A General and Efficient Approach for the Construction of RNA Oligonucleotides Containing a 5´-Phosphorothiolate Linkage

Nan-Sheng Li[1,*], John K. Frederiksen[1,†], Selene C. Koo[1], Jun Lu[1], Timothy J. Wilson[2], David M.J. Lilley[2,*], and Joseph A. Piccirilli[1,*]

[1]The University of Chicago, Center for Integrative Science, Departments of Biochemistry & Molecular Biology and Chemistry, 929 E. 57th Street, Room W406, Chicago, IL 60637, USA.

[2]Cancer Research UK Nucleic Acid Structure Research Group, MSI/WTB Complex, The University of Dundee, Dow Street, Dundee DD1 5EH, U.K.

[†]Present address: Department of Pathology and Laboratory Medicine, The University of Rochester Medical Center, 601 Elmwood Avenue, Box 626, Rochester, NY 14642.

*To whom correspondence should be addressed. E-mails: nli@uchicago.edu, d.m.j.lilley@dundee.ac.uk, jpicciri@uchicago.edu

Supplementary Data

Table S1: Synthesis and applications of oligonucleotides containing a 3´-S linkage

Oligonucleotide	Synthetic route	Applications	Reference
3´-S			
d($T_{3'-S}$T)	Coupling of 5´-deoxy-5´-*O*-monomethoxytrityl-3´-mercaptothymidine with 3´-*O*-acetylthymidine in the presence of (tetrazol-1-yl)$_2$POCH$_2$CH$_2$CN and 2,6-lutidine		(1)
	Solid phase synthesis using thymidine 3´-*S*-phosphoramidite		(2)
d(GCACG$T_{3'-S}$TGCACG)	Solid phase synthesis using thymidine 3´-*S*-phosphoramidite	Thio analog of a thymine photodimer	(3)
d(CCCUCU$_{3'-S}$A)	Solid phase synthesis using ribo- and deoxyribouridine 3´-*S*-phosphoramidites	Metal ion-dependent cleavage by the *Tetrahymena* group I ribozyme	(4)
		Metal ion-dependent cleavage by Klenow fragment of *E. coli* DNA polymerase I	(5)
$U_{3'-S}$U	Addition of 2´,3´-*O*-bis(benzoyl)-uridine 5´-*H*-phosphonate to 2´,5´-*O*-bis(Fpmp)-3´-(*o*,*p*-dinitrophenyldisulfanyl) uridine	RNA cleavage studies	(6)
$I_{3'-S}$U	Addition of 2´,3´-*O*-bis(*tert*-butyldimethylsilyl)-uridine 5´-*H*-phosphonate to 9-[5-*O*-(monomethoxytrityl)-3-deoxy-3-*S*-(5-nitropyridyl-2-disulfanyl)-2-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]hypoxanthine	Metal ion-dependent cleavage by the *Tetrahymena* group I ribozyme; test substrate for T4 PNK, snake venom PDE, and ribonuclease T_2	(7,8)
		Sugar pucker conformational analysis by NMR	(9)
d(GATT$_{3'-S}$GCTAGGC)	Solid phase synthesis using thymidine 3´-*S*-phosphoramidite	Mechanistic studies of the *E. coli* RuvC protein	(10)
(dU$_{3'-S}$) GUGAGUACUC CCUCUCAAAA	Solid phase synthesis using riboinosine and deoxyribouridine 3´-*S*-	Metal ion dependence of pre-mRNA splicing	(11)

$AI_{3'\text{-}S}CUCGCGGUU$	phosphoramidites		
$(C_{2'\text{-}OMe})_3UCdU_{3'\text{-}S}rA$	Solid phase synthesis using 2´-deoxy-3´-thiouridine phosphoramidites	Metal ion-dependent cleavage by the *Tetrahymena* group I ribozyme	(12,13)
R_P,S_P-$(C_{2'\text{-}OMe})_3UCdU_{3'\text{-}S}rA_{5'\text{-}PS}$			(13)
$UC_{3'\text{-}S}GAGCGGUCU$	Solid phase synthesis using 3´-thiouridine and thiocytidine phosphoramidites	Metal ion-dependent cleavage by the ai5γ group II intron	(14)
$U_{3'\text{-}S}ACUAUGUAU$			
$UC_{3'\text{-}S}ACUAUGUAU$			
$CGGGAU_{3'\text{-}S}ACUAUG$			(15)
$GACAI_{3'\text{-}S}GAUCCAAGAGUACU$	Solid phase synthesis using 3´-thioinosine phosphoramidite	Metal ion dependence of pre-mRNA splicing	(16)
d(CCTAAATT$_{3'\text{-}S}$TGCC) and others	Solid phase synthesis using thymidine 3´-*S*-phosphoramidite	Sugar pucker conformational analysis by NMR	(17,18)
d(AAACGTCGCACTTCGC$_{3'\text{-}S}$ TAGGCAGCCTGCATCCAGG)	Solid phase synthesis using 2´-deoxy-3´-thiocytidine phosphoramidite	Mechanistic studies of *E. coli* DNA T:G-mismatch endonuclease	(19)
d(TGTGTATTGTCT$_{3'\text{-}S}$ATAG)	Solid phase synthesis using thymidine 3´-*S*-phosphoramidite	Mechanistic studies of TrwC conjugative relaxase	(20)
d(TGTGTATTGTCT$_{3'\text{-}S}$ ATAGCCCAGATTTAAGGA)			
d(GCGCACCGAAAGGTGCGTATTG TCT$_{3'\text{-}S}$ATAG)			

Abbrievations: d, deoxyribo-; r, ribo-; Fpmp, 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl; I, inosine; PNK, polynucleotide kinase; PDE, phosphodiesterase; PS, nonbridging phosphorothioate.

Table S2: Synthesis and applications of oligonucleotides containing a 5´-S linkage

Oligoncleotide	Synthetic route	Applications	Reference
5´-S			
$U_{5'\text{-}SH}(U_{5'\text{-}S})_nU_{5'\text{-}S,2',3'\text{-}P}$	Treatment of 5´-thiouridine-2´,3´-cyclic phosphate with diphenyl phosphorochloridate and base		(21)
$d(TT_{5'\text{-}S})$	Thymidine 3´-thiophosphate attack on 5´-iodo-5´-deoxythymidine		(22)
$d(TT_{5'\text{-}S}T_{5'\text{-}S,3'\text{-}O\text{-}PS})$	Repeated addition of 5´-*O*-tosylthymidine-3´-*O*-cyanoethylphosphorothioate mononucleotides to a terminal thymidine 3´-*O*-thiophosphate		(23)
$d[T(T_{5'\text{-}S})_{12}]$		Test substrates for T4 PNK and DNA polymerase, *E. coli* DNA polymerase I, snake venom PDE, and S1 nuclease	(24)
d(TCCGTTGAAGCCTGCTTTT$_{5'\text{-}S}$ TTATACTAACTTGAGC)	Solid phase synthesis using 5´-*S*-trityl deoxythymidine phosphoramidite	Suicide substrate for DNA topoisomerase I	(25)
$UU_{5'\text{-}S}$	Uridine 3´-*H*-phosphonate attack on 5´-deoxy-5´-(*o*-nitrophenyldisulfanyl)uridine	RNA cleavage studies	(26)
	Uridine 3´-*O*-thiophosphate attack on 5´-iodo-5´-deoxyuridine	RNA cleavage studies	(27)
d(ACGGTCTCA$_{5'\text{-}S}$CGAGC)	Solid phase synthesis using 5´-*S*-trityl-2´-deoxyadenosine and 2´-*O*-Cee-cytidine phosphoramidites	RNA cleavage studies; metal ion-dependent cleavage by the hammerhead ribozyme	(28,29)
d(ACGGTCT)r(C)d(A$_{5'\text{-}S}$CGAGC)			
GCCGUCC$_{5'\text{-}S$CCCG	Solid phase synthesis using "5´-thiol amidite"	Metal ion-dependent cleavage by the hammerhead ribozyme	(30)
d(AGCCCTTACTT$_{5'\text{-}S}$ TGACGGTATATCT) (and others)	Solid phase synthesis using 5´-*S*-(4,4´-dimethoxytrityl)-2´-deoxy-5´-thiothymidine phosphoramidite	Detection and construction of DNA arrays based on incorporation of selective cleavage sites	(31)
$UUC_{2'\text{-}O\text{-}o\text{-}NBn}d(G_{5'\text{-}S})GGUCGGC$	Solid phase synthesis using 5´-*S*-trityl-2´-deoxyguanosine phosphoramidite	General acid catalysis by the HDV ribozyme	(32)
d(GGGCAT)r(C)d(C$_{5'\text{-}S}$ TGGATTCCACTCGCC)	Enzymatic ligation of 5´-thiophosphorylated d(CTGGATTCCACTCGCC) with d(GGGCAT)r(C)	General acid catalysis by the hammerhead ribozyme	(33)

Abbreviations: d, deoxyribo-; r, ribo-; PS, nonbridging phosphorothioate; PNK, polynucleotide kinase; PDE, phosphodiesterase; Cee, 1-(2-chloroethoxy)ethyl; *o*-NBn, *ortho*-nitrobenzyl; HDV, hepatitis delta virus.

References

1. Cosstick, R. and Vyle, J.S. (1988) Synthesis and Phosphorus Sulfur Bond-Cleavage of 3´-Thiothymidylyl(3´-5´)Thymidine. *J. Chem. Soc. Chem. Comm.*, 992-993.
2. Cosstick, R. and Vyle, J.S. (1990) Synthesis and Properties of Dithymidine Phosphate Analogs Containing 3´-Thiothymidine. *Nucleic Acids Res.*, **18**, 829-835.
3. Murata, T., Iwai, S. and Ohtsuka, E. (1992) Synthesis of a Dodecadeoxyribooligonucleotide Containing a 3´-Thio Analog of Thymidine Photodimer. *Heterocycles*, **33**, 529-531.
4. Piccirilli, J.A., Vyle, J.S., Caruthers, M.H. and Cech, T.R. (1993) Metal ion catalysis in the Tetrahymena ribozyme reaction. *Nature*, **361**, 85-88.
5. Curley, J.F., Joyce, C.M. and Piccirilli, J.A. (1997) Functional evidence that the 3 '-5 ' exonuclease domain of *Escherichia coli* DNA polymerase I employs a divalent metal ion in leaving group stabilization. *J. Am. Chem. Soc.*, **119**, 12691-12692.
6. Liu, X.H. and Reese, C.B. (1996) 3´-Thiouridylyl-(3´•5´)-uridine. *Tetrahedron Lett.*, **37**, 925-928.
7. Weinstein, L.B., Jones, B.C.N.M., Cosstick, R. and Cech, T.R. (1997) A second catalytic metal ion in a group I ribozyme. *Nature*, **388**, 805-808.
8. Weinstein, L.B., Earnshaw, D.J., Cosstick, R. and Cech, T.R. (1996) Synthesis and characterization of an RNA dinucleotide containing a 3´-*S*-phosphorothiolate linkage. *J. Am. Chem. Soc.*, **118**, 10341-10350.
9. Beevers, A.P.G., Witch, E.M., Jones, B.C.N.M., Cosstick, R., Arnold, J.R.P. and Fisher, J. (1999) Conformational analysis of 3´-*S*-PO_3-linked ribo- and deoxyribodinucleoside monophosphates. *Magn. Reson. Chem.*, **37**, 814-820.
10. Shah, R., Cosstick, R. and West, S.C. (1997) The RuvC protein dimer resolves Holliday junctions by a dual incision mechanism that involves base-specific contacts. *Embo J.*, **16**, 1464-1472.
11. Sontheimer, E.J., Sun, S. and Piccirilli, J.A. (1997) Metal ion catalysis during splicing of premessenger RNA. *Nature*, **388**, 801-805.
12. Shan, S., Yoshida, A., Sun, S., Piccirilli, J.A. and Herschlag, D. (1999) Three metal ions at the active site of the Tetrahymena group I ribozyme. *Proc. Natl. Acad. Sci. USA*, **96**, 12299-12304.
13. Yoshida, A., Sun, S. and Piccirilli, J.A. (1999) A new metal ion interaction in the Tetrahymena ribozyme reaction revealed by double sulfur substitution. *Nat. Struct. Biol.*, **6**, 318-321.
14. Sontheimer, E.J., Gordon, P.M. and Piccirilli, J.A. (1999) Metal ion catalysis during group II intron self-splicing: parallels with the spliceosome. *Genes Dev.*, **13**, 1729-1741.

15. Gordon, P.M., Sontheimer, E.J. and Piccirilli, J.A. (2000) Kinetic characterization of the second step of group II intron splicing: role of metal ions and the cleavage site 2´-OH in catalysis. *Biochemistry*, **39**, 12939-12952.
16. Gordon, P.M., Sontheimer, E.J. and Piccirilli, J.A. (2000) Metal ion catalysis during the exon-ligation step of nuclear pre-mRNA splicing: extending the parallels between the spliceosome and group II introns. *RNA*, **6**, 199-205.
17. Beevers, A.P., Fettes, K.J., Sabbagh, G., Murad, F.K., Arnold, J.R., Cosstick, R. and Fisher, J. (2004) NMR and UV studies of 3´-S-phosphorothiolate modified DNA in a DNA : RNA hybrid dodecamer duplex; implications for antisense drug design. *Org. Biomol. Chem.*, **2**, 114-119.
18. Beevers, A.P., Fettes, K.J., O'Neil, I.A., Roberts, S.M., Arnold, J.R., Cosstick, R. and Fisher, J. (2002) Probing the effect of a 3´-S-phosphorothiolate link on the conformation of a DNA:RNA hybrid; implications for antisense drug design. *Chem. Commun. (Camb.)*, 1458-1459.
19. Elliott, S.L., Brazier, J., Cosstick, R. and Connolly, B.A. (2005) Mechanism of the Escherichia coli DNA T:G-mismatch endonuclease (Vsr protein) probed with thiophosphate-containing oligodeoxynucleotides. *J. Mol. Biol.*, **353**, 692-703.
20. Gonzalez-Perez, B., Lucas, M., Cooke, L.A., Vyle, J.S., de la Cruz, F. and Moncalian, G. (2007) Analysis of DNA processing reactions in bacterial conjugation by using suicide oligonucleotides. *Embo J.*, **26**, 3847-3857.
21. Michelson, A.M. (1962) Polynucleotides. Part IV. Synthesis of Oligonucleotide Analogues Substituted in the Sugar Portion. *J. Chem. Soc.*, 979-982.
22. Cook, A.F. (1970) Nucleoside S-alkyl phosphorothioates. IV. Synthesis of nucleoside phosphorothioate monoesters. *J. Am. Chem. Soc.*, **92**, 190-195.
23. Chladek, S. and Nagyvary, J. (1972) Nucleophilic reactions of some nucleoside phosphorothioates. *J. Am. Chem. Soc.*, **94**, 2079-2085.
24. Rybakov, V.N., Rivkin, M.I. and Kumarev, V.P. (1981) Some substrate properties of analogues of oligothymidylates with p-s-C5´ bonds. *Nucleic Acids Res.*, **9**, 189-201.
25. Burgin, A.B., Jr., Huizenga, B.N. and Nash, H.A. (1995) A novel suicide substrate for DNA topoisomerases and site-specific recombinases. *Nucleic Acids Res.*, **23**, 2973-2979.
26. Liu, X.H. and Reese, C.B. (1995) Uridylyl-(3´□5´)-(5´-Thiouridine) - an Exceptionally Base-Labile Di-Ribonucleoside Phosphate Analog. *Tetrahedron Lett.*, **36**, 3413-3416.
27. Thomson, J.B., Patel, B.K., Jimenez, V., Eckart, K. and Eckstein, F. (1996) Synthesis and Properties of Diuridine Phosphate Analogues Containing Thio and Amino Modifications. *J. Org. Chem.*, **61**, 6273-6281.
28. Kuimelis, R.G. and McLaughlin, L.W. (1997) Application of a 5´-bridging phosphorothioate to probe divalent metal and hammerhead ribozyme mediated RNA cleavage. *Bioorg. Med. Chem.*, **5**, 1051-1061.
29. Kuimelis, R.G. and McLaughlin, L.W. (1995) Cleavage properties of an oligonucleotide containing a bridged internucleotide 5´-phosphorothioate RNA linkage. *Nucleic Acids Res.*, **23**, 4753-4760.

30. Zhou, D.M., Usman, N., Wincott, F.E., MatulicAdamic, J., Orita, M., Zhang, L.H., Komiyama, M., Kumar, P.K.R. and Taira, K. (1996) Evidence for the rate-limiting departure of the 5´-oxygen in nonenzymatic and hammerhead ribozyme-catalyzed reactions. *J. Am. Chem. Soc.*, **118**, 5862-5866.
31. Jahn-Hofmann, K. and Engels, J.W. (2004) Efficient Solid Phase Synthesis of Cleavable Oligodeoxynucleotides Based on a Novel Strategy for the Synthesis of 5´-*S*-(4,4´-Dimethoxytrityl)-2´-deoxy-5´-thionucleoside Phosphoramidites. *Helv. Chim. Acta*, **87**, 2812-2828.
32. Das, S.R. and Piccirilli, J.A. (2005) General acid catalysis by the hepatitis delta virus ribozyme. *Nat. Chem. Biol.*, **1**, 45-52.
33. Thomas, J.M. and Perrin, D.M. (2009) Probing general acid catalysis in the hammerhead ribozyme. *J. Am. Chem. Soc.*, **131**, 1135-1143.

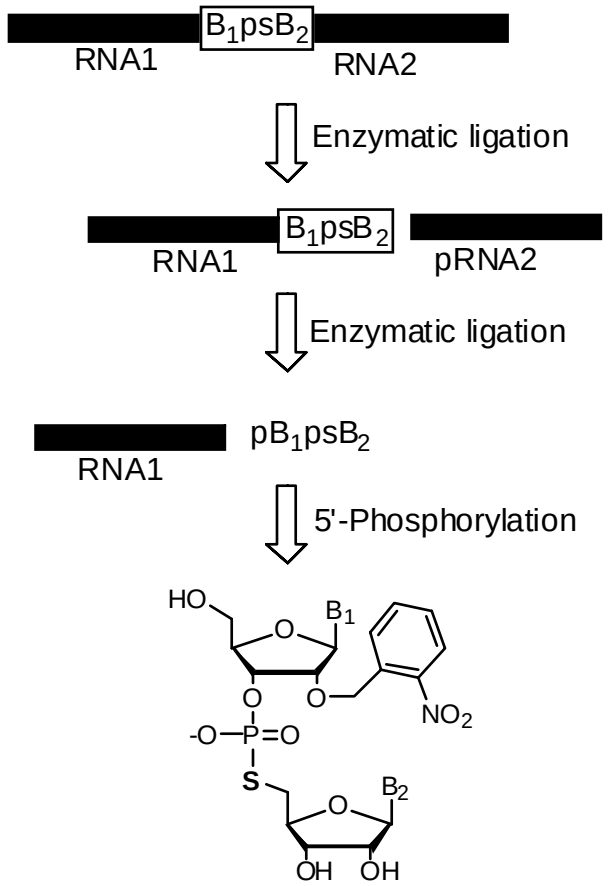


Figure S1. Retrosynthetic scheme for the construction of RNA oligonucleotides containing a 5′-phosphorothiolate linkage.

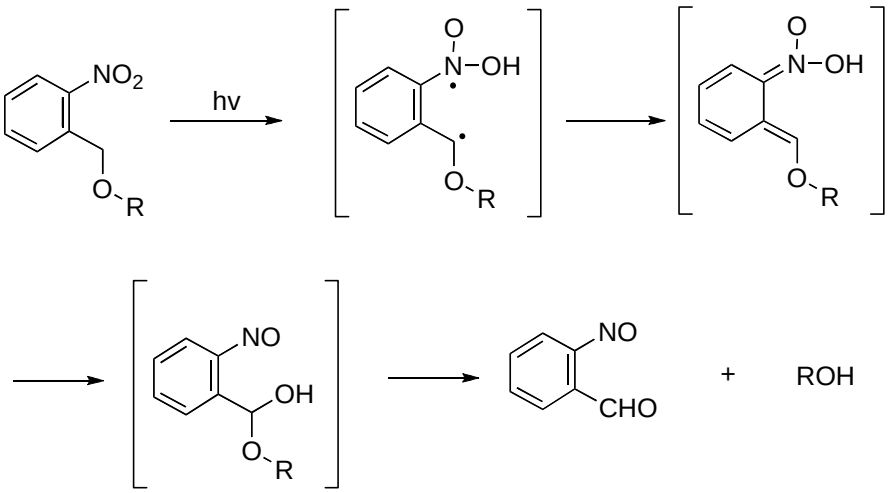


Figure S2. Mechanism of the UV light-mediated removal of the *o*-nitrobenzyl group to form ROH.

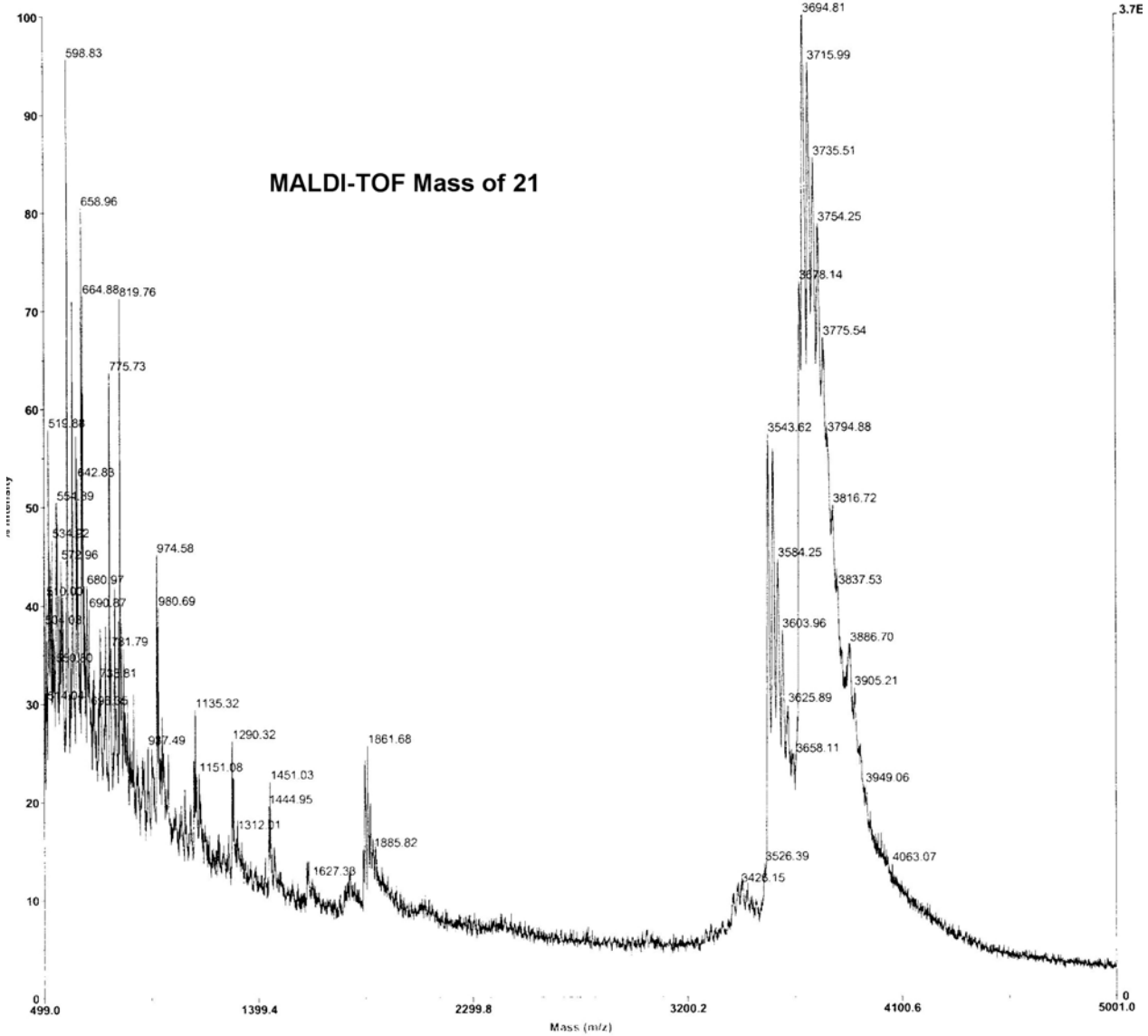


Figure S3: MALDI-TOF Mass of **21**: Calcd Mass: 3694.5, MALDI-TOF Mass: 3694.8.

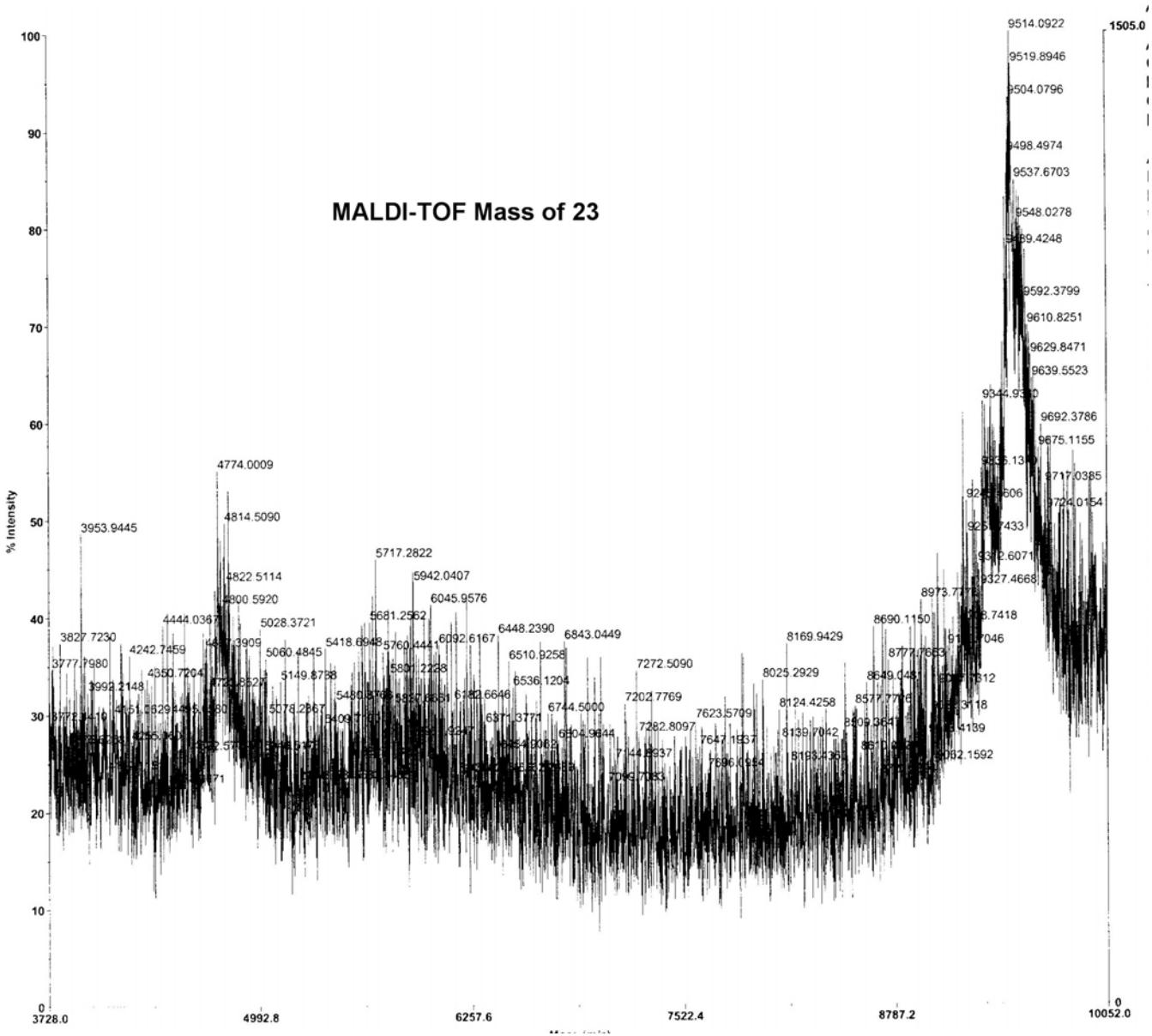


Figure S4: MALDI-TOF Mass of **23**: Calcd Mass: 9515.3, MALDI-TOF Mass: 9514.1.

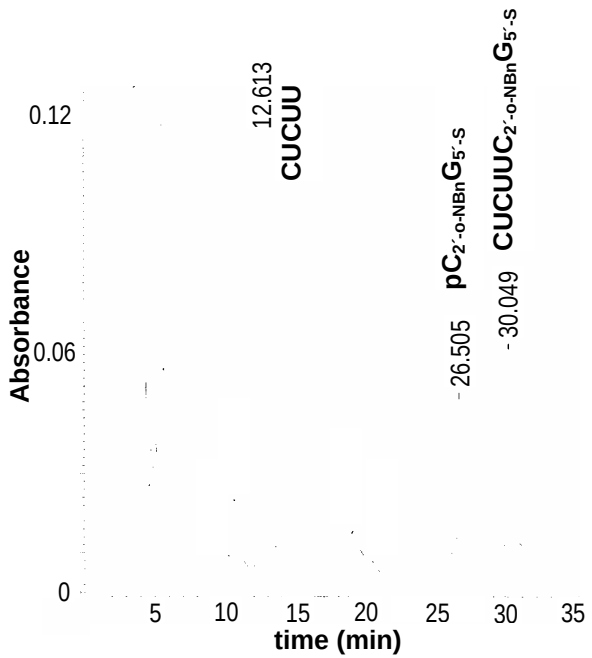
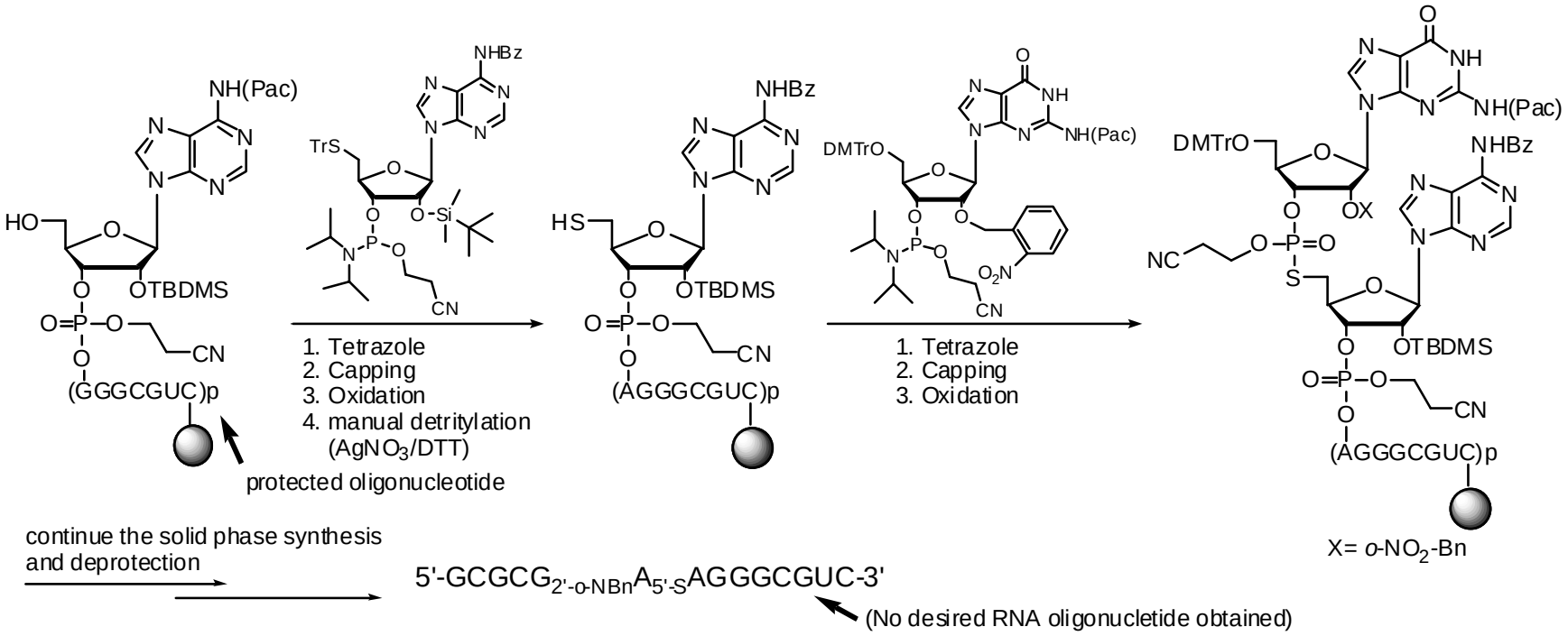
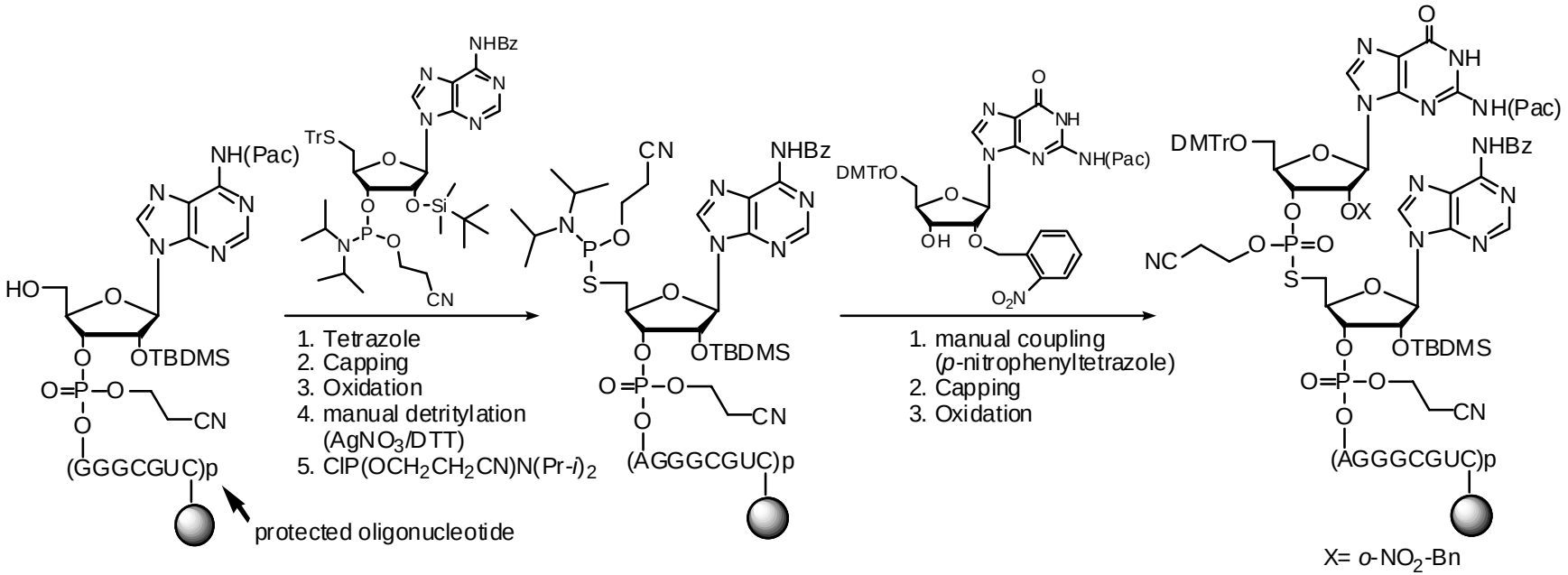


Figure S5. HPLC trace of crude reaction mixture after the first ligation step. HPLC conditions: C18 reversed-phase column, 6-16% acetonitrile / 94-84% 0.1 M TEAA pH 7.0 over 35 min. Peak identities are confirmed by migration of purified peaks on a denaturing gel.

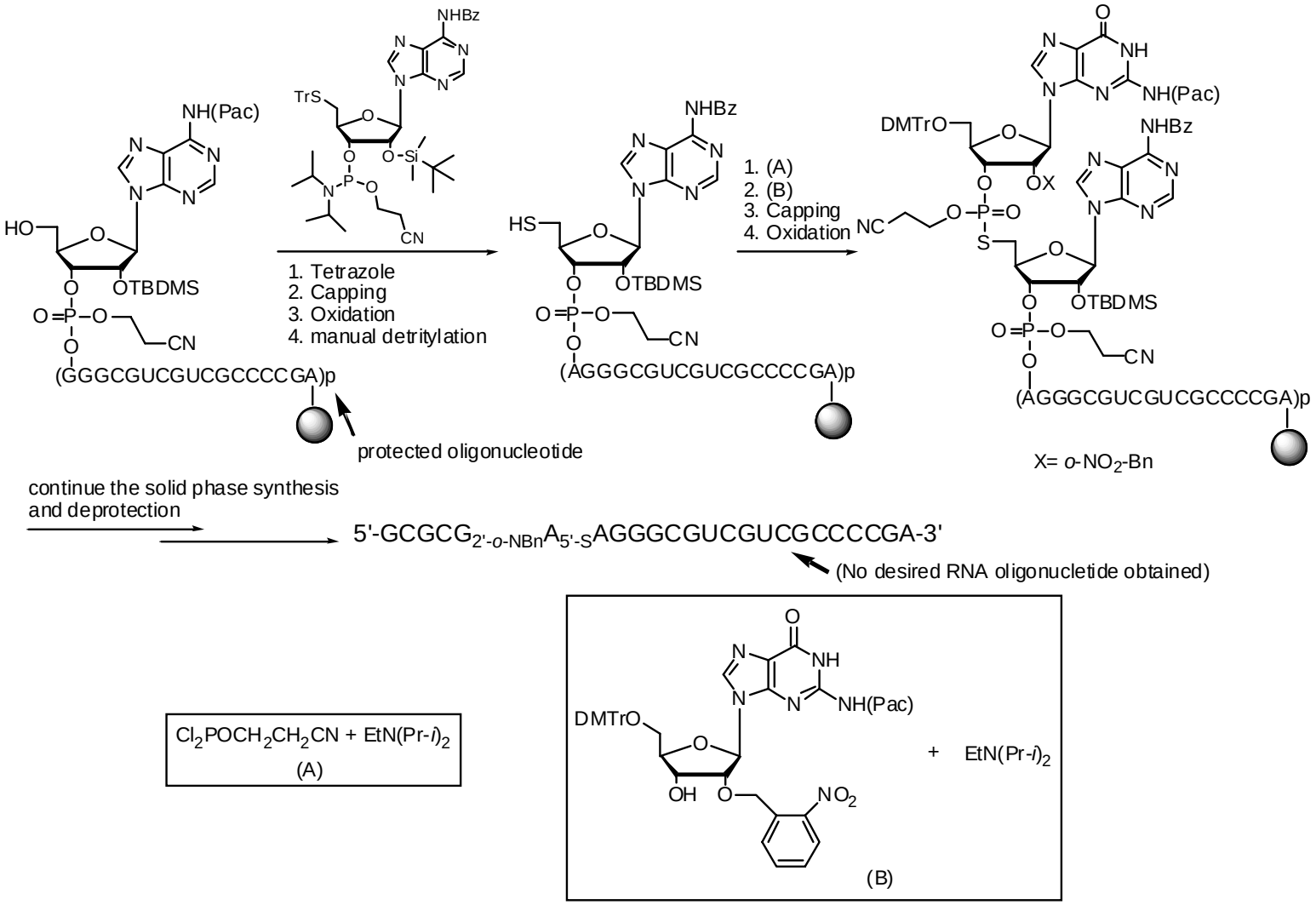
Scheme S1: Synthesis of 5'-GCGCG$_{2'\text{-}o\text{-}NBn}A_{5'\text{-}S}$AGGGCGUC-3'



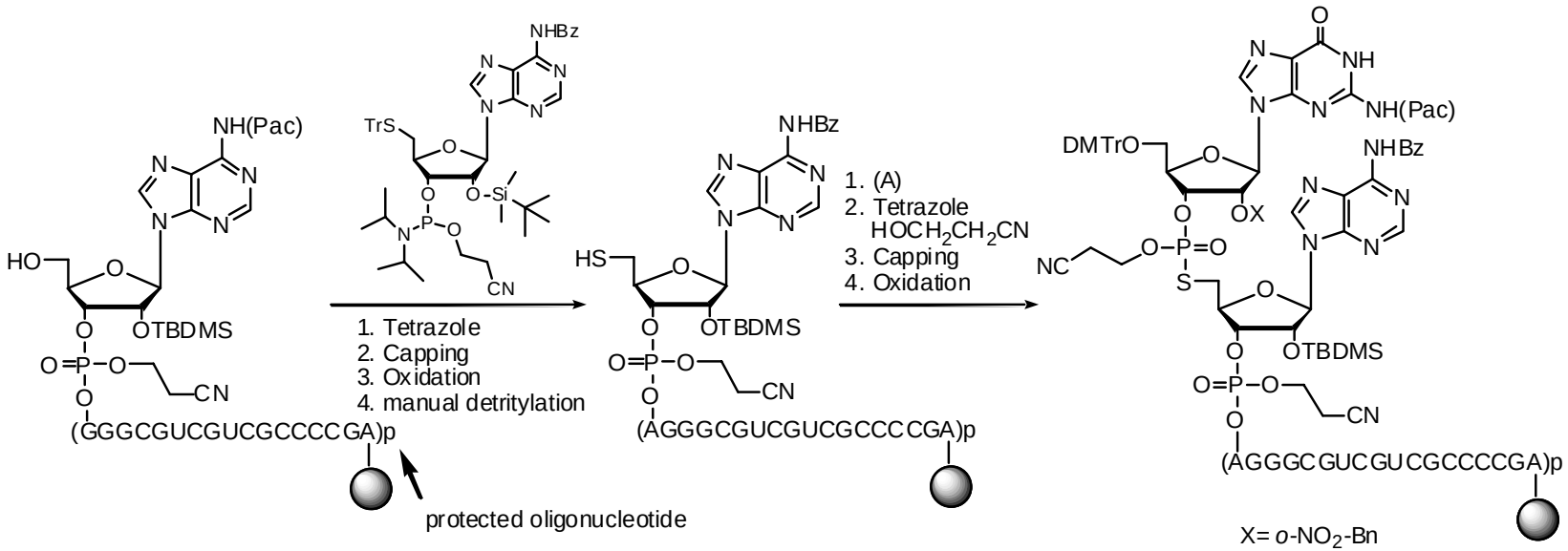
Scheme S2: Synthesis of 5'-GCGCG$_{2'\text{-}o\text{-}NBn}A_{5'\text{-}S}$AGGGCGUC-3'



Scheme S3: Synthesis of 5'-GCGCG$_{2'\text{-}o\text{-}NBn}A_{5'\text{-}S}$AGGGCGUCGUCGCCCCGA-3'



Scheme S4: Synthesis of 5'-GCGCG$_{2'\text{-}o\text{-}NBn}A_{5'\text{-}S}$AGGGCGUCGUCGCCCCGA-3'



continue the solid phase synthesis and deprotection

5'-GCGCG$_{2'\text{-}o\text{-}NBn}A_{5'\text{-}S}$AGGGCGUCGUCGCCCCGA-3'

(No desired RNA oligonucleotide obtained)