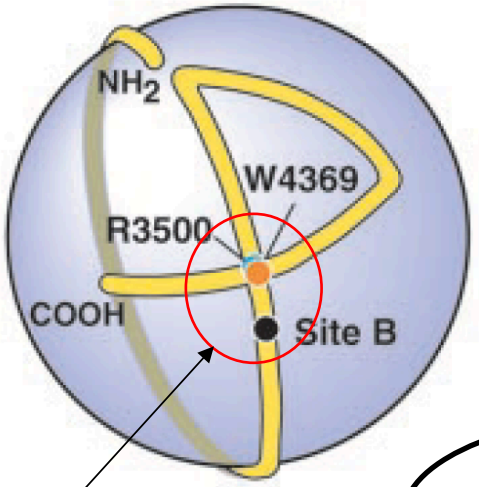
- Second-generation antisense drug released by Isis Pharmaceuticals in 2013
- Treatment of Homozygous familial hyperchoresterolaemia
- Apolipoprotein B (ApoB-100)

1) Crooke, S. T.; Geary, R. S. *Br. J. Clin. Pharmacol*. **2013**. *76*, 269–276.

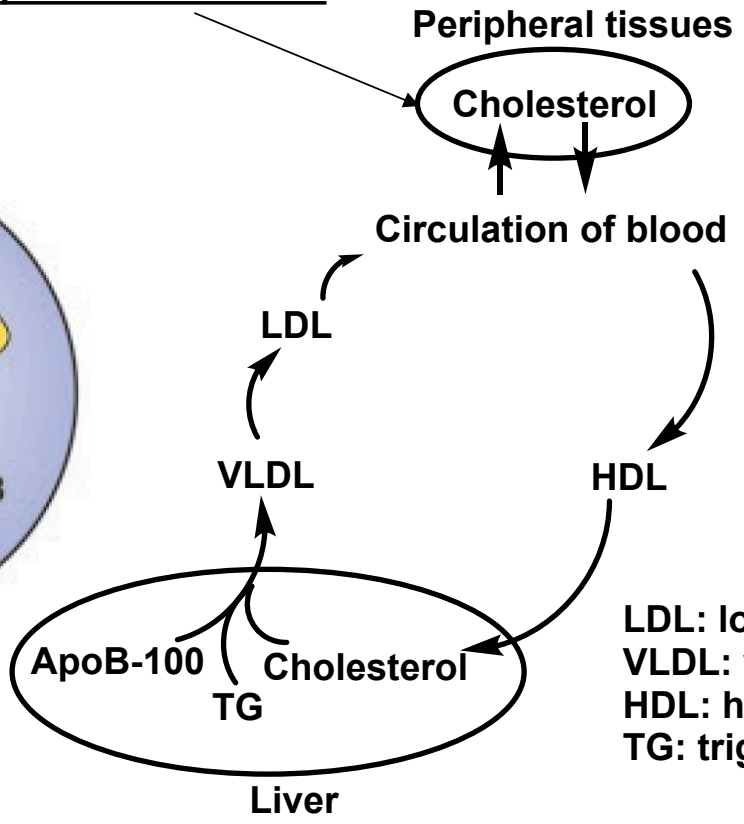
Overview of Cholesterol Metabolism

Existence of LDL receptor on membrane

1)



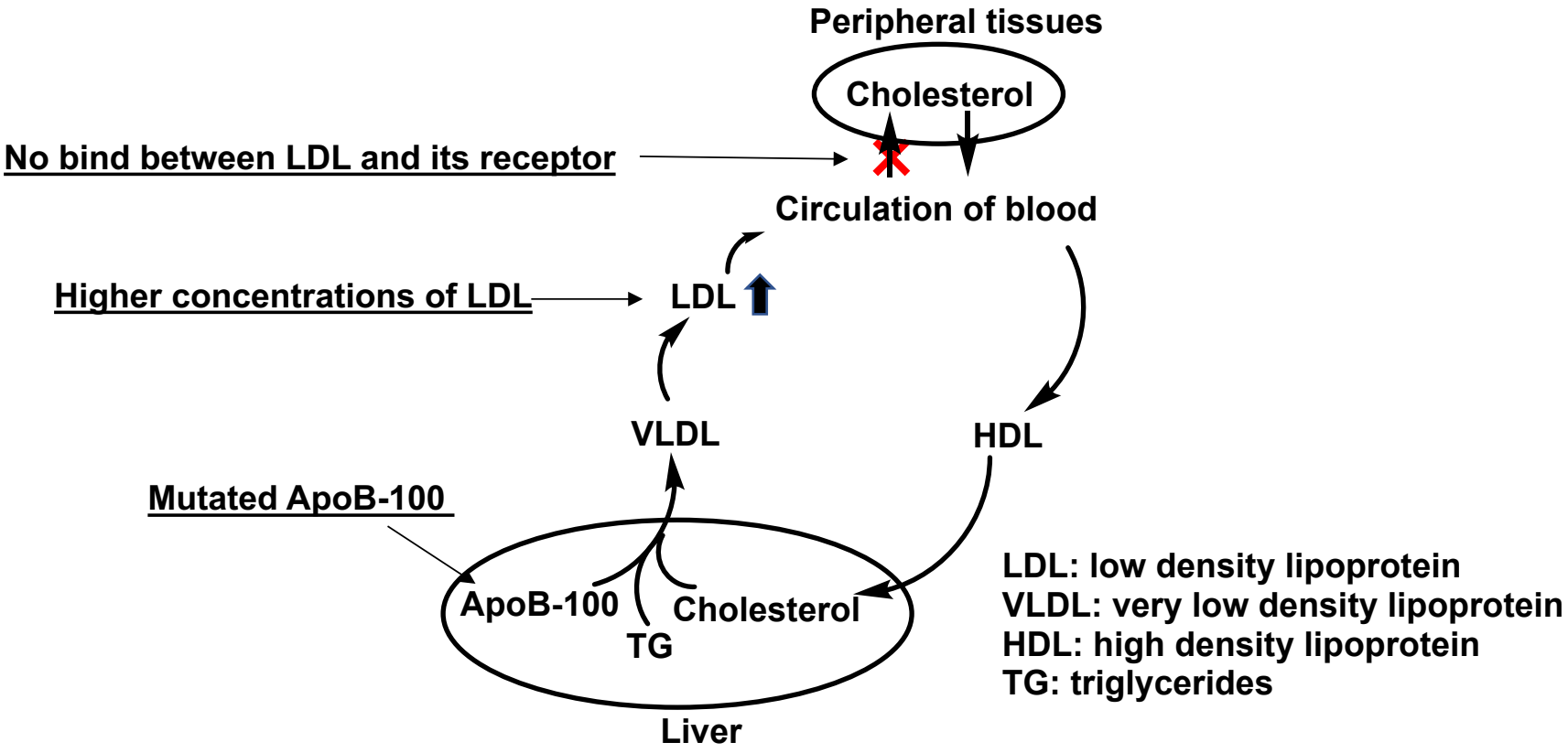
Essential residue for interaction between LDL and its receptor[2)]



LDL: low density lipoprotein
VLDL: very low density lipoprotein
HDL: high density lipoprotein
TG: triglycerides

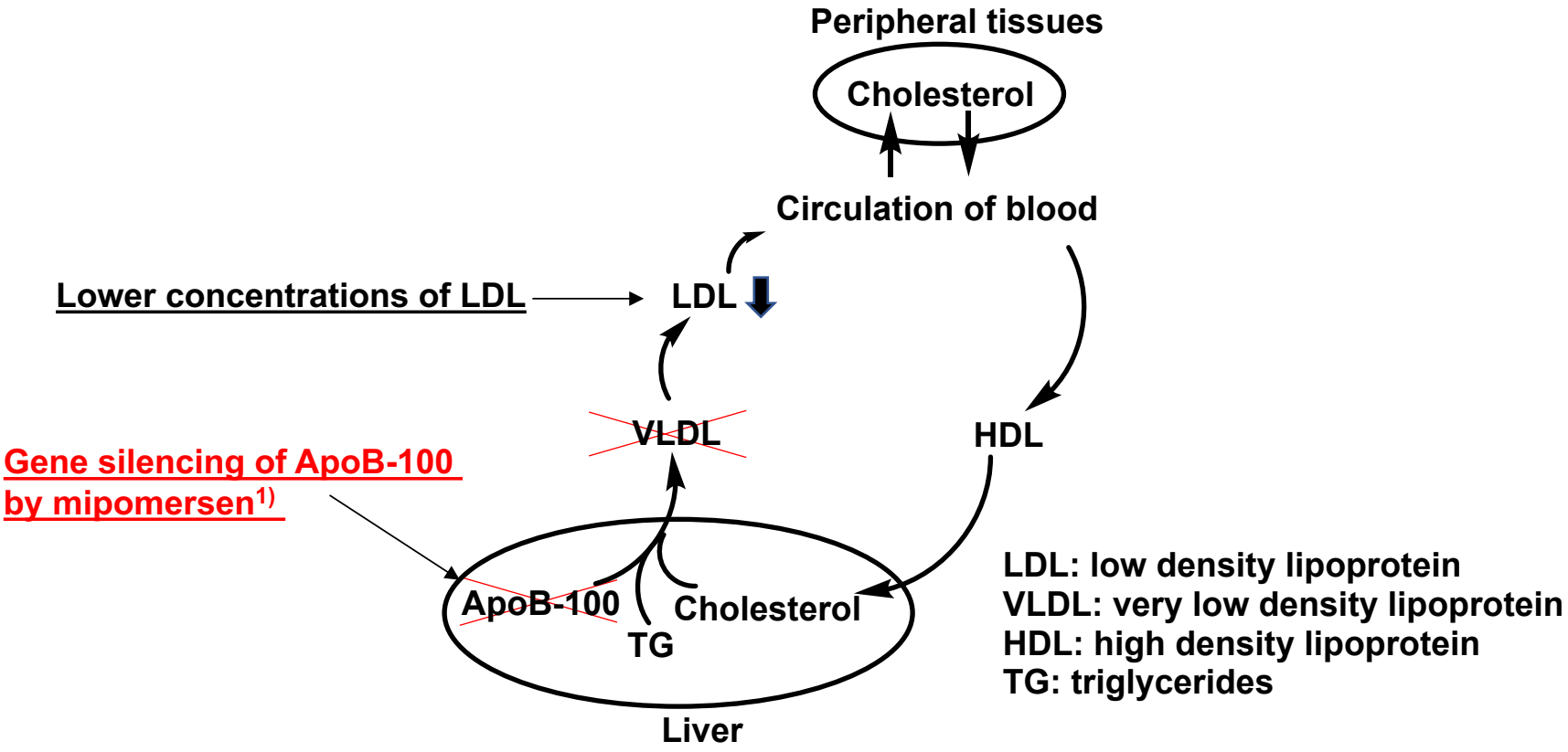
1) Names, K.; Aberg, F.; Gylling, H.; Isoniemi, H. *World J. Hepatol*. **2016**, *8*, 924-932.
2) Jan, B.; Ulf, E.; Bo, A.; Petter, N. E.; Thomas, L. *J. Biol. Chem*. **2001**, *12*, 9214-9218.

Homozygous Familial Hyperchoresterolaemia 1)



1) Jan, B.; Ulf, E.; Bo, A.; Petter, N. E.; Thomas, L. *J. Biol. Chem*. **2001**, *12*, 9214-9218.

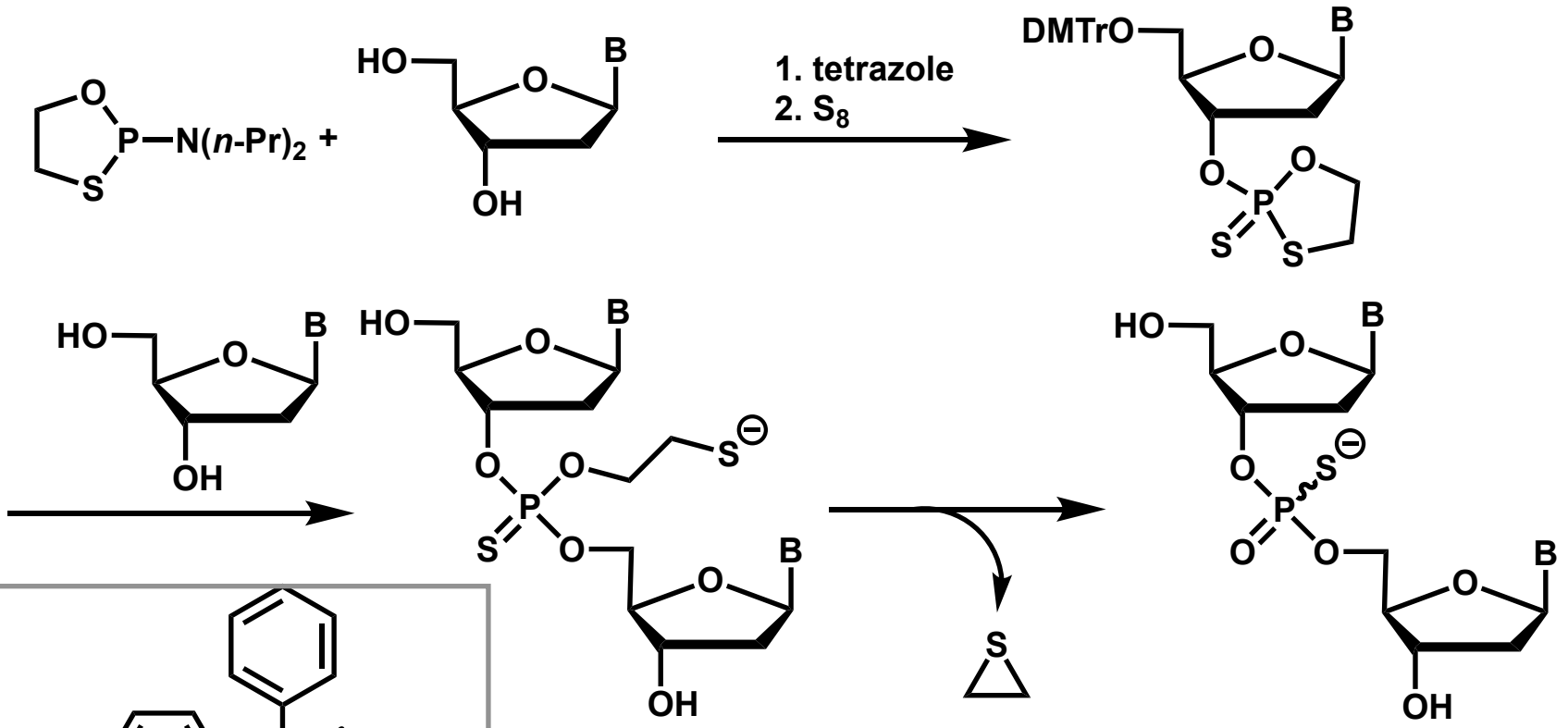
Treatment of Homozygous Familial Hyperchoresterolaemia



1) Crooke, S. T.; Geary, R. S. *Br. J. Clin. Pharmacol*. **2013**. *76*, 269-276.

The First Chemical Synthesis of Stereopure PS-Modified Oligonucleotide

1)



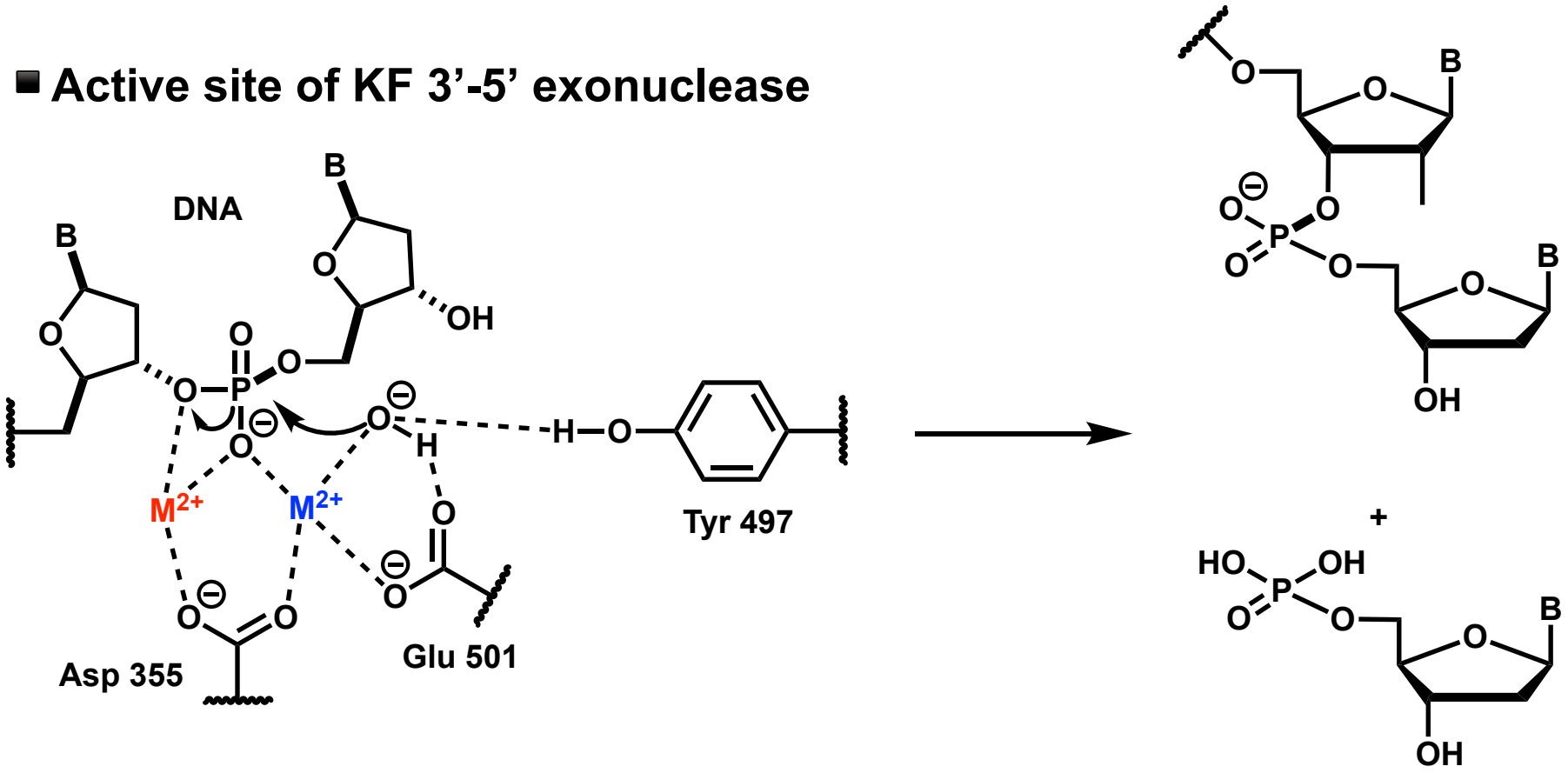
- **Oxathiaphospholanes was used**
- **8-nucleotide-long sequence was obtained**

1) Stec, W. J.; Grajkowski, A.; Koziolkiewicz, M.; Uznanski, B. *Nucleic Acid Res*. **1991**, *19*, 5883-5888.

Two-Metal-Ion Mechanism for Phosphoryl Transfer in KF 3'-5' Exonuclease

1)

- Active site of KF 3'-5' exonuclease



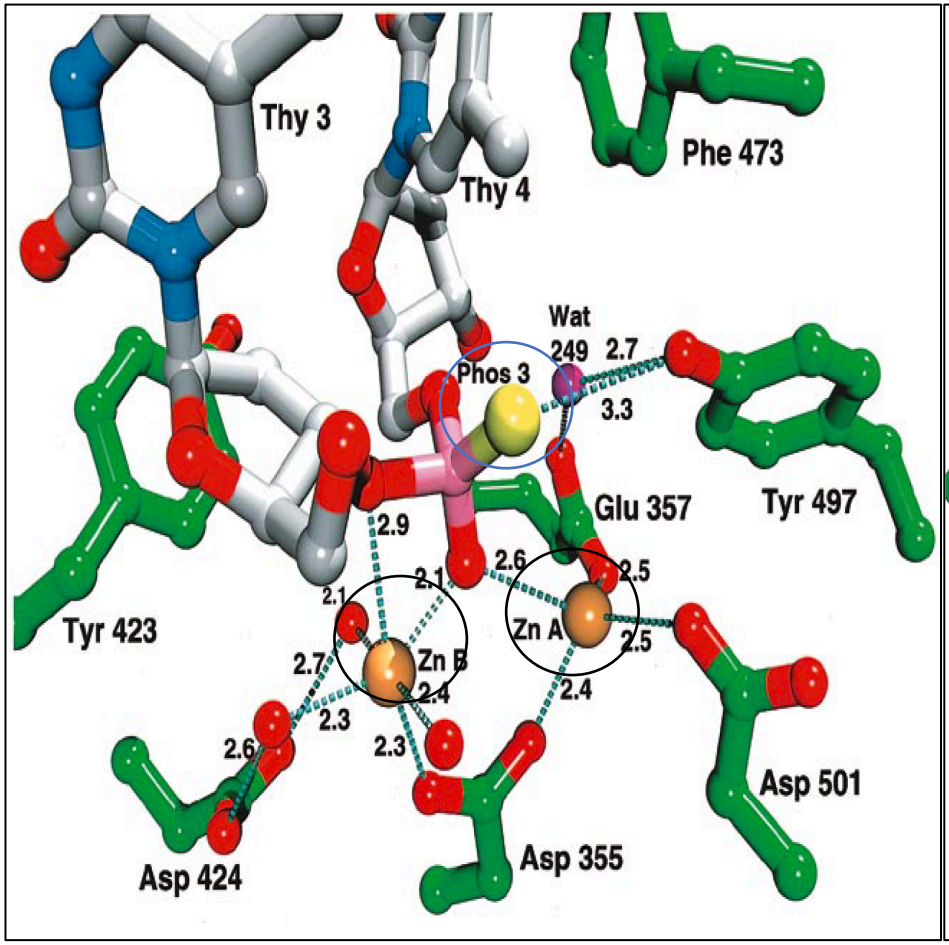
- **Metal ion A (blue) activates nucleophile attacks**
- **Metal ion B (red) stabilizes the leaving 3' oxygen**

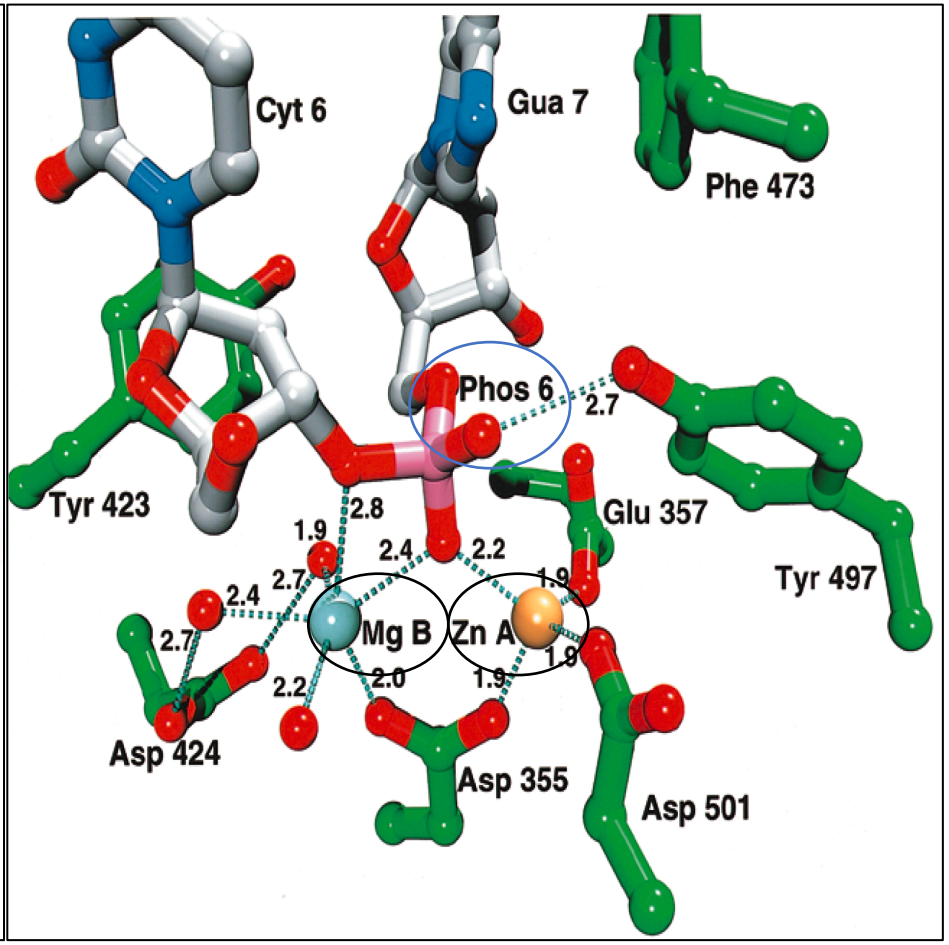
1) Brautigam, T. A.; Steitz, T. A. *J. Mol. Biol*. **1998**, *277*, 363-377.

Similarity between R_p Isomer and All-Oxygen Structure

1)

- Active site of KF 3'-5' exonuclease bound to PS R_p diastereomer
- Active site of KF 3'-5' exonuclease bound to phosphodiester





1) Brautigam, T. A.; Steitz, T. A. *J. Mol. Biol*. **1998**, *277*, 363-377.

Active Site of S_p Isomer

1)

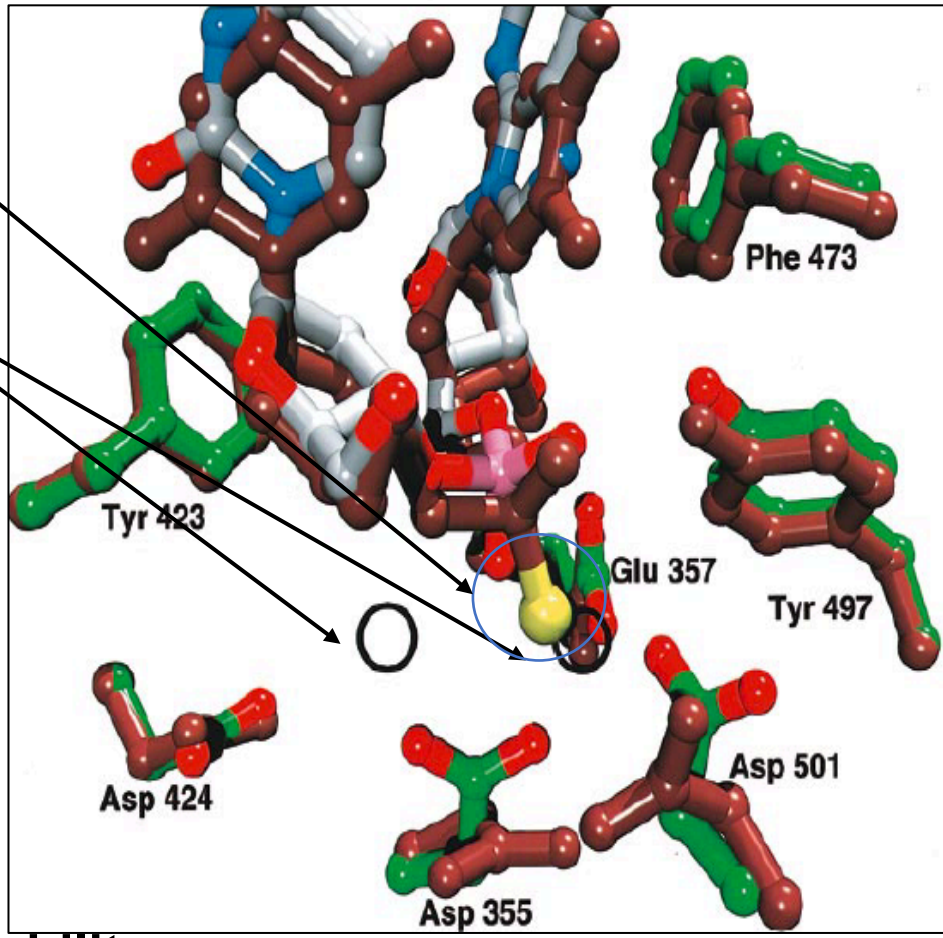
Sulfur atom of S_p isomer occupied space

<u>Absence of metal ions A and B</u>

- Nucleophile attack was not activated
- Leaving 3' oxygen was not stabilized

Only S_p isomer obtained nucleoase stability

- Active site of KF 3'-5' exonuclease bound to PS S_p diastereomer



1) Brautigam, T. A.; Steitz, T. A. *J. Mol. Biol*. **1998**, *277*, 363-377.

S_p and R_p Linkage

- **Different properties between S_p and R_p**

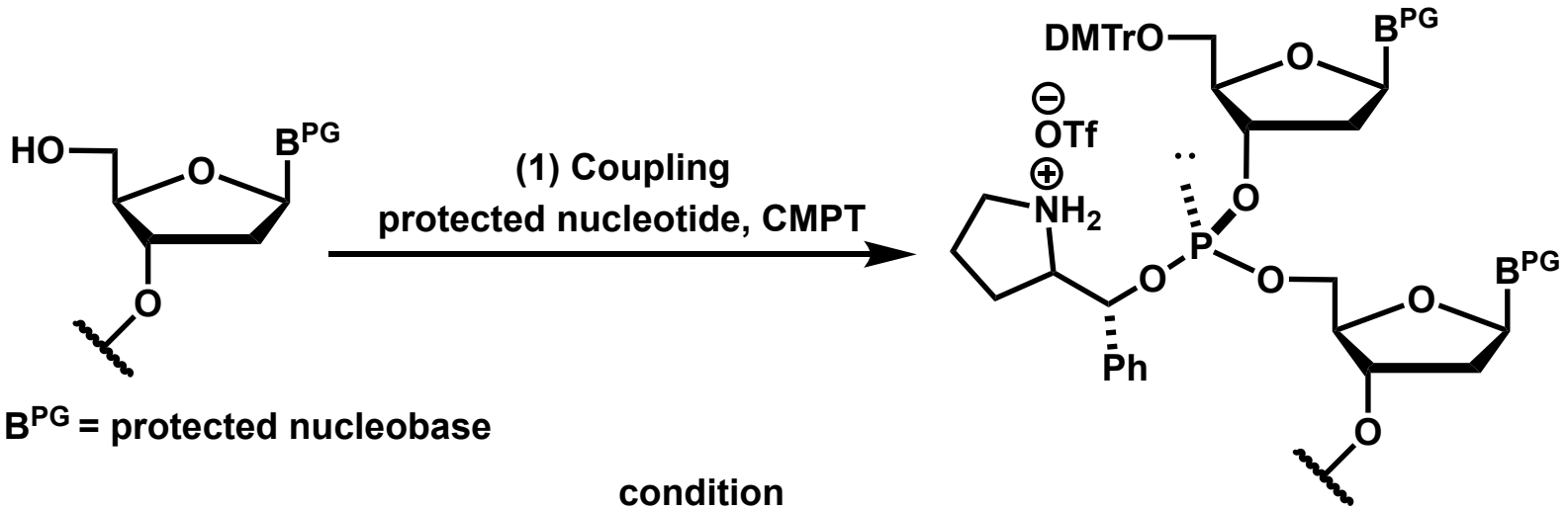
- **S_p isomer shows better nuclease stability[1)]**
- **R_p PS DNA activate RNase H [2)]**
- **R_p isomer has better affinity for target mRNA[3)]**

- **Mixture of R_p/S_p achieve balance between activity and stability[4)]**
- **Sequence having activity and stability was needed**

1) Koziolkiewicz, M.; Wojcik, M.; Kobylanska, A.; Karwowski, B.; Rebowska, B.; Guga, P.; Stec, W. J. *Antisense Nucleic Acid Drug Dev.* **1997**. *7*, 43-48.
2) Koziolkiewicz, M.; Krakowlak, A.; Kwinkowski, M.; Boczkowska, M.; Stec, W. J. *Nucleic Acid Res.* **1995**. *23*, 5000-5005.
3) Tang, J.; Roskey, A.; Li, Y.; Agrawal, S. *Nucleosides Nucleotides* **1995**, *14*, 985-990.
4)Wan, W. B.; Migawa, T. M.; Vaswuez, G.; Murray, M. H.; Nichols, G. J.; Gaus, H.; Berdeja, A.; Lee, S.; Hart, E. C.; Lima, F. W.; Swayze, E. E.; Seth, P. P. *Nucleic Acid Res*. **2014**, *42*, 13456-13468.

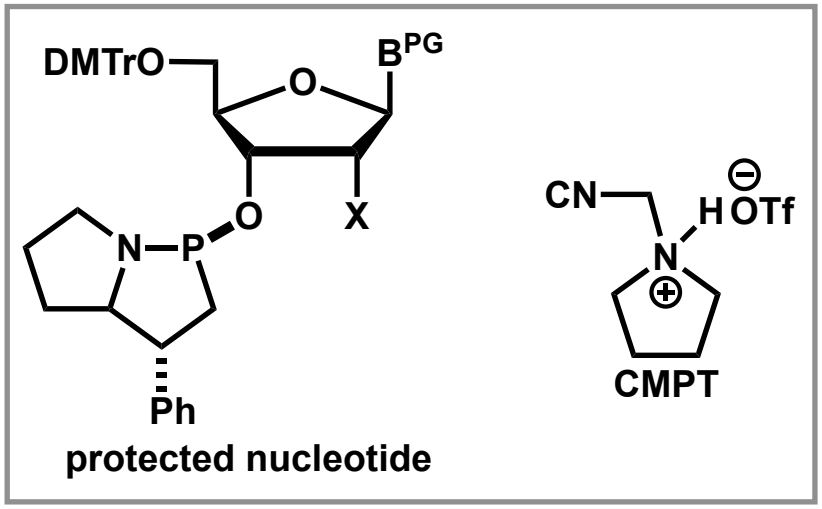
Attempts of Stereo-Controlled Synthesis

1)



B^{PG} = protected nucleobase

condition



1) Oka, N.; Yamamoto, M.; Sato, T.; Wada, T. *J. Am. Chem. Soc*. **2008**, *130*, 16031-16037.

Attempts of Stereo-Controlled Synthesis

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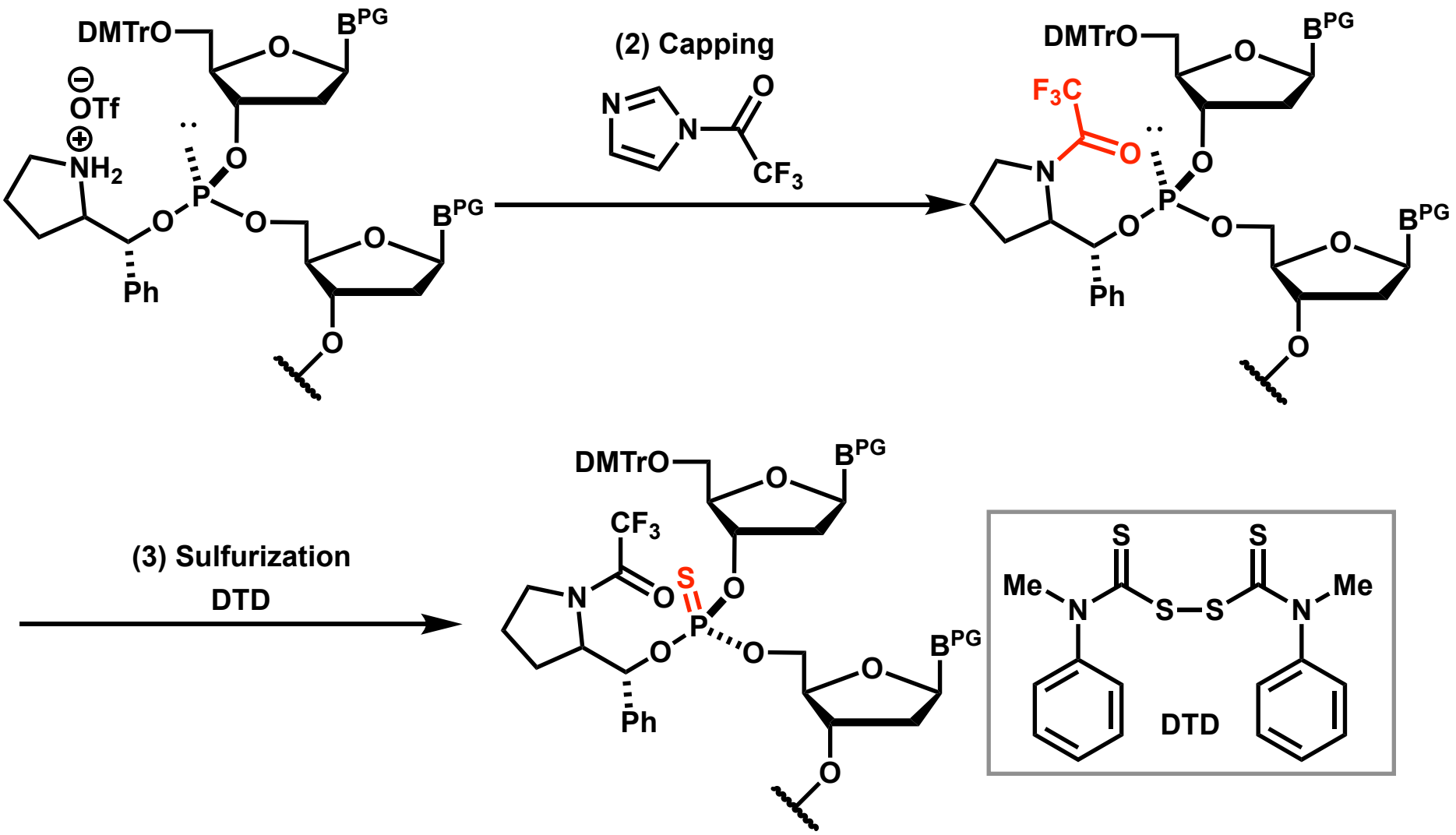

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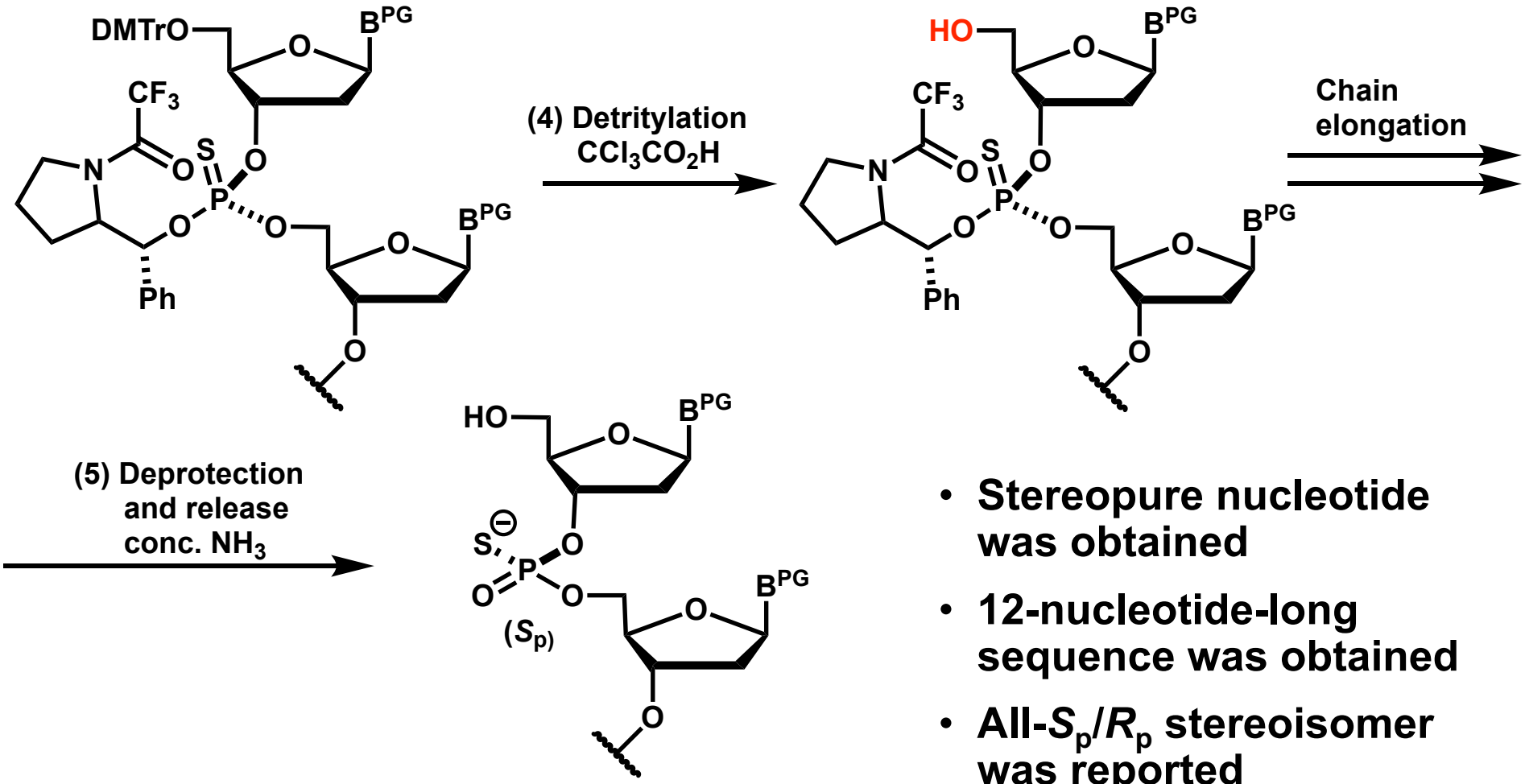

- **Stereopure nucleotide was obtained**
- **12-nucleotide-long sequence was obtained**
- **All-S_p/R_p stereoisomer was reported**

1) Oka, N.; Yamamoto, M.; Sato, T.; Wada, T. *J. Am. Chem. Soc*. **2008**, *130*, 16031-16037.

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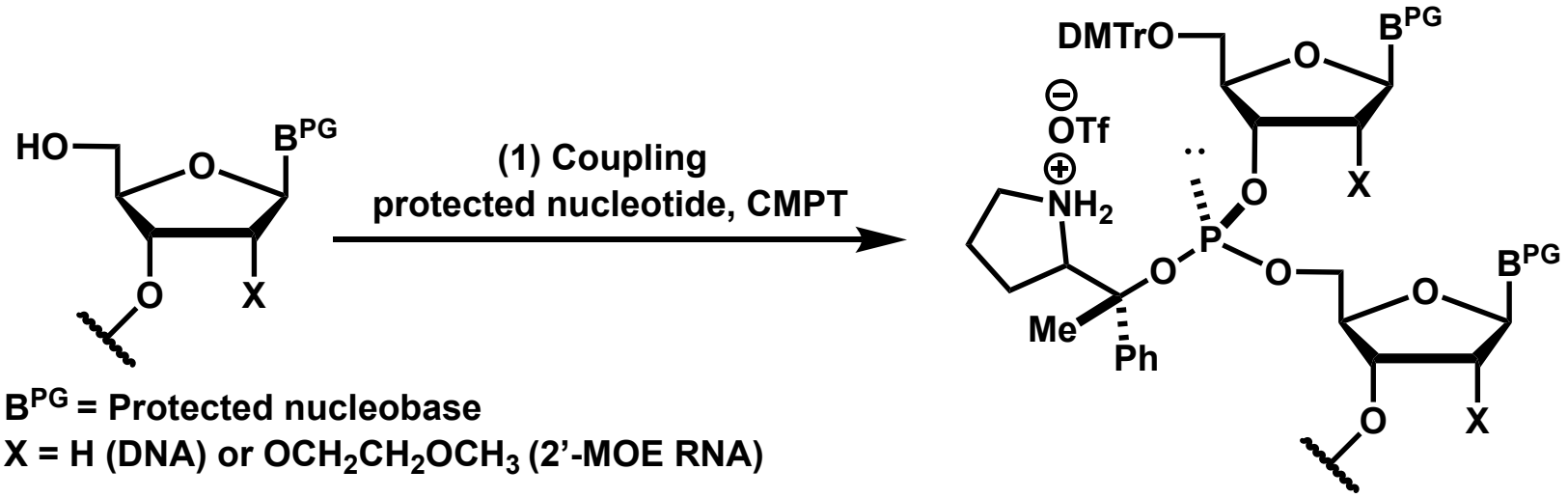
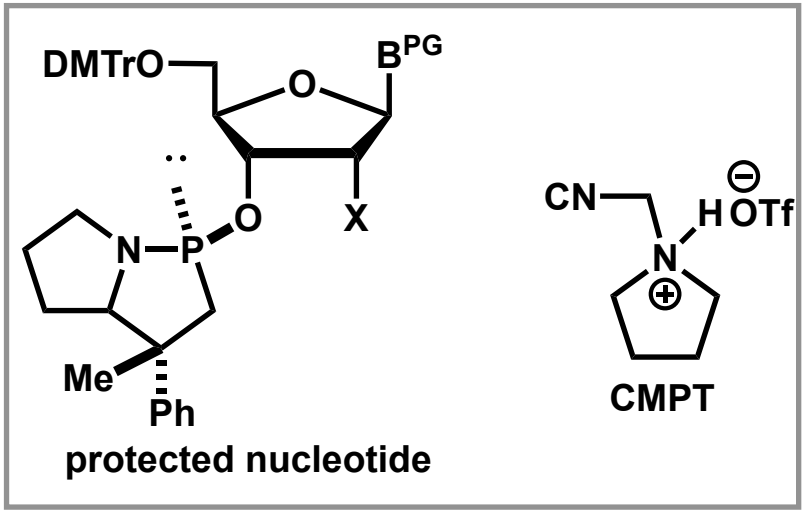
Stereo-Controlled Synthesis (1)



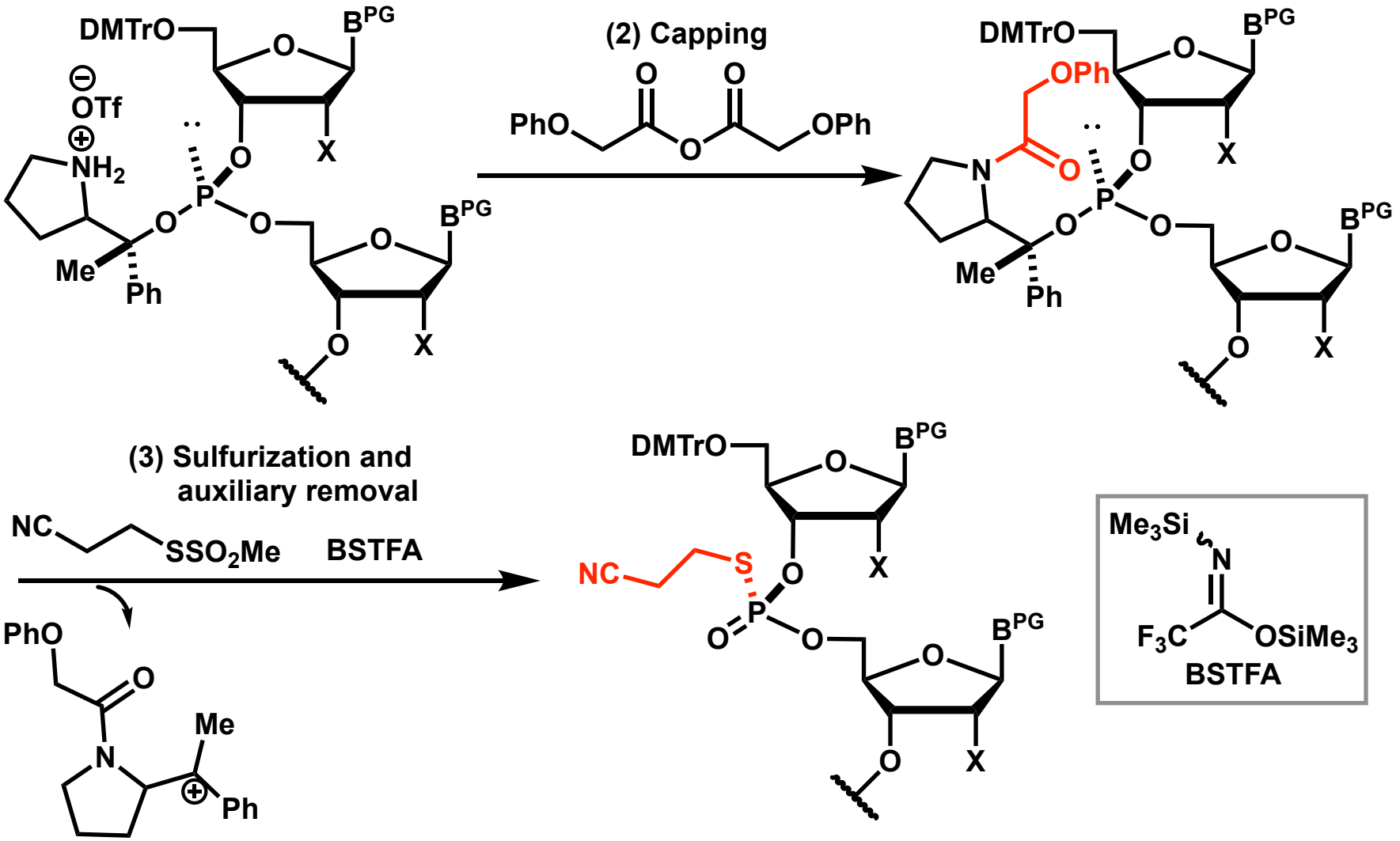
B^{PG} = Protected nucleobase

X = H (DNA) or $OCH_2CH_2OCH_3$ (2'-MOE RNA)

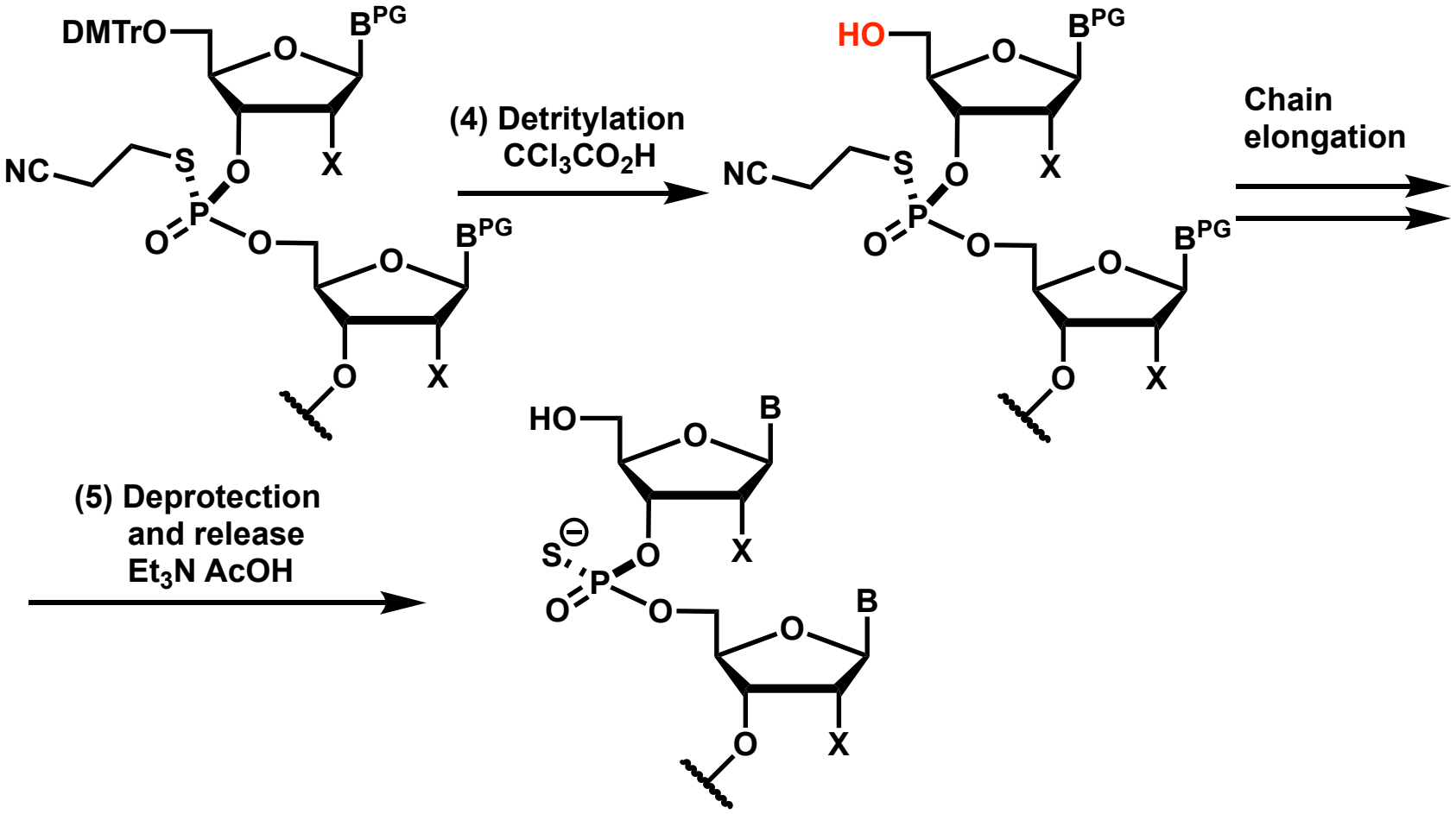
condition



Stereo-Controlled Synthesis (2)

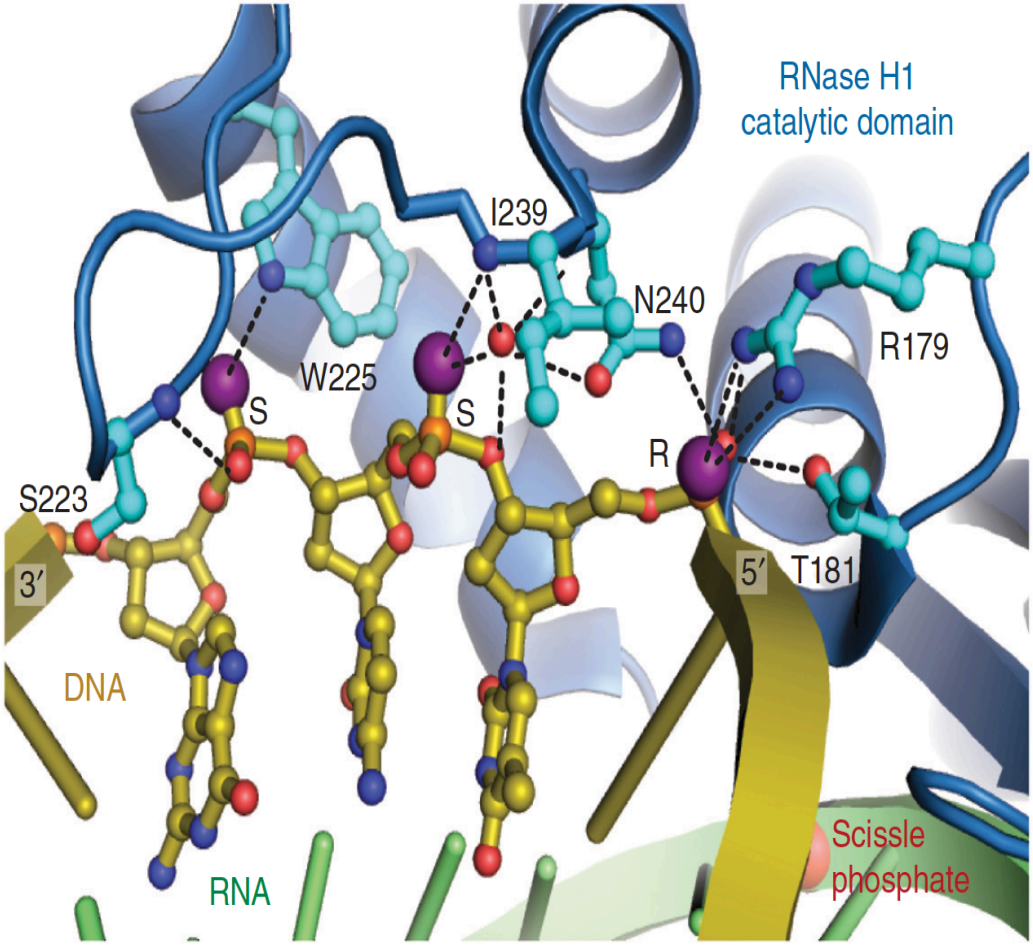


Stereo-Controlled Synthesis (3)

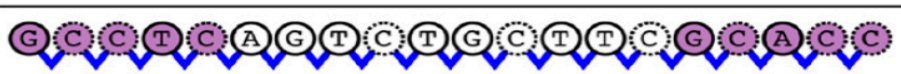
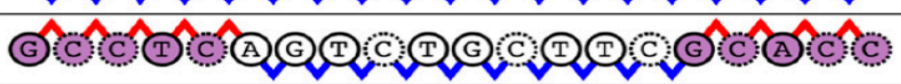


X-Ray Structure of RNase H1 Bound to RNA-DNA Heteroduplex

- Pro-S_p/R_p O contact amino acids in a stereochemically different manner
- The 3'-$S_pS_pR_p$-5' sequence would be well-contacted



Stereochemically Pure Design

ASO	Sequence	Number of stereoisomer	Tm (°C)	V0 (μM/min)
Mipomersen		524,288	80.2	0.05
WV-1		1	84.7	---
WV-2		1	74.7	---
WV-3		1	78.8	---
WV-4		1	80.0	---
WV-5		1	81.6	0.13
WV-6		1	78.3	---
WV-7		1	68.2	---

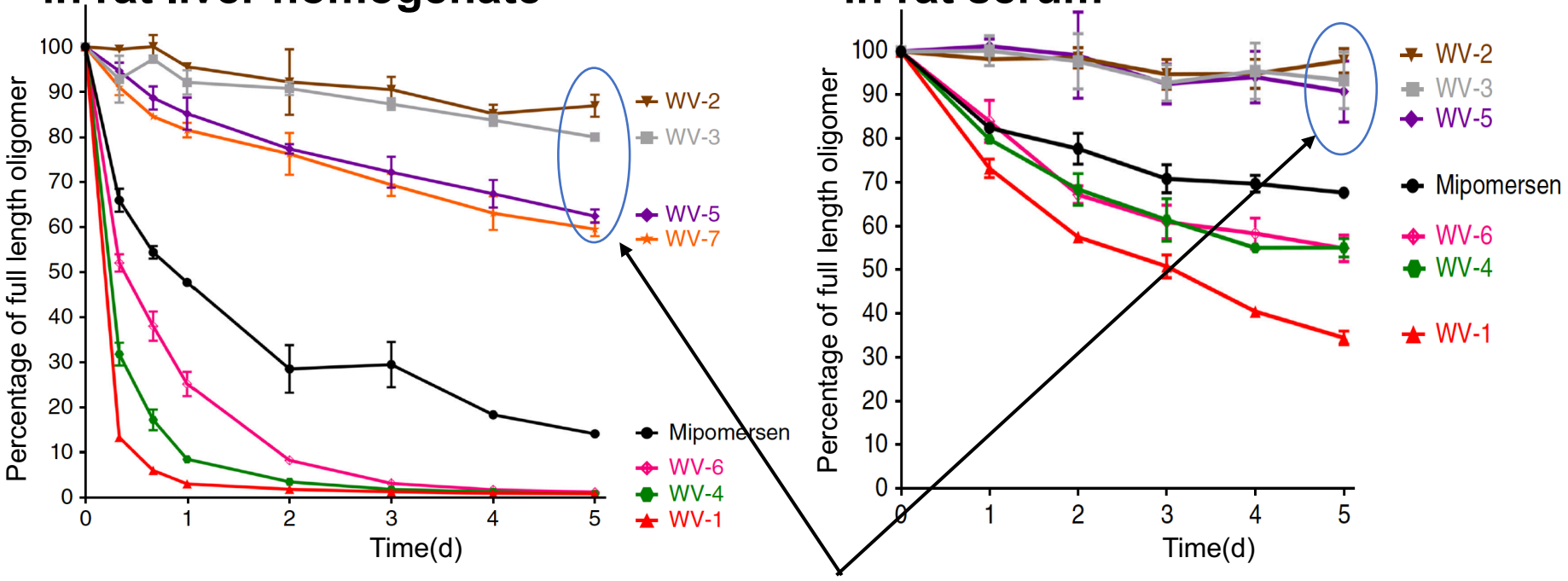
◇ Stereorandom ^ *Rp* v *Sp*

○ DNA ◌ 5-Methyl DNA ⬭ 2′-Methoxyethyl (MOE) ⬭ 5-Methyl 2′-Methoxyethyl (MOE)

- **WV-1– WV-4 was designed to test the effect of stereochemically uniform in the 2'-MOE ends and DNA core**
- **WV-5 and WV-6 was designed based on X-ray observation**
- **WV-7 was designed to test the stability of S_p linkage**

Stability of ASOs Affected by Stereochemistry of PS Linkage *in vitro*

- Time-dependent ASOs degradation in rat liver homogenate
- Time-dependent ASOs degradation in rat serum

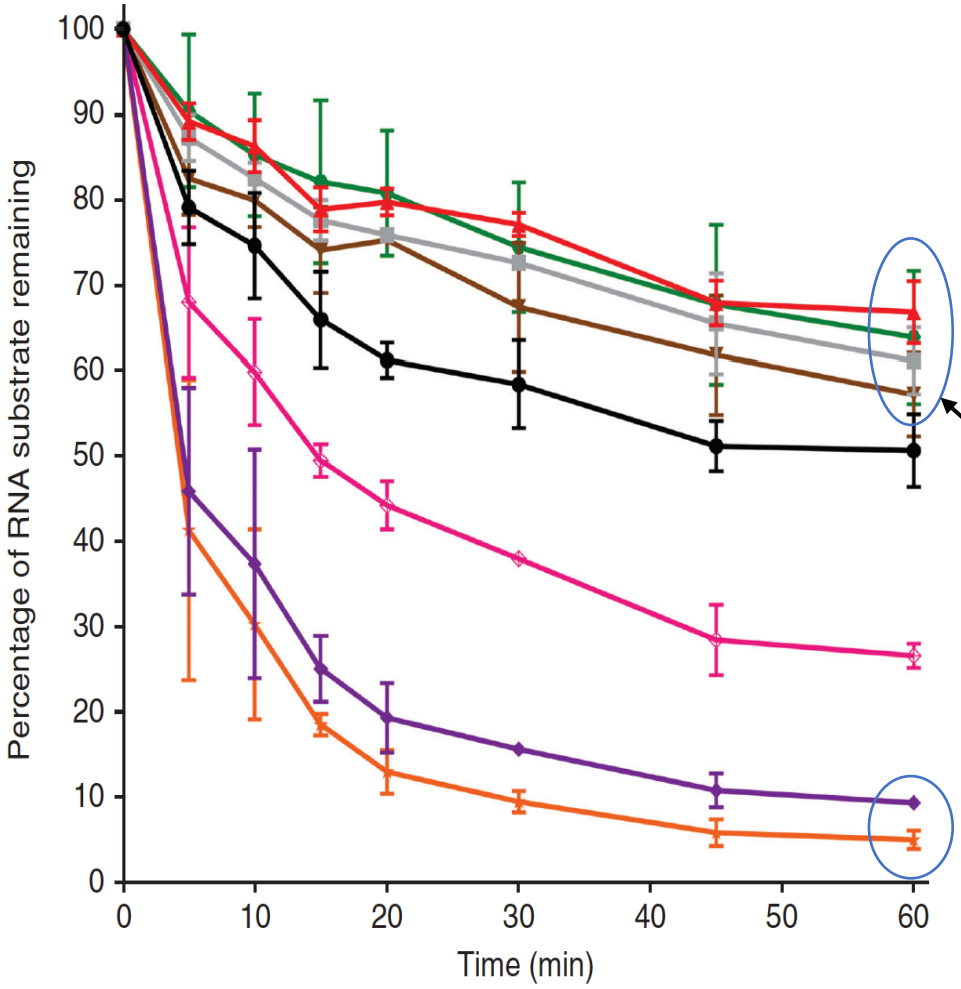


Upper group majorly contained S_p linkage

- S_p linkage, especially in DNA core, increased ASOs stability
- S_p linkage stabilized ASOs

Activity of RNase H Affected by Stereochemistry of PS Linkage *in vitro*

- Time-dependent RNA degradation *in vitro*



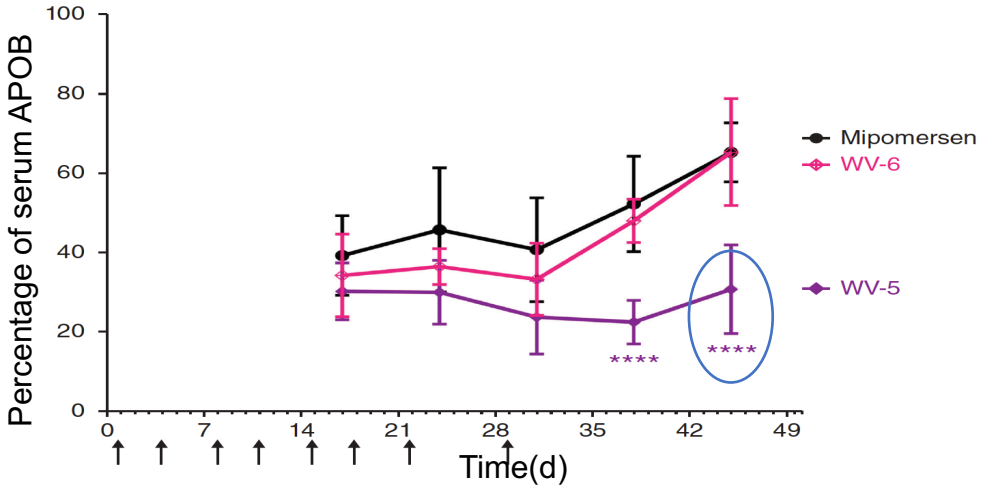
- **SSR sequence promoted RNase H activity**
- **<u>SSR code increased stability and activity of RNase H</u>**

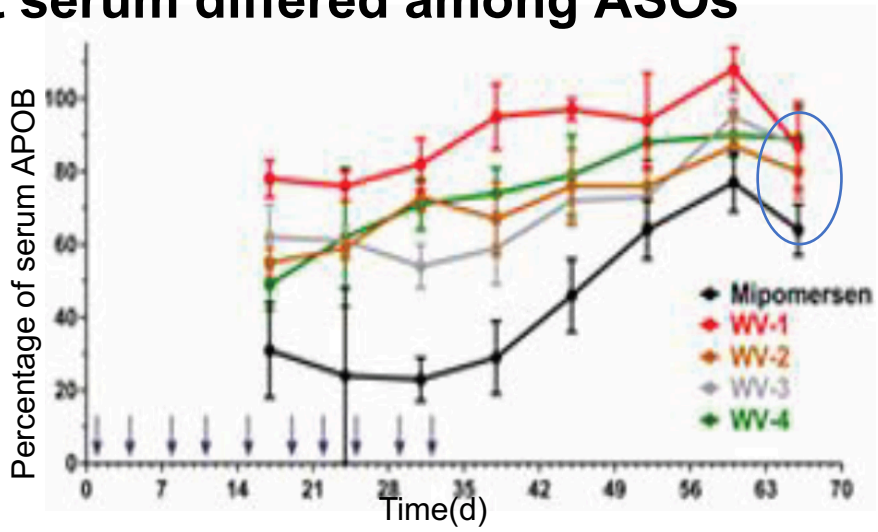
All-S_p/R_p isomer decreased activity

<u>Containing SSR sequence in DNA core</u>

Potency and Durability of Responses *in vivo*

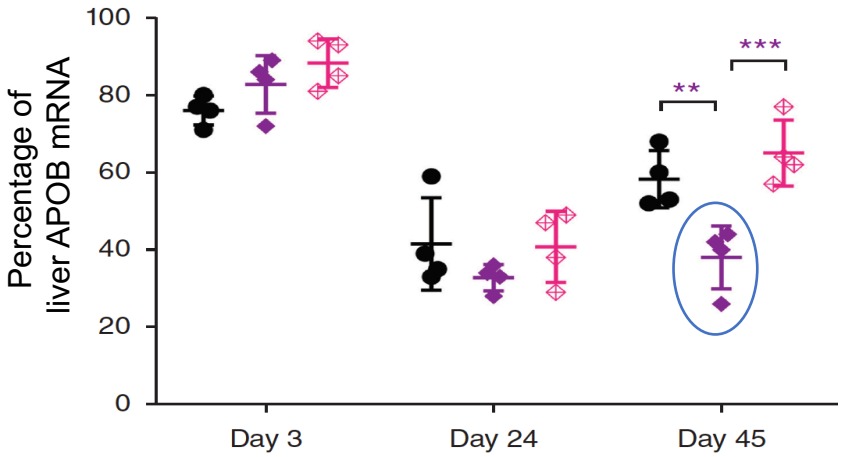
- Durability of responses in rat serum differed among ASOs



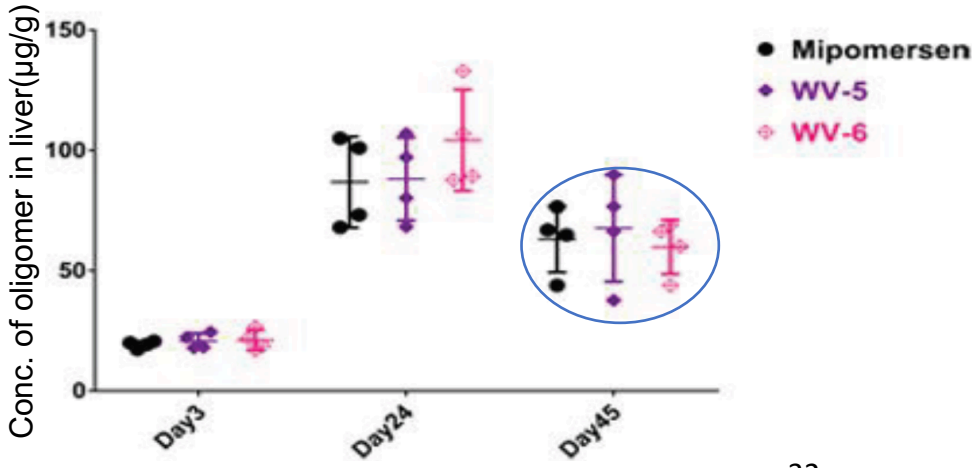


Dose: IP injection, 10 mg/kg

- Durability of responses in rat liver



- Persistence of ASOs in rat liver



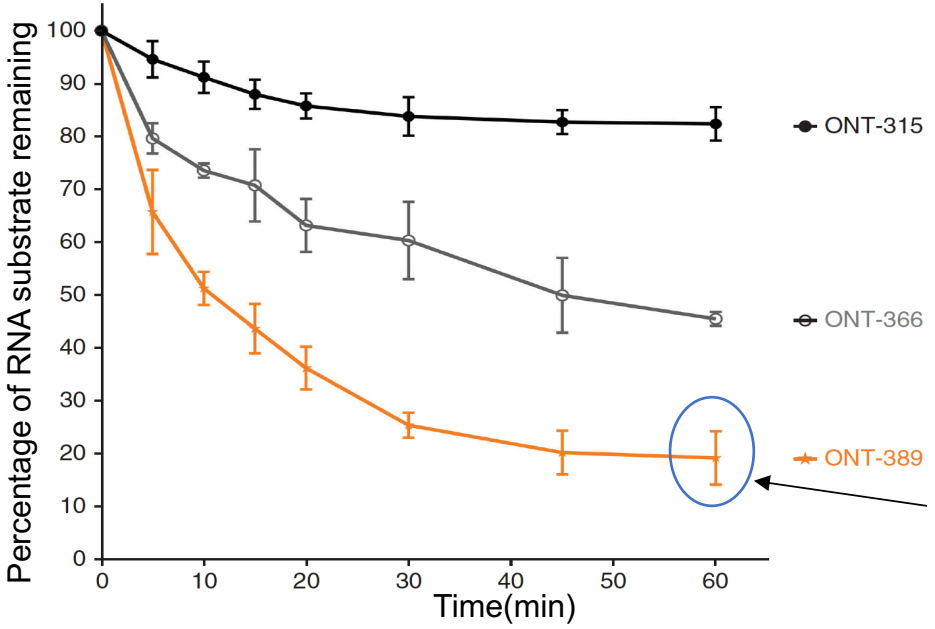
Sequence-Independent Effect of SSR Code (1)

ASO	Sequence	Number of stereoisomer
ONT-315	TGAGATGCCTGGCTGCCATA	524,288
ONT-366	TGAGATGCCTGGCTGCCATA	524,288
ONT-389	TGAGATGCCTGGCTGCCATA	1

Stereorandom Rp Sp

DNA 5-Methyl DNA 2'-Methoxyethyl (MOE) 5-Methyl 2'-Methoxyethyl (MOE)

- **Time-dependent RNA degradation *in vitro***

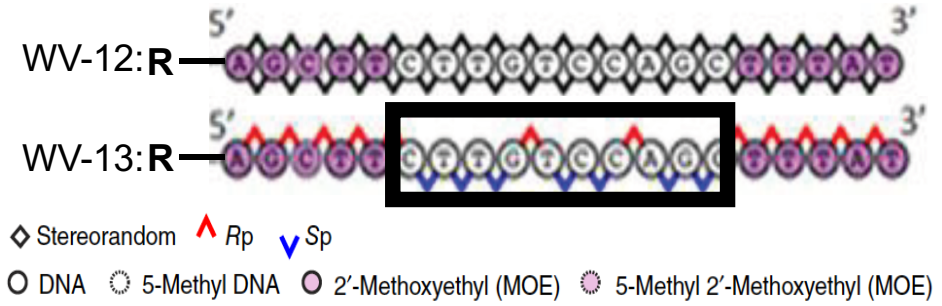


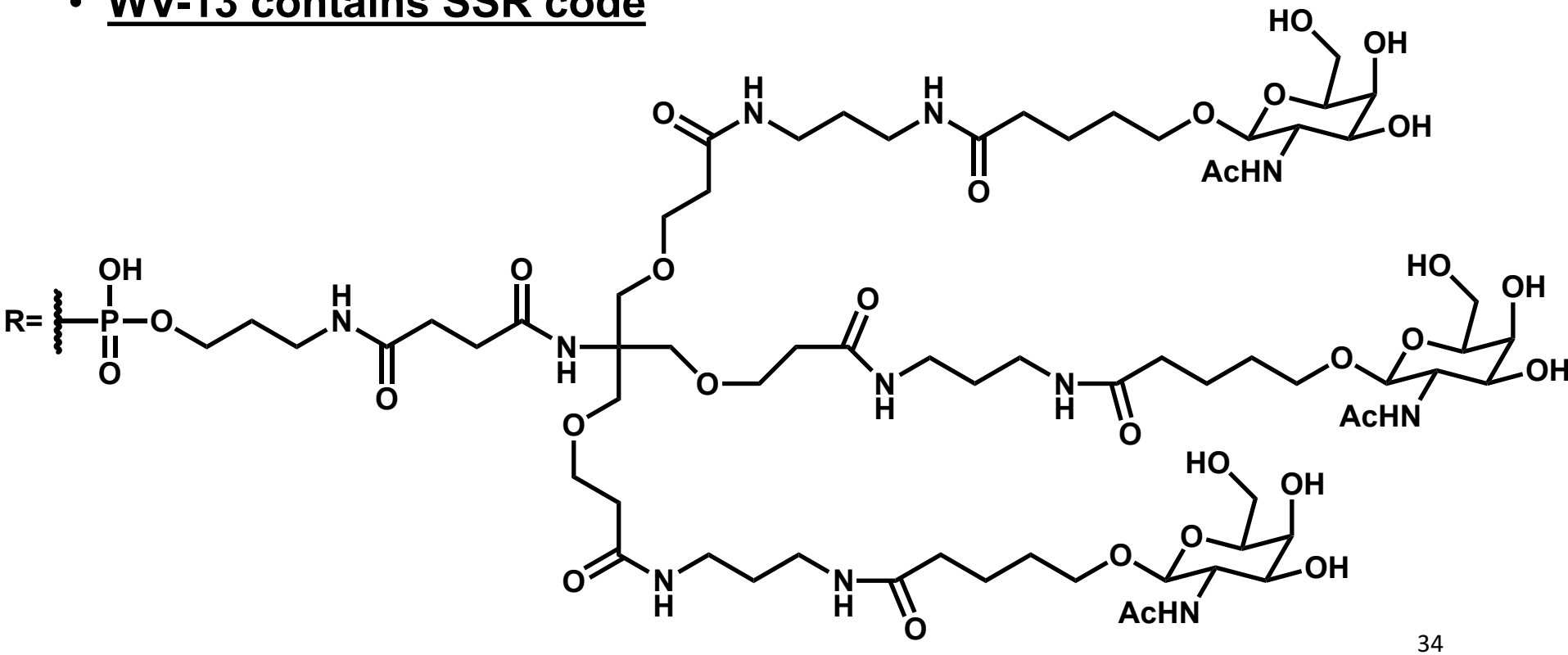
- **ONT targeted human FOXO1 mRNA**
- **<u>SSR code enhanced RNase H activity</u>**

<u>Containing SSR code in DNA core</u>

Sequence-Independent Effect of SSR Code (2)

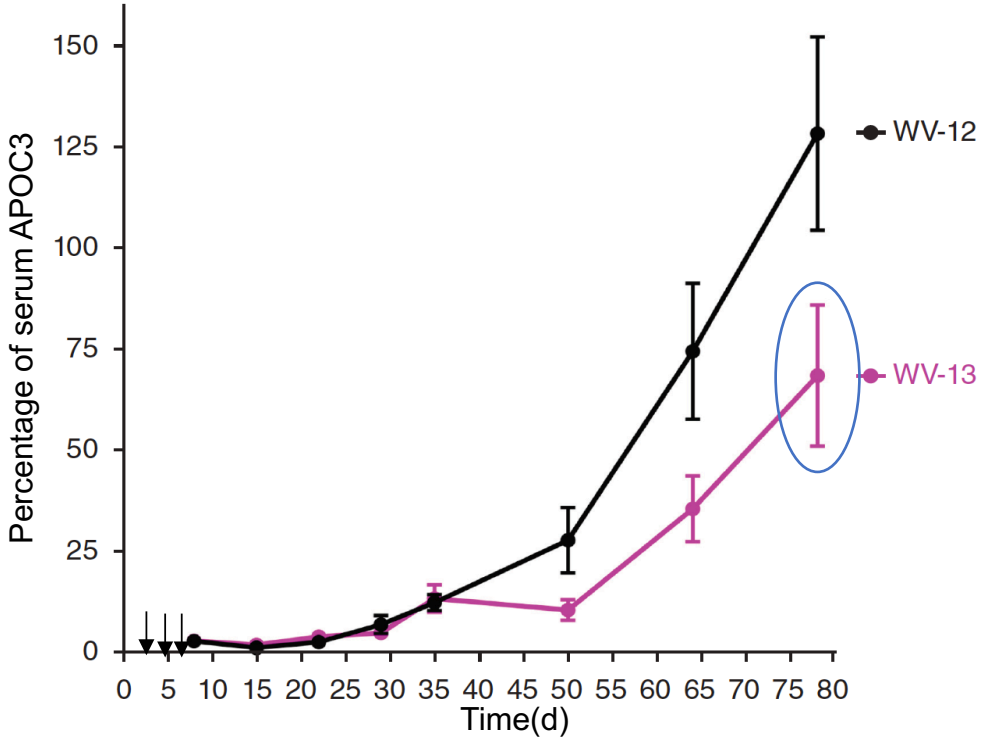
- **WV-12 targeted human ApoC mRNA**
- **Chemical modification improved delivery to liver**
- **<u>WV-13 contains SSR code</u>**

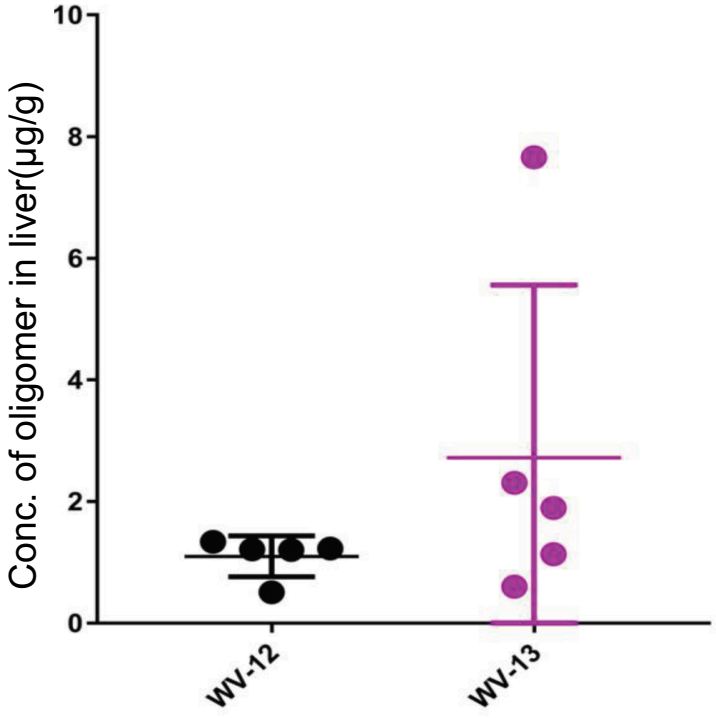




Sequence-Independent Effect of SSR Code (3)

- Durability of responses in rat serum
- Persistence of ASOs in rat liver





Dose: Subcutaneous injection, 5 mg/kg

- **Persistence of WV-12 and 13 was not significantly different**
- **WV-13 yielded a more durable response than WV-12**

Summary

Summary of main paper

- Synthetic method of a stereo-controlled 20-nucleotide-long sequence was established
- SSR code could yield more therapeutic efficacy than stereorandom mixture

Future perspective

- Discovery of the mechanism of interaction between PS linkage and enzymes is needed for progress of PS linkage-related research field
- SSR code is also applicable to anti-miRNA