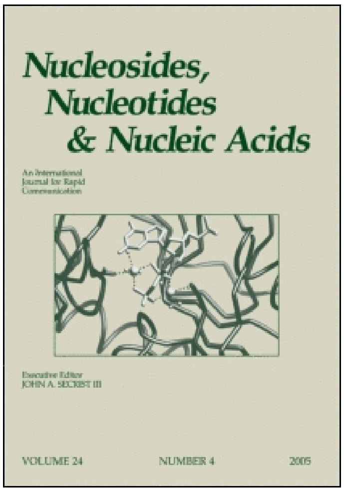

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Synthesis, characterization, and biological properties of small branched RNA fragments containing chiral (R_p and S_p) 2',5'-phosphorothioate linkages

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SYNTHESIS, CHARACTERIZATION, AND BIOLOGICAL PROPERTIES OF SMALL BRANCHED RNA FRAGMENTS CONTAINING CHIRAL (R_P AND S_P) 2′,5′-PHOSPHOROTHIOATE LINKAGES

Rawan Mourani and Masad J. Damha □ *Department of Chemistry, McGill University, Montreal, Quebec, Canada*

□ *Synthetic branched RNA fragments were prepared to examine the stereochemical requirements for hydrolysis of RNA lariats by the yeast debranching enzyme (yDBR). Specifically, two branched trinucleoside diphosphates and a tetranucleoside triphosphate containing a 2′,5′-linked phosphorothioate linkage of defined stereochemistry, namely R_p-A(2′ps5′G)pC, S_p-A(2′ps5′G)pC and S_p-ApA(2′ps5′G)pC, were prepared via solution-phase methods. Unlike the all-phosphodiester control, A(2′p5′G)pC, the R_p-thioated trimer was not cleaved by yDBR, demonstrating that changing the pro-R_p oxygen at the 2′,5′-phosphodiester bond averts hydrolysis by the enzyme. In contrast, the S_p branched compounds (trimer and tetramer) were cleaved yDBR, albeit with reduced efficiency relative to the corresponding all-phosphodiester branched compounds. Furthermore, the small branched RNAs (5 nt) were not cleaved as efficiently as a 18-nt bRNA, suggesting that the enzyme appears to have a stronger preference for larger bRNA substrates. The non-hydrolyzable branched RNA fragments prepared during these studies may be promising candidates for the future co-crystallization and X-ray analyses of DBR:bRNA complexes.*

Keywords Branched RNA; Debranching enzyme

INTRODUCTION

The cellular transformation of a precursor messenger RNA (pre-mRNA) into its mature or functional form, mRNA, proceeds by way of a splicing reaction, in which "exon" sequences are joined together to form the mature linear RNA. Typically, the "introns" or intervening sequences are excised in the shape of small circles with tails, better known as RNA "lariats"

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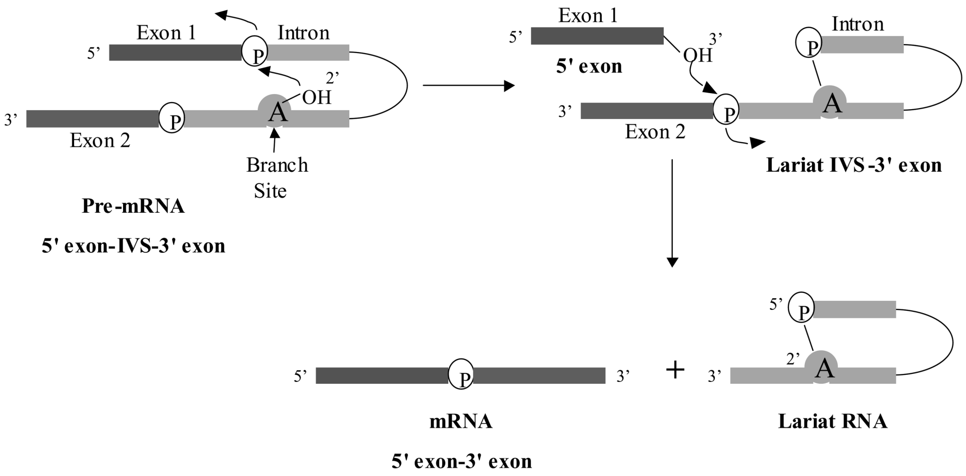


FIGURE 1 Cis splicing of pre-mRNA proceeds via lariat RNA intermediates.

(Figure 1).[1] The branchpoint of the molecule comprises a conserved adenosine residue containing vicinal 2′,5′ and 3′,5′-phosphodiester linkages. In many eukaryotic organisms, pre-mRNA splicing is not autocatalytic but occurs in the "spliceosome,"[2–4] a multi-protein complex consisting of *s*mall *n*ucleolar *r*ibo*n*ucleo*p*rotein units (*sn*RNPs), which themselves are intricate assemblages of polypeptides and *s*mall *n*uclear RNAs (*sn*RNAs). Assembly of these factors on the pre-mRNA occurs in a multistep fashion that involves binding and association of the snRNPs along the transcript to fold and orient the strand while holding the reactive centers in close proximity to aid in the subsequent transesterification steps.

Branched RNA molecules were first isolated from total nuclear RNA by Wallace and Edmonds in 1983.[5] Their low abundance relative to the mature mRNA suggested that the molecules were being hydrolyzed soon after their formation, probably to yield the corresponding linearized RNA. Accordingly, a novel 2′,5′-phosphodiesterase activity was observed by Ruskin and Green in 1985 in HeLa cell extracts.[6] This "debranching" activity specifically hydrolyzes 2′,5′-phosphodiester linkages in branched molecules to generate linear RNA species. *S. cerevisiae* Dbr1 ("DBR") is a 405-amino acid RNA debranching enzyme first isolated and characterized by Boeke and Chapman in 1991.[7] The only role the lariat debranching enzyme was believed to have was to provide a method for linearizing the lariats for further degradation. However, recent studies have shown that introns, along with DBR, appear to play more essential and vital roles *in vivo*.[8–10] For example, *s*mall *n*ucle*o*lar RNA introns (*sno*RNAs) participate in splicing through processing and modification of pre-rRNA and rRNA nucleotides, repectively.[9] The active snoRNA species are generated upon debranching of the circularized precursor RNA lariat in *S. cerevisiae; dbr1* deletion mutants produce the intronic snoRNA as "trapped" lariat forms, with concomitant reductions in cellular levels of mature intronic snoRNAs.[8] The function of DBR may be more crucial in higher eukaryotic cells, which contain a greater number of larger introns in most genes. Accordingly, Boeke and Nam demonstrated that *S. pombe* mutants with a defective *dbr* gene showed severe growth defects and altered cell morphologies relative to the wild-type yeast strains, whereas *S. cerevisiae* displayed no significant phenotypic defects, presumably owing to a relatively simpler genomic organization (*ca.* 40% and 2.5% intron content, respectively).[10] These lines of evidence suggest DBR to be crucial for cell viability in higher eukaryotes (*e.g.*, mammalian cells) that therefore merit further exploration of the substrate specificity and properties of this unique phosphodiesterase.

We have previously shown that yDBR can digest various branched nucleic acids (bNA) but exhibits a strong preference for purines over pyrimidines at the branchpoint as well as the 2′-position.[11–14] Conversely, the nucleotide at the 5′-position has only a negligible influence on debranching efficacy, given that branched oligonucleotides lacking the 5′-nucleotide were still highly susceptible to efficient debranching by the enzyme.[13,14] In a separate study examining the role of substrate stereochemistry on debranching activity, Padgett and coworkers[15] showed an inability for the HeLa cell RNA debranching enzyme to hydrolyze an RNA lariat containing an R_p phosphorothioate at the 2′,5′-linkage. However, the susceptibility of an S_p 2′, 5′-linkage towards debranching by the yeast or mammalian enzymes is unknown at present.

With the recent studies and discoveries on DBR, more is known and appreciated about its cellular importance, especially in higher organisms. However, not much information currently exists regarding its precise mechanism of action, structure, or substrate specificity. It is thus the focus of this work to explore the solution syntheses of the first branched RNA fragments containing R_p and S_p stereochemistry at the 2′,5′-phosphorothioate linkage (Figure 2). Both isomers of the branched phosphorothioate were used as substrates to further elucidate the particular substrate specificity of this important enzyme.

MATERIALS AND METHODS

Materials

Pyridine was continuously refluxed over barium oxide under nitrogen and distilled prior to use. Tetrahydrofuran (THF) was refluxed over sodium and benzophenone under nitrogen until a purple color persisted. Acetonitrile (CH_3CN), used for organic synthesis, was refluxed over phosphorus pentoxide under inert atmosphere before use, while CH_3CN used for nucleoside phosphoramidite coupling, in solution or on solid phase, was further refluxed and distilled over calcium hydride (CaH_2). Dichloromethane

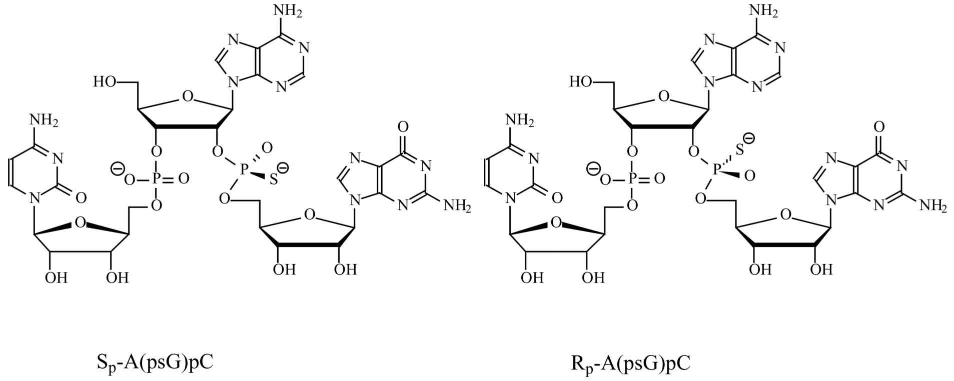


FIGURE 2 Structure of the stereoisomeric R_p- and S_p-branched trinucleoside diphosphates.

(DCM) was refluxed over CaH_2. N,N-dimethylformamide (DMF), collidine (2,3,6-trimethylpyridine), and diisopropylethylamine (DIPEA) were all dried by refluxing over calcium hydride (CaH_2), distilled under reduced pressure, and stored over 4Å molecular sieves. Triethylamine (TEA) was distilled over calcium hydride (CaH_2) and was stored over 4Å molecular sieves.

Methods

Gravity and flash silica gel column chromatography were performed on Merck Kieselgel 60 (200–400 mesh) silica gel (VWR). Reactions were analyzed and monitored using Merck Kieselgel 60F 254 silica analytical TLC aluminum sheets (0.2 × 20 cm × 20 cm). Preparative TLC to purify compounds was carried out using glass plates (20 cm × 20 cm) coated with a thick layer of Kieselgel 60 GF 254 silica gel. Ammonium salts produced by the desilylation reaction via TBAF were removed using Dowex 50W-X8 cation exchange resin (Na^+ form, 20–50 mesh). The resin was regenerated by washing with 0.1 N HCl, followed by 0.1 N NaOH, followed by autoclaved H_2O until the filtrate was of neutral pH. It was then autoclaved and packed into a column (5 × 1.5 cm) and equilibrated with autoclaved H_2O prior to usage.[16]

NMR spectra were obtained at ambient temperature using Varian Mercury-200, Mercury-300, Mercury-400, and UNITY-500 spectrophotometers. ^1H NMR spectra were referenced to the solvent deuterium signal, whereas 85% H_3PO_4 was used as an external reference for ^{31}P NMR (δ 0 ppm). UV/VIS spectra were collected using a Varian CARY1 UV/VIS spectrophotometer at ambient temperature (Varian: Mulgrave, Victoria, Australia). The data was computed using Cary WinUV version 2 software (Varian Ltd.). Spectra were obtained in 95% ethanol for protected nucleoside derivatives, and water for the completely deprotected oligonucleotides.

Circular dichroic spectra were recorded using a JASCO J-710 spectropolarimeter at ambient temperature.[17] The spectra were recorded using an average of five scans between 180 and 350 nm, at a rate of 50 nm/min and sampling wavelength of 0.2 nm. The buffer used was 50 mM Na_2HPO_4, 100 mM HCl, at pH 7.25.

FAB-MS spectra were collected using KRATOS MS25RFA high-resolution spectrometer under Xenon gas with a beam energy of ~7 KV. Nitrobenzyl alcohol (NBA) matrix was used. ESI and APCI spectra were collected using a Finnigan LCQ Duo, ThermoQuest.

MALDI-TOF was performed using a Kratos mass spectrometer. The oligomers (0.5 OD dissolved in 5 μL H_2O) were mixed with 5 μL of matrix, and 1 μL of the solution was used per analysis. The matrix used contained 10 mg/mL 6-Aza-2-thiothymine in a solution of 1:1 CH_3CN/ammonium acetate (20 mM) as described elsewhere.[18]

HPLC analysis and purifications were carried out on a Waters HPLC equipped with an M486 Tunable UV detector and a W600E multisolvent delivery system, dual pump heads (225 μL), a UK6 injector, and a temperature controller. The system is interfaced to a PC running Millennium® software version 3.20. Solvents were filtered through a 0.45-μm filter and degassed with helium prior to use. For oligonucleotide purification, an anion exchange 7.5 × 75 mm Protein-Pak™ DEAE 5PW HPLC column was used. The mobile phases consisted of: Solvent A: H_2O (doubly distilled and deionized using the Millipore system), Solvent B: 1 M $LiClO_4$. Chromatograms were run using a gradient of 0–10% Solvent B over 60 min with a flow rate of 1.2 mL/min at ambient temperature. For analytical runs, 0.3–0.7 O.D. of sample was injected; for preparative runs, 3–7 O.D. was injected. Enzymatic digestion analyses were performed using a reverse-phase HP Hypersil ODS-C_{18} column (4.6 × 250 mm, 5 μm) from Waters with a flow rate of 1.2 mL/min. The mobile phases comprised of: Solvent A: 50 mM TEAA (triethylammonium acetate), Solvent B: CH_3CN. Chromatograms were obtained utilizing a gradient of 0–10% Solvent B over 10 min, followed by 10–35% Solvent B over 15 min.[19] Under these conditions, the order of elution of nucleosides was: rC (5.9 min), rI (12.3 min; resulting from deamination of rA), rG (12.7), and rA (15.2 min). The deprotected isomers of the phosphorothioate dimers were resolved on an HB Zorbax RX-C_{18} semi-preparative reverse-phase column (9.4 × 250 mm, 5 μm) at r.t. with a flow rate of 4 mL/min. The mobile phases consisted of, Solvent A: 50 mM TEAA, solvent B: CH_3CN. The gradient utilized 0–20% Solvent B over 60 min at a temperature of 22°C.

Monomer Synthesis

N^2-Isobutyrylguanosine (2a). Guanosine (35.3 mmol, 10 g) was dried by coevaporation (anhydrous pyridine) and suspended in anhydrous pyridine (160 mL) to which chlorotrimethylsilane (TMSCl; 7.8 eq, 265 mmol, 33.6 mL) was added dropwise at 0°C. The mixture was stirred at r.t. for 1.25 h, cooled to 0°C, and isobutyric anhydride (5 eq, 176.5 mmol, 29 mL) was added, after which stirring was resumed overnight at r.t. Cold H_2O (150 mL) was added to the reaction followed by 28% NH_4OH (65 mL) at 0°C and the mixture was stirred at r.t. for 50 min, at which time it was concentrated to an oil, dissolved in water, and washed with ethyl acetate. Purification was carried out via crystallization from H_2O, to yield a white powder in 75% isolated yield. ^{1}H NMR (DMSO-d_6, 400 MHz): 8.24 (s, 1, H_8), 5.77 (d, 1, $H_{1'}$), 4.40 (m, 1, $H_{2'}$), 4.41 (m, 1, $H_{3'}$), 3.88 (dt, 1, $H_{4'}$), 3.59–3.52 (m, 2, $H_{5'}$, $H_{5''}$), 2.75 (m, 1, CH-*i*Bu), 0.99 (dd, 6, CH_3-*i*Bu).

N^2-Isobutyryl-5′-*O*-(4,4′-dimethoxytrityl) guanosine (2b). N^2-Isobutyrylguanosine (**2a**) (24.4 mmol, 8.6 g) was dried by coevaporation with anhydrous pyridine and then suspended in (80 mL) anhydrous pyridine. The mixture was cooled to 0°C before adding, dropwise, a mixture of dimethoxytrityl (DMT) chloride (1.2 eq, 29 mmol, 9.9 g) and 4-dimethylaminopyridine (DMAP, catalytic amount, 40 mg) in pyridine (25 mL) under a nitrogen atmosphere. The mixture was allowed to warm to r.t. and was stirred overnight before it was stopped by adding 5% $NaHCO_3$ (100 mL). The crude product was then extracted with ethyl acetate, and the combined organic layers dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo* to afford a dark brown oil. The crude mixture was purified via flash silica gel column chromatography CH_2Cl_2/MeOH/NEt_3 (gradient 99.5:0:0.5 to 96.5:3:0.5), to recover N^2-iBu-guanosine as a white foam in 80% yield (12.7 g). TLC: CH_2Cl_2/MeOH 9:1, R_f 0.36. MS (FAB-nitrobenzyl alcohol, calc. M^+: 655), found: MH^+: 656, DMT^+: 303. ^{1}H NMR (DMSO-d_6, 400 MHz): 8.11 (s, 1, H_8), 7.31–7.20–6.80 (overlapping m, 13, DMT), 5.84 (d, 1, $H_{1'}$), 5.62 (d, 1, $OH_{2'}$), 5.19 (d, 1, $OH_{3'}$), 4.51 (m, 1, $H_{2'}$), 4.19 (m, 1, $H_{3'}$), 4.01 (dt, 1, $H_{4'}$), 3.70 (s, 6, OCH_3), 3.23–3.15 (m, 2, $H_{5'}$, $H_{5''}$), 2.74 (m, 1, CH-*i*Bu), 1.09 (dd, 6, CH_3-*i*Bu).

N^2-Isobutyryl-2′,3′-*O*-dibenzoyl-5′-*O*-(4,4′-dimethoxytrityl)guanosine (2c). Compound (**2b**) (3 mmol, 2 g) was dried by coevaporation with anhydrous pyridine, suspended in anhydrous pyridine (40 mL), and cooled to 0°C before adding benzoyl chloride (2.4 eq, 7.3 mmol, 0.85 mL) dropwise. The mixture was allowed to warm to r.t. and was stirred for 3.5 h before it was stopped by adding 20 mL of H_2O at 0°C and stirring at r.t. for 5 min. The crude product was dissolved in CH_2Cl_2 (100 mL) and washed with H_2O. The organic phase was dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. The crude product was purified by flash silica gel column chromatography using CH_2Cl_2/MeOH (gradient 0–3% MeOH), to yield the desired nucleoside (containing some of the the corresponding N^2-benzoyl nucleoside; see compound 2d below) as a white foam (77%, 2 g). TLC: CH_2Cl_2/MeOH 9/:, R_f 0.54. MS (APCI-methyl alcohol, calc. M^+ for product with N^2-*i*Bu guanine: 863, calc. M^+ for product with N^2-Bz: 897), found: M^+: 863, 896 in ratio of 10:1, respectively, DMT^+: 303.

N^2-Isobutyryl-O^6-*p*-nitrophenylethyl-2′,3′-*O*- dibenzoyl-5′-*O*-(4,4′-dimethoxytrityl)guanosine (2d). To a stirred solution of (**2c**) (0.69 mmol, 0.6 g), triphenylphosphine (Ph_3P; 3 eq, 2.08 mmol, 0.53g) and $2-p$-nitrophenylethanol (3 eq, 2.08 mmol, 0.34 g) in anhydrous THF, was added diethyl azodicarboxylate (DEAD; 2.25 eq, 1.56 mmol, 0.24 mL) dropwise and the reaction was stirred overnight at r.t. The reaction was concentrated to an oil and extracted from 5% $NaHCO_3$ with CH_2Cl_2. The organic phase was dried over anhydrous Na_2SO_4, and the solvent removed under reduced pressure. The crude product was purified by flash silica gel column chromatography using 100% CH_2Cl_2 until the impurities were flushed out before using a gradient containing 1–2% MeOH (yield: 0.48 g, 69%). TLC: CH_2Cl_2/MeOH 19:1, R_f 0.77; CH_2Cl_2/MeOH 29:1, R_f 0.6; Toluene/EtOAc 1:1, R_f 0.58; Et_2O/CH_2Cl_2/EtOH 21:76:3, R_f 0.79. MS (FAB-nitrobenzyl alcohol, calc. M^+ for product with N^2-*i*Bu guanine: 1013, calc. M^+ for product with N^2-Bz guanine: 1047), found: MH^+: 1014, 1048 in ratio of ca. 3:1, respectively.

N^2-Isobutyryl-O^6-*p*-nitrophenylethyl-2′,3′-*O*-dibenzoylguanosine (2e). To compound (**2d**) (0.56 mmol, 0.48 g) was added *p*-toluenesulfonic acid-MeOH/CH_2Cl_2 (12 mL; prepared by stirring 3 g of *p*-TSA, 30 mL MeOH and 120 mL CH_2Cl_2) at r.t. After 15 min the reaction was diluted with CH_2Cl_2 and washed with 5% $NaHCO_3$. The organic phase was dried over anhydrous Na_2SO_4, followed by solvent removal *in vacuo* to afford a dark yellow crude residue. The pure product was precipitated from stirred hexanes and filtered to give **2e** contaminated with N^2-benzoyl-O^6-*p*-nitrophenylethyl-2′, 3′-*O*-di-benzoylguanosine as a white residue, *ca.* 3:1 ratio (85% combined yield, 0.28 g). TLC: CH_2Cl_2/MeOH 9:1, R_f 0.59; CH_2Cl_2/acetone 3:1, R_f 0.4. MS (FAB-nitrobenzyl alcohol, calc. M^+ for product with N^2-*i*Bu: 710, calc. M^+ for product with N^2-Bz: 744), found: MH^+: 711, 745 in ratio of 3:1, respectively.

N^4-Benzoyl-2′,3′-*O*-dibenzoyl-5′-*O*-(4,4′-dimethoxytrityl)cytidine (3c). N^4-Benzoyl-5′-*O*-dimethoxytritylcytidine (5.0 mmol, 3.27 g) was dried by coevaporation (anhydrous pyridine), suspended in anhydrous pyridine (60 mL) and the mixture was cooled to 0°C prior to the dropwise addition of BzCl (3.5 eq, 17.6 mmol, 2.04 mL). The mixture was allowed to warm to r.t. and stirred for 3.5 h before it was quenched with H_2O (30 mL) at 0°C and stirring continued at r.t. for 5 min. The crude product was dissolved in CH_2Cl_2 (120 mL) and washed with H_2O. The organic phase was dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. The crude product was purified by flash silica gel column chromatography using CH_2Cl_2/MeOH (gradient 0–3% MeOH), to give a pale yellow foam (86% yield, 3.73g). TLC: CH_2Cl_2/MeOH 9:1, R_f 0.74. MS (FAB-nitrobenzyl alcohol, calc. M^+: 857), found: MH^+: 858, DMT^+: 303.

N^4-Benzoyl-2′,3′-*O*-dibenzoylcytidine (3d). To compound (**3c**) (3.76 mmol, 3.24 g) was added a solution consisting of *p*-toluenesulfonic acid/MeOH/CH_2Cl_2 (76 mL; prepared by dissolving *p*-TSA (3 g) in MeOH (30 mL) and CH_2Cl_2 (120 mL) at r.t. After 15 min, the reaction was diluted with CH_2Cl_2 and washed with 5% $NaHCO_3$. The organic phase was dried (anhydrous Na_2SO_4), filtered and the solvent removed *in vacuo* to afford a dark yellow crude residue. The pure product was precipitated from a cold solution of stirred hexanes and was filtered to yield a white solid (79% yield, 1.51 g). TLC: CH_2Cl_2/MeOH 9.5:0.5, R_f 0.40; Hexanes/EtOAc/$NEt_3$2.7 : 7 : 0.5, R_f 0.19. MS (FAB-nitrobenzyl alcohol, calc. M^+: 555), found: MH^+: 556.

N^4-Benzoyl-2′,3′-*O*-dibenzoylcytidine-5′-*O*-[β-cyanoethyl-*N,N*-diisopropyl] phosphoramidite (3e). To a stirred solution of (**3d**) (2.71 mmol, 1.5 g), DIPEA (4 eq, 10.84 mmol, 1.86 mL) and DMAP (0.1 eq, 0.271 mmol, 33 mg) in anhydrous THF (24 mL) was added *N,N*-diisopropylamino-β-cyanoethylphosphonamidic chloride (1.2 eq, 3.25 mmol, 0.72 mL) dropwise at 0°C. The reaction was allowed to go to r.t. and was stirred for 2.5 h. The mixture was diluted with EtOAc (150 mL, prewashed with 5% $NaHCO_3$) and the crude product was washed with saturated brine solution (4 × 60 mL). The organic phase was dried over anhydrous Na_2SO_4 and the solvent removed under reduced pressure to afford a light yellow oil. Purification proceeded by flash silica gel column chromatography using EtOAc/Hexanes/NEt_3 (gradient 4:5.5:0.5 to 5:4.5:0.5), to afford a pure **3e** as a white foam (86% yield, 1.76 g). TLC: EtOAc/hexanes/NEt_3 7:2.5:0.5, R_f 0.38; CH_2Cl_2/MeOH 9.5:0.5, R_f 0.65. MS (FAB-nitrobenzyl alcohol, calc. M^+: 755), found: MH^+: 656. ^{31}P NMR (in acetone–d_6): 150.4, 149.9 ppm.

2′,5′-Linked Ap_sG Triesters (R_p-4 and S_p-4). To an Argon purged glass hypovial with an oven-dried magnetic stirring bar was added guanosine **2e** (0.128 mmol, 91 mg), *N*-Bz, 5′,3′-*O*-protected adenosine-2′-*O*-phosphoramidite (**1**; 1.2 eq, 0.154 mmol, 0.15 g) and 1*H*-tetrazole (4.4 eq, 0.565 mmol, 40 mg). The reaction was started by injecting anhydrous acetonitrile (1.5 mL) under nitrogen, and stirred at r.t. until complete consumption of the starting material was observed after 2 h. To this mixture was added a solution of 3-ethoxy-1,2,4-dithiazoline-5-one (EDITH) (1.2 eq, 0.154 mmol, 25 mg) in anhydrous acetonitrile (1 mL) under an inert atmosphere to

TABLE 1 R_f Values of Oligoribonucleotides

	R_f Value in CH_2Cl_2/MeOH, 9:1	
Compound	R_p Isomer	S_p Isomer
4	0.47	0.63
5	0.4	0.42
6	0.35	0.33
7	0.44	0.45
8	0.28	0.31
9	0.26	0.27
10	0.25	0.29

sulfurize the phosphite triester intermediate. The mixture was stirred at r.t. for 10 min and the solvent was evaporated. The crude product was dissolved in CH_2Cl_2 and washed with 5% $NaHCO_3$. The organic phase was dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure to obtain a yellow solid. The crude dimer was purified by flash silica gel column chromatography in order to separate the two isomers using CH_2Cl_2/Et_2O 5:5 (79% total yield, 109 mg (53%) of the first isomer (**R_p-4**), 53 mg (26%) of the second isomer (**S_p-4**). TLC: CH_2Cl_2/Et_2O 9:1, R_f value for **S_p-4** and **R_p-4** dimers are 0.16, 0.34; CH_2Cl_2/MeOH 9:1, R_f 0.47, 0.63; CH_2Cl_2/Acetone 3:1, R_f 0.4, 0.53, respectively. Refer to Tables 1 and 2 for the R_f and ^{31}P NMR values. MS (FAB-nitrobenzyl alcohol, calc. M^+ for product with N^2 – *i*Bu guanine: 1629, calc. M^+ for product with N^2-Bz guanine: 1663), found for the R_p isomer: MH^+: 1630, 1664 in ratio of 2.3:1, respectively, and DMT^+: 303; and for the S_p isomer: MH^+: 1630, 1664 in ratio of 2.5:1, respectively, and DMT^+: 303.

2′,5′-Linked Diester R_p-Ap$_s$G (R_p-5). R_p-2′,5′-ApG triester (**R_p-4**) (0.062 mmol, 101 mg) was dissolved in 3.5 mL TEA/CH_3CN (4:6 v/v) and the reaction was stirred at r.t. for 4 h. The solvent was then evaporated and the crude dimer redissolved in CH_2Cl_2 and washed with 5% $NaHCO_3$. The

TABLE 2 ^{31}P-NMR Data of Oligoribonucleotides

Oligomer # in NMR solvent	^{31}P NMR (ppm)	
	R_p Isomer	S_p Isomer
4 in acetone–d_6	69.4	68.8, 68.9
5 in acetone–d_6	59.4, 59.5	60.2, 60.5
6 in acetone–d_6	59.5, 59.8	—
7 in CH_2Cl_2-d_2	59.6, −0.3, −1.1	—
8 in CH_2Cl_2-d_2	61.1, 1.1	60.2, 1.0

*Isolated as mixture of N-Bz and N-iBu guanine bases, which converge to one compound upon N-deacylation (see Materials and Methods).

organic phase was dried over anhydrous Na_2SO_4, and the solvent evaporated under reduced pressure generating the crude phosphorothioate diester which was used without further purification in the ensuing reaction (97% yield, 94 mg). Refer to Tables 1 and 2 for the R_f and ^{31}P NMR values. MS (FAB-nitrobenzyl alcohol, calc. M^+ for product with N^2-*i*Bu guanine: 1575, calc. M^+ for product with N^2-Bz guanine: 1609), found: MH^+: 1576, 1610 in ratio of 2.8:1, respectively. DMT^+: 303.

2′,5′-Linked diester Sp-Ap$_s$G (S$_p$-5). S$_p$-2′,5′-Ap$_s$G triester (**S$_p$-4**) (0.031 mmol, 50 mg) was dissolved in 1.5 mL TEA/CH_3CN (4:6 v/v) and the reaction was stirred at r.t. for 3 h. The solvent was then evaporated and the crude dimer redissolved in CH_2Cl_2 and washed with 5% $NaHCO_3$. The organic phase was dried over anhydrous Na_2SO_4 and the solvent evaporated under reduced pressure generating the crude phosphorothioate diester which was used without further purification in the ensuing reaction (98% yield, 47.3 mg). Refer to Tables 1 and 2 for the R_f and ^{31}P NMR values. MS (FAB-nitrobenzyl alcohol, calc. M^+ for product with $N^2 - i$Bu guanine: 1575, calc. M^+ for product with N^2-Bz guanine: 1609), found: MH^+: 1576, 1610 in ratio of 1.7:1, respectively. DMT^+: 303.

2′,5′-Linked diester R$_p$-Ap$_s$G (R$_p$-6). R$_p$-2′,5′-Ap$_s$G diester (**R$_p$-5**), (0.049 mmol, 77 mg) was dissolved in excess TBAF solution (1.0 M in THF) which had been pH adjusted to 7.0 by adding glacial acetic acid. The reaction was stirred at 45°C overnight before it was quenched with H_2O and the crude product extracted with CH_2Cl_2 and dried under reduced pressure. The crude dimer was purified by flash silica gel column chromatography using EtOAc/MeOH (gradient 0–2% MeOH) (70% yield, 50 mg). Refer to Tables 1 and 2 for the R_f and ^{31}P NMR values. MS (ESI-MeOH-negative mode, calc. M^+ for product with N^2-*i*Bu guanine: 1461, calc. M^+ for product with N^2-Bz guanine: 1495), found: M-H^-: 1460, 1494 in ratio of *ca.* 2.4:1, respectively.

2′,5′-Linked diester S$_p$-Ap$_s$G (S$_p$-6). S$_p$-2′,5′-Ap$_s$G diester (**S$_p$-5**) (0.03 mmol, 47.3 mg) was dissolved in excess TBAF solution (1.0 M in THF) that had been pH adjusted to 7.0 by adding glacial acetic acid. The reaction was stirred at 42°C for 19 h before it was quenched with H_2O and the crude product extracted with CH_2Cl_2, and dried under reduced pressure. The crude dimer was purified by flash silica gel column chromatography using EtOAc/MeOH (gradient 0–2% MeOH) (82% yield, 36 mg). Refer to Tables 1 and 2 for the R_f and ^{31}P NMR values. MS (ESI-MeOH-negative mode, calc. M^+ for product with N^2-*i*Bu guanine: 1461, calc. M^+ for product with N^2-Bz guanine: 1495), found: M-H^-: 1460, 1494 in ratio of *ca.* 1.3:1, respectively.

Branched trimer R_p-A(p_sG)pC (R_p-7). To an Ar-purged glass hypovial with a magnetic stirring bar, was added R_p dimer (**R_p-6**) (0.027 mmol, 40 mg), cytidine-5′-phosphoramidite (**3e**) (6 eq, 0.164 mmol, 127 mg) and tetrazole (6 eq, 0.164 mmol, 12.6 mg). The reaction was initiated by injecting anhydrous acetonitrile (0.75 mL) and stirred at r.t. for 3 h in a dry nitrogen box. 2,4,6-collidine (6 eq, 0.164 mmol, 21.7 μL) was added followed by addition of aqueous iodine solution (0.1 M in THF/water 3:1.5, excess) in order to oxidize the phosphite triester intermediate. After 5 min, the reaction was diluted with CH_2Cl_2 and washed with 5% sodium bisulfite. The organic phase was washed with 5% $NaHCO_3$, dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure to obtain a foam. The crude trimer was purified by flash silica gel column chromatography using CH_2Cl_2/MeOH/0.5%TEA (gradient 0–2% MeOH) (82% total yield, 47.5 mg). Refer to Tables 1 and 2 for the R_f and ^{31}P NMR values.

Branched trimer S_p-A(p_sG)pC (S_p-7). To an Argon purged glass hypovial with a magnetic stirring bar, was added S_p dimer (**S_p-6**) (0.021 mmol, 30 mg), cytidine-5′ -phosphoramidite (**3e**) (6 eq, 0.123 mmol, 90 mg) and tetrazole (6 eq, 0.123 mmol, 8.7 mg). The reaction was initiated by injecting anhydrous acetonitrile (0.60 mL) and stirred at r.t. for 1.25 h in a dry nitrogen box. 2,4,6-collidine (6 eq, 0.123 mmol, 16.25 μL) was added followed by addition of aqueous iodine solution (0.1 M in THF/water 3:1.5, excess) in order to oxidize the phosphite triester intermediate. After 5 minutes the reaction was diluted with CH_2Cl_2 and washed with 5% sodium bisulfite. The organic phase was washed with 5% $NaHCO_3$, dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure to obtain a foam. The crude trimer was purified by flash silica gel column chromatography using CH_2Cl_2/MeOH/0.5%TEA (gradient 0–2% MeOH) (75% total yield, 32.2 mg). Refer to Tables 1 and 2 for the R_f and ^{31}P NMR values.

Branched trimers A(p_sG)pC R_p-8 and S_p-8. These were obtained by treatment of **R_p-7** and **S_p-7** with triethylamine/acetonitrile as described above for the synthesis of dinucleoside phosphorothioates **R_p-5** and **S_p-5**. Characterization data (R_f and ^{31}P NMR) are provided in Tables 1 and 2.

Branched trimer R_p-A(p_sG)pC (R_p-9). Trimer **R_p-8** (0.011 mmol, 23 mg) was dissolved in 2.5 mL CH_2Cl_2 and 2.5 mL (*p*-TSA/MeOH/CH_2Cl_2), prepared by stirring 0.5 mg *p*-TSA in 5 mL MeOH and 20 mL CH_2Cl_2, and the reaction was stirred at r.t. for 25 min. It was diluted with CH_2Cl_2, washed with 5% $NaHCO_3$, dried over anhydrous Na_2SO_4, and the solvent was dried under reduced pressure. The crude trimer was purified by flash silica gel column chromatography using CH_2Cl_2/MeOH (gradient 0–2% MeOH). (>90% yield) Refer to Table 1 for the R_f values. MS (ESI-MeOH-negative mode, calc. M^+ for product with N^2-*i*Bu guanine: 1775, calc. M^+ for product with N^2-Bz guanine: 1809), found: M-H^-: 1775, 1809 in ratio of 2.75:1, respectively.

Branched trimer S_p-A(p_sG)pC (S_p-9). Trimer **S_p-8** (0.010 mmol, 22 mg) was dissolved in 3 mL CH_2Cl_2 and 0.6 mL (*p*-TSA/MeOH/CH_2Cl_2), prepared by stirring 0.5 mg *p*-TSA in 5 mL MeOH and 20 mL CH_2Cl_2, and the reaction was stirred at r.t. for 10 min. It was diluted with CH_2Cl_2, washed with 5% $NaHCO_3$, dried over anhydrous Na_2SO_4, and the solvent was dried under reduced pressure. The crude trimer was purified by flash silica gel column chromatography using CH_2Cl_2/MeOH (gradient 0–2% MeOH). (93% yield, 17.4 mg) Refer to Table 1 for the R_f values. MS (ESI-MeOH-negative mode, calc. M^+ for product with N^2-*i*Bu guanine: 1775, calc. M^+ for product with N^2-Bz guanine: 1809), found: M^-: 1775, 1809 in ratio of ca. 3:1, respectively.

Branched tetramer S_p-ApA(p_sG)pC (S_p-10). To an Argon purged glass hypovial with a magnetic stirring bar was added S_p-trimer **(Sp-9)** (0.01 mmol, 17.4 mg),5′-O-dimethoxytrityl-2′-O-t-butyldimethylsilyladenosine-3′-O-[β-cyanoethyl-N,N-diisopropyl]phosphoramidite (6 eq, 0.059 mmol, 59 mg) and tetrazole (18 eq, 0.176 mmol, 11 mg). The reaction was commenced by injecting anhydrous acetonitrile (0.5 mL) and stirred at r.t. for 2.5 h in a dry nitrogen box. 2,4,6-Collidine (6 eq, 0.059 mmol, 7.8 μL) was then added followed by addition of aqueous iodine solution (0.1 M in THF/H_2O 3:1.5, excess) to oxidize the phosphite triester intermediate. After 5 min, the reaction was diluted with CH_2Cl_2 and washed with 5% sodium bisulfite. The organic phase was washed with 5% $NaHCO_3$, dried over anhydrous Na_2SO_4, and the solvent removed under reduced pressure to attain a foam. The crude tetramer was partially purified by flash silica gel column chromatography using CH_2Cl_2/MeOH/0.5%TEA (gradient 0–2% MeOH) (67% yield, 17.9 mg). Refer to Table 1 for the R_f values. MS (MALDI-TOF, calc. M^- for product without the cyanoethyl protecting group: 2626), found: M-2H^-: 2633 = 2626 + 7 (Li cation).

Deprotection of Branched Oligonucleotides

The procedure can be illustrated by the deprotection of the S_p-A(p_sG)pC trimer **S_p-7**.

Detritylation. S_p-trimer **S_p-7** (5 mg) was dissolved CH_2Cl_2 (0.5 mL) followed by the addition of trifluoroacetic acid (12 μL) at r.t. The solution turned orange immediately (ditrityl cation), indicating the desired detritylation reaction. After 7 min, CH_2Cl_2 was added and the resulting mixture was washed with 5% $NaHCO_3$. The organic phase was collected and dried over anhydrous Na_2SO_4 to afford, after evaporation under reduced pressure, a dark yellow crude residue. In the case of the S_p-tetramer **S_p-10**, detritylation was conducted by treatment with p-toluenesulfonic acid (p-TSA) as follows: **S_p-10** (6.5 mg) was dissolved in CH_2Cl_2 (0.3 mL) and *p*-toluenesulfonic acid-MeOH/CH_2Cl_2(0.3 mL) was added at r.t.; the latter solution prepared by stirring 0.52 g *p*-TSA, 5.2 mL MeOH and 20.8 mL CH_2Cl_2. Work-up was carried out as described above for the detritylation of **S_p-7**.

Simultaneous removal of cyanoethyl, benzoyl, and isobutyryl groups. The material obtained after step (a) above was dissolved in 1.5 mL conc. aqueous ammonia (NH_4OH) and 0.5 mL EtOH, and the reaction was stirred at r.t. for 48 h. After cooling the sample in an acetone/dry ice bath, ammonia was removed under low vacuum, followed by removal of ethanol and H_2O using a high vacuum Speed-Vac® concentrator.

Simultaneous removal of tert-butyldimethylsilyl and p-nitrophenylethyl groups. The residue obtained above was treated with 1.0 M TBAF/THF (560 μL) for 4 h at r.t. Water was added and the resulting mixture passed through a column filled with Dowex Na^+ ion exchange resin. The eluent (10 mL) was quantitated on a UV spectrometer to yield 42 A_{260} units (42 O.D.) of crude S_p-trimer [A(p_sG)pC].

Oligonucleotide Analysis, Purification, and Desalting

R_p- and S_p-Ap$_s$G phosphorothioates (and enzymatic digests) were analyzed and resolved by reverse-phase HPLC. Fully deprotected branched trimers were purified via anion exchange HPLC, whereas tetramer S_p-ApA(p_sG)pC was purified via polyacrylamide gel electrophoresis (PAGE) as described below.

Polyacrylamide Gel Electrophoresis (PAGE). The tetranucleotide S_p-ApA(p_sG)pC (32 O.D. units) was analyzed and purified by denaturing polyacrylamide gel electrophoresis (24%, 7 M urea).[20] The separate bands were visualized under UV lamp (254 nm) and the desired (slowest moving) band excised with a scalpel blade, and crushed in a sterile 10-mL polypropylene tube. Autoclaved H_2O (5 mL) was added to the tube and it was shaken at r.t. for 24 h in order to extract the oligomer from the gel matrix. The tube was centrifuged and the oligomer-containing supernatant evaporated under reduced pressure on a Speed-Vac® concentrator.

Desalting. The samples purified by anion exchange HPLC and PAGE were desalted using Sep-Pak reverse-phase C_{18} cartridges (Waters). The cartridges were flushed with 10 mL MeOH then equilibrated with 10 mL of doubly distilled and autoclaved H_2O. The oligomers were dissolved in 1 mL of 50 mM triethylammonium acetate (TEAA) and were slowly loaded into the cartridges to collect the first fraction in a 1-mL microtube. The cartridge was flushed further with 50 mM TEAA (3 mL) in order to elute the salts. The oligomers were eluted from the column in 10 fractions of 1 mL each using a solution of 50 mM TEAA:MeOH (7:3). The individual fractions were analyzed by diluting the samples in 1 mL of sterile water, and measuring the UV absorbance profile at 260 nm. Typically, the compound of interest was found in the first two or three fractions.

In Vitro Enzymatic Studies

Enzymatic Digestion via Snake Venom Phosphodiesterase (SVPDE). SVPDE from *Crotalus adamanteus,* a 3′-exonuclease (0.027 units/mg), was dissolved in 2 mL buffer A (0.11 M Tris, 0.11 M NaCl, and 15 mM $MgCl_2$ with pH 8.9) to give a final concentration of 0.00135 U/μL. The residual nucleoside 5′-monophosphates were converted to their constituent nucleosides via in situ treatment with alkaline phosphatase from calf intestinal mucosa (AP, 2000 units/mL), which was kept in the storage buffer provided (10 mM tris-HCl (pH 8.0), 50 mM KCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 50% glycerol). AP (5 μL) was added to 10 μL of its 10X buffer (100 mM Tris-acetate, 100 mM magnesium acetate, and 500 mM potassium acetate) and 85 μL of H_2O, to give a 20-fold dilution of enzyme. 90 μL of this solution was added to each assay. The oligomers (0.2 O.D. each) were dissolved in 30 μL buffer B (50 mM Tris-HCl and 10 mM $MgCl_2$ with pH 8). The SVPDE (0.002 U) and AP (9 U) enzymes were added, and the reactions were incubated at 37°C for the allotted times before they were analyzed using HPLC as described above. Under these conditions, the branched compounds and corresponding hydrolysis products had the following retention times: ApG diester (15.5 min), Rp-A(ps)G, (17.1 min), and Sp-A(ps)G (17.7 min), Rp-A(psG)pC (18.4 min), and Sp-A(psG)pC (18.3 min), rC (5.9 min), rI (12.3 min; resulting from deamination of rA), rG (12.7 min), and rA (15.2 min).

3′ End^{32} P labeling. Labeling the branched oligomers and linear control molecules at their 3′-termini was conducted in a similar method to Uhlenbeck *et al.* using the enzyme T4 RNA ligase and [5′-^{32}P]cytidine 3′, 5′-biphosphate.[21] The oligomers (0.01 OD units) were dissolved in 0.5 μL of the 10 × T4 RNA Ligase buffer (500 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 100 mM DTT, and 10 mM ATP), 0.5 μL deionized DMSO-d_6, 0.5 μL acetylated bovine serum albumin (BSA; 0.1 mg/mL), 0.5 μL (5 U) T4 RNA ligase (10 units/μL), 2 μL [5′-^{32}P]cytidine 3′,5′-biphosphate (3,000 Ci/mmol), to a final volume of 5 μL with sterile water. The reaction mixtures were incubated at 37°C overnight, and the enzyme inactivated by heating at 70°C for 10 min. Their labeling reactions were taken up in gel loading dye (5 μL), purified from any unlabeled material on a 24% denaturing polyacrylamide gel, and visualized by autoradiography (Kodak X-Omat AR film). The labeled oligonucleotides were excised from the gel and extracted by crushing and soaking the gel section in H_2O at 37°C overnight, and desalted via size-exclusion chromatography on Sephadex G-5 columns. The labeled oligonucleotides were stored as dry pellets at –20°C until needed.

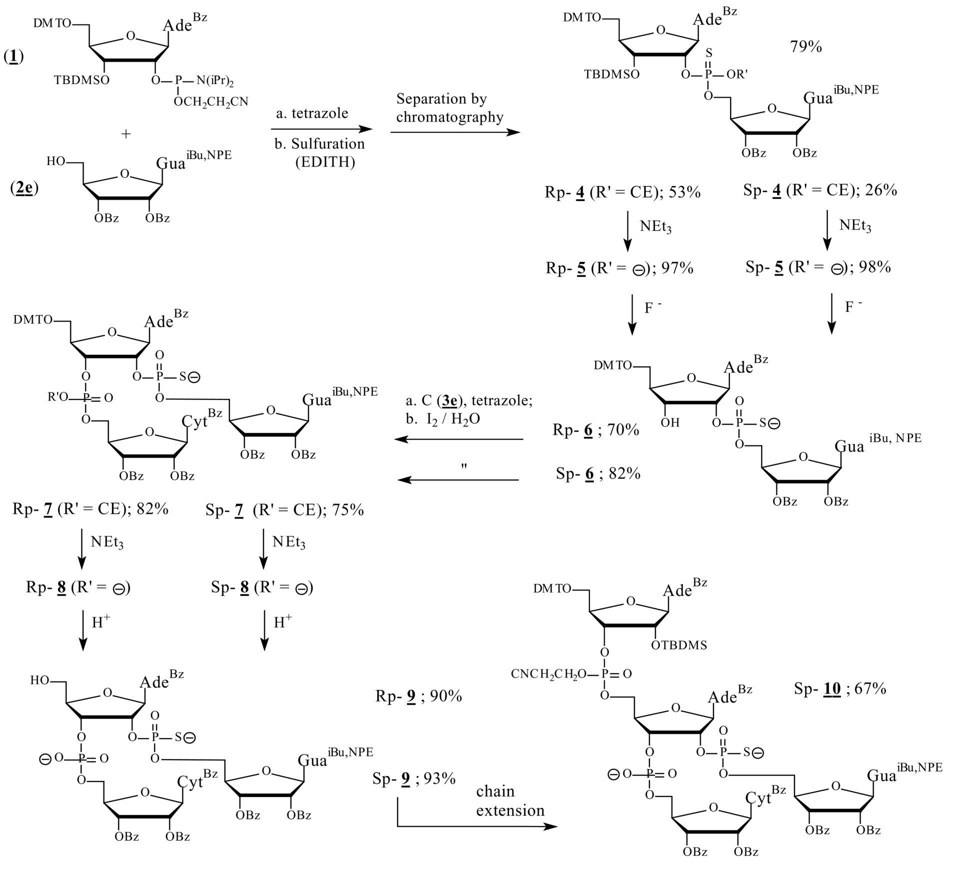
Debranching of synthetic oligomers (purified yDBR). Branched compounds labeled in the above manner were hydrolyzed at the 2′, 5′-linkage using purified His-tagged yeast lariat debranching enzyme (yDBR), a generous gift of Dr. J.D. Boeke (Johns Hopkins University). The3′ end labeled oligomers were dissolved in 2.5 μL yDBR debranching buffer (final concentration of 20 mM HEPES, pH 7.6, 125 mM KCl, 0.5 mM $MgCl_2$, 1 mM DTT, 10% glycerol), 0.5–1 μL yDBR (50–100 ng), and H_2O to obtain a final volume of 7.5 μL per reaction. The debranching reactions were incubated at 37°C, inactivated by heating at 70°C for 10 min, and lyophilized before loading dye was added and the mixtures were analyzed using 24% denaturing PAGE and visualized by autoradiography.

RESULTS AND DISCUSSION

The general scheme for the synthesis of the branched 2′,5′-phosphorothioate oligonucleotides commences with the coupling and sulfurization of the adenosine 2′-amidite (**1**) with the properly protected guanosine monomer (**2e**) to yield the 2′,5′-phosphorothioate dimer A(p_sG) (**4**). Separation of the two isomers at this stage was next accomplished by chromatographic resolution according to established protocols,[22] followed by selective removal of the TBDMS protection from the adenosine 3′-hydroxyl function. The cytidine 5′-amidite (**3**) was subsequently coupled to either dimer (R_p and S_p isomers separately) at the 3′ terminus, followed by oxidation to give a 3′,5′-phosphodiester linkage, yielding the V-shaped trimers A(p_sG)pC (**7**). The 5′-DMT was last removed from the adenosyl branching residue, and the deprotected trimer coupled with an adenosine 3′-amidite to extend the chain in the 5′-direction and yield the Y-tetramer (**10**) (Scheme 1).

Synthesis of Branched 2′,5′-Phosphorothioate RNA

Phosphorothioate-linked dimer (**4**) was synthesized by sulfurizing the intermediary phosphite triester using 3-ethoxy-1,2,4-dithiazoline-5-one (EDITH).[23–25] The Beaucage reagent (3*H*-1,2-benzodithiole-3-one 1,1-dioxide),[26,27] also afforded the dimer, although some oxidized



SCHEME 1 Chemical synthesis of branched RNAs.

phosphotriester dimer formed as well (data not shown). A non-stereoselective method was employed for the synthesis of the phosphorothioate dimer since both isomers were required for probing yDBR stereospecificity, although R_p isomer(**4**) was produced to a greater extent than S_p isomer (**4**) (*ca.* 2:1 respectively, reaction repeated three times). Stereochemistry was enzymatically assigned using snake venom phosphodiesterase (SVPDE; see below) and ^{31}P-NMR, in which a relatively larger chemical shift of the R_p versus the S_p isomer was observed (Figure 3). Additional evidence for the presence of both chemical moieties in each of the two chiral samples was provided via mass spectrometric analysis.

Desilylation of the dimers was first attempted by treating dimer (**4**) with tetra-*n*-butylammonium fluoride (TBAF) as a solution in THF. However, this led to the removal of the O^6-*p*-nitrophenylethyl protecting group due to the basic nature of the solution. Furthermore, the acidity of triethylamine trihydrofluoride (TREAT-HF) promoted cleavage of the dimethoxytrityl-protecting group. Therefore, an alternative method with neutral conditions

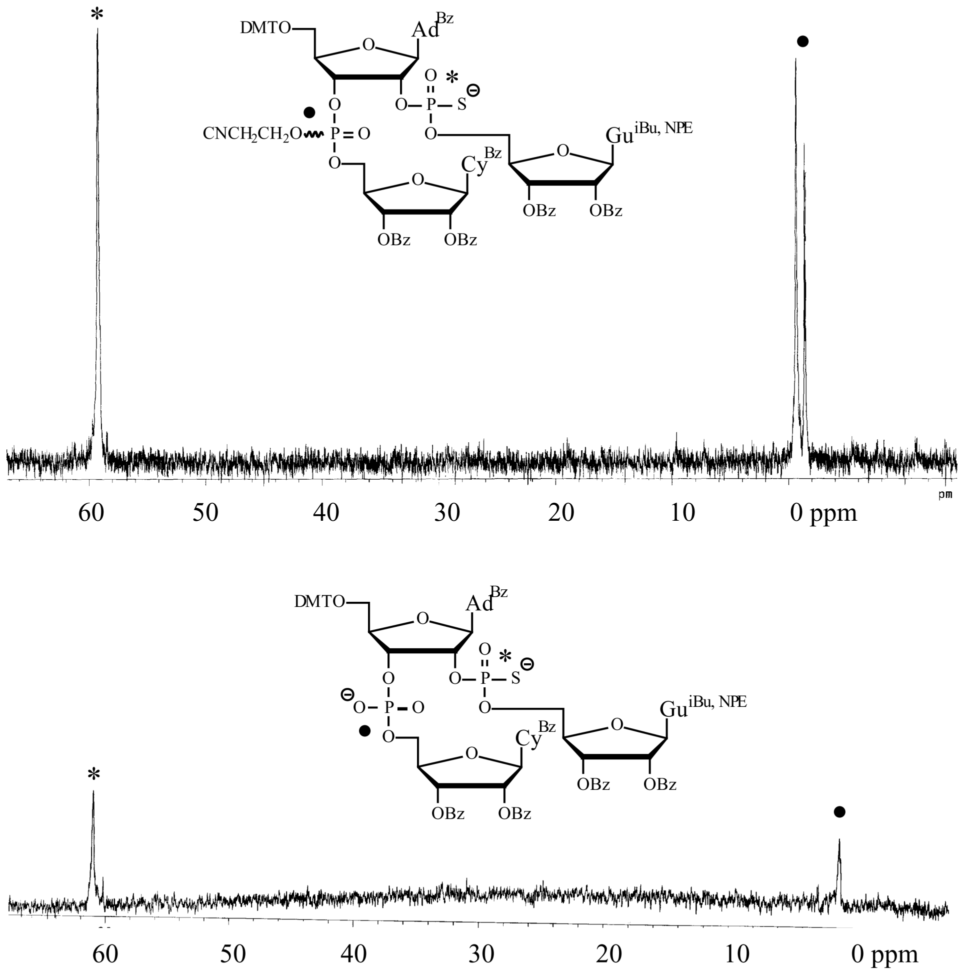


FIGURE 3 ^{31}P-NMR spectra of R$_p$-A(p$_s$G)pC (**7** & **8**) in CH_2Cl_2-d_2, (A) before and (B) after the removal of the cyanoethyl group at the 3′,5′-internucleotide linkage. The phosphorothiolate and phosphate linkages resonate at ca. 60 and 1 ppm, respectively.

was developed in order to remove the silyl group selectively. The method of choice involved treatment of the dimer with a "preneutralized" solution of 1.0 M TBAF in THF using AcOH. This method was very clean and quantitative, and as confirmed by mass spectroscopy (ESI) led to the selective removal of the TBDMS group.

After the removal of the silyl group the dimer was coupled to cytidine 5′-phosphate monomer to yield the V-shaped trimer (**7**). In addition to NMR, MS, and HPLC, UV profiles provided a good additional characterization tool to differentiate between the A(p$_s$G) dimer and the A(p$_s$G)pC trimer, where the trimer absorbs at an additional wavelength, *ca.* 312 nm, due to the presence of cytidine.

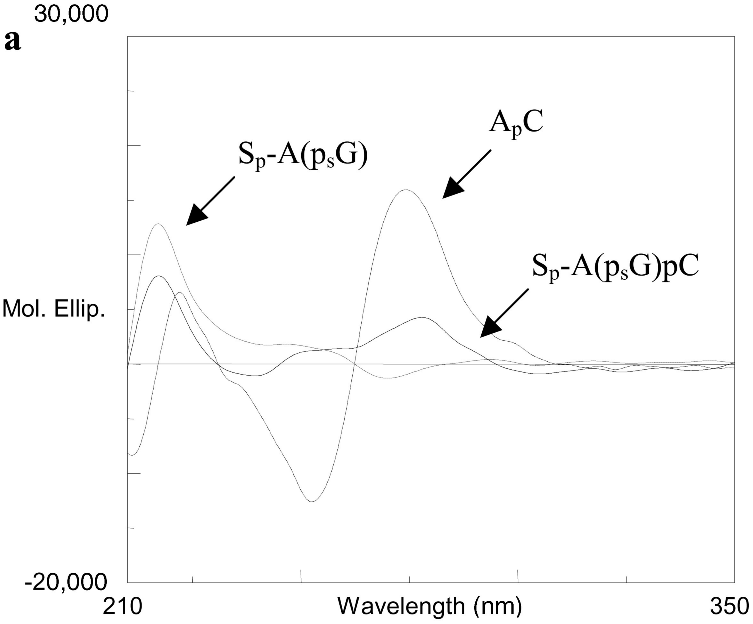
During the coupling of the trimer to the adenosine phosphoramidite, and due to the very similar R_f values of the protected trimer and tetramer, it was not possible to determine by TLC whether all of the V-trimer had been consumed. However, HPLC analyses following deprotection of the products confirmed the nearly complete consumption of S_p-V-trimer. The synthesized tetramer was verified by mass spectroscopy (MALDI-TOF), HPLC, and CD (Figure 4).

The CD spectra of the phosphorothioate oligonucleotides (V-trimers, and S_p-Y-shaped tetramer), and some of the corresponding $2', 5'$ and $3', 5'$ dinucleoside monophosphates and trinucleoside diphosphates were obtained and analyzed to gain further information on possible base interactions.[28–30] The CD spectra of the two A(p_sG) isomers have similar peak positions to the corresponding A(pG) phosphodiester dinucleotide reported by Damha,[31] but are less intense in magnitude. The CD spectra of R_p and S_p phosphorothioate V-trimers show a non-conservative cotton effect (Figure 4a, b). These types of non-conservative spectra are usually exhibited by base-stacked dimers containing guanosine bases due to the presence of at least two overlapping $\pi \rightarrow \pi^*$ bonds from the guanosine monomers in the spectral region ranging from 210 to 300 nm.[32,33] As a result, interpreting CD spectra of oligomers containing guanosine can prove quite difficult. However, this observation indicates that the guanine of the 2′-nucleotide residue in the phosphorothioate V-trimers are involved in base stacking interactions. The low intensity band observed in the $2', 5'$-A(p_sG) signature is retained in the branched phosphorothioate trimers, which also possess similar CD patterns to the phosphodiester V-trimers A(pG)pC reported by Damha,[33] and in the phosphorothioate S_p-Y-tetramer's case (Figure 4c). This similarity in intensity between the branched phosphorothioate oligomers and $2', 5'$-A(p_sG), is possibly a second indication to the involvement of the 2′-guanosine residues in stacking interactions. The CD profiles of both phosphorothioate V-trimers and phosphorothioate S_p-Y-tetramer appear to be intermediate between the corresponding $2', 5'$- and $3', 5'$-linked dinucleotide monophosphates. This suggests that two relatively equal populations may exist for the phosphorothioate V-trimers, in which the branched adenine base forms a stack with the chromophore of the 2′-residues in one population, and with the 3′-residues in the second. This characteristic is absent in the spectra of normal phosphodiester V-shaped trimers, in which a predominant population of stacked branchpoint adenines with the adjacent 2′-residue occurs.[30]

Stereochemical Assignment of the Phosphorothioate Isomers Using Snake Venom Phosphodiesterase (SVPDE)/Alkaline Phosphatase (AP)

Stereochemical assignment of the phosphorothioate isomers was accomplished via treatment with snake venom exonuclease (phosphodiesterase

I; SVPDE). This enzyme stereoselectively hydrolyzes only the R_p isomer of either 3′, 5′- or 2′, 5′- phosphorothioates linkages.[21,34] Since little is known about the behavior of SVPDE toward branched phosphorothioate



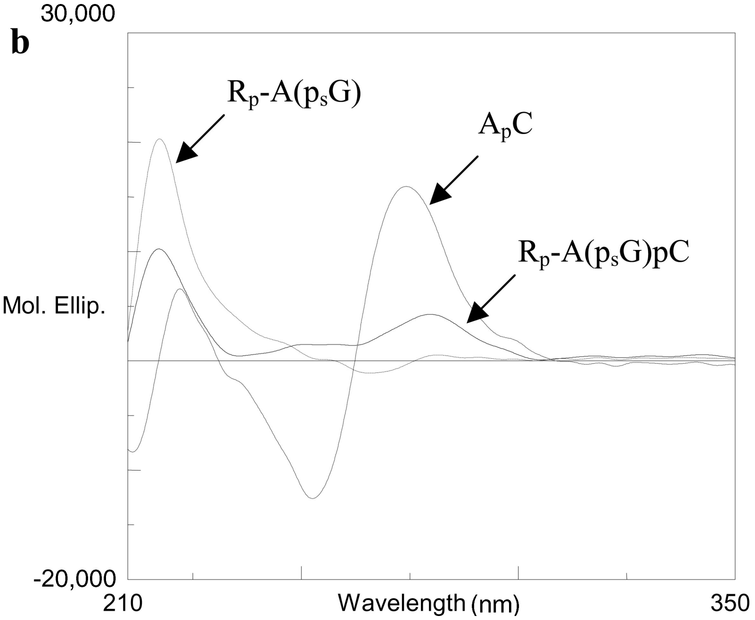


FIGURE 4 (a)–(c) CD spectra of unprotected di, tri and tetranucleotides in 50 mM Na2HPO4, 100 mM HCl, at pH 7.25. (*Continued*)

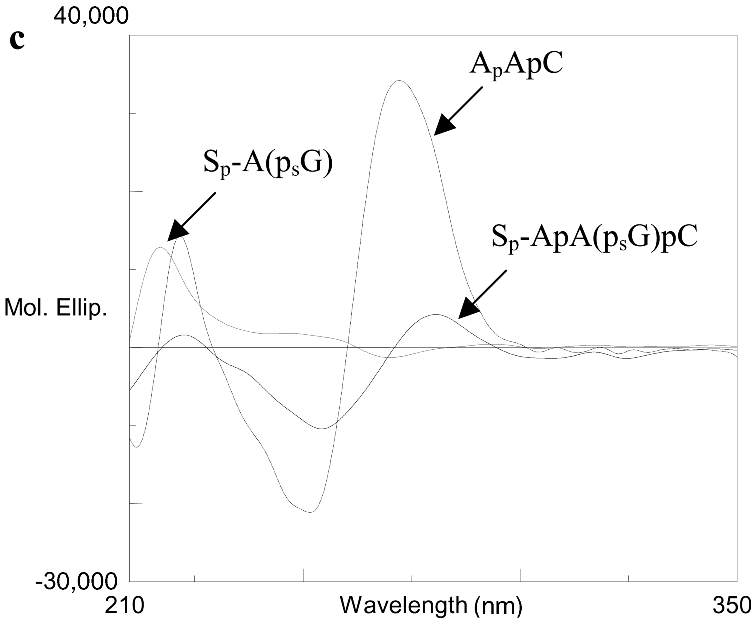


FIGURE 4 (Continued).

oligonucleotides, the enzymatic characterization was first carried out on the two separated isomers of 2′, 5′-Ap(s)G **4**.

The SVPDE/AP assays were carried out using as control an ApG diester, and the two isomers of A(p_s)G. The phosphodiester linkage of ApG was fully hydrolyzed within 4 h. However, the faster eluting phosphorothioate dimer on reverse-phase HPLC showed more than 50% hydrolysis after 4 h, while the slower eluting dimer showed no signs of hydrolysis even after 24 h. Thus, the faster eluting dimer has an R_p configuration and the slower eluting dimer has an S_p configuration (see Materials and Methods).

The SVPDE/AP assays were also conducted using V-trimers [5′-A(p_sG)pC] as substrates in order to confirm the stereochemistry of the two isomers, and to verify the enzyme's ability to hydrolyze branched oligoribonucleotides. The S_p-V-trimer showed full hydrolysis of the 3′, 5′-phosphodiester bond after 2 h, yielding deoxycytidine 5′-monophosphate and S_p-configured Ap$_s$G dimer as products. Conversely, HPLC analysis of the R_p-V-trimer digestion profiles with SVPDE showed a relatively broader product distribution, corresponding to deoxycytidine 5′-monophosphate and intact R_p dimer, as well as the constituent mononucleosides arising from partial hydrolysis of the R_p-linkage. These results demonstrate the enzyme's ability to stereoselectively hydrolyze R_p-branched phosphorothioate oligonucleotides.

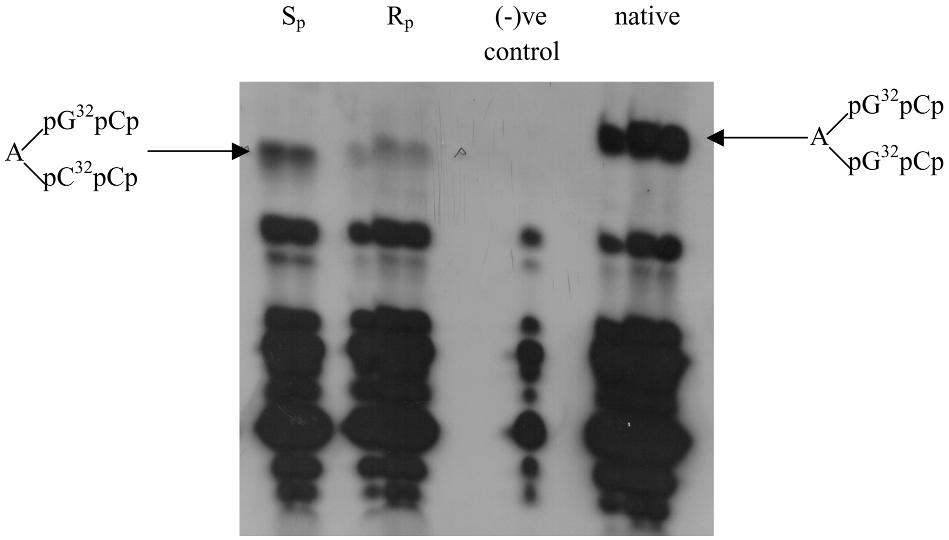


FIGURE 5 ^{32}pCp Labeling of branched trinucleotides. The (−)ve control contains ^{32}pCp and ligase without branched RNA substrate.

3′-End Labeling with ^{32}P

T4 PNK-mediated incorporation of a 5′-phosphorus radiolabel within V-branched compounds using $\gamma-^{32}$P-[ATP] as a phosphate source is exceedingly difficult, owing to the absence of a 5′-nucleotide tract in these substrates.[35,36] These oligonucleotides were therefore 3′ end labeled using T4 RNA ligase and 5′-^{32}P-cytosine-3′, 5′-bisphosphate (*i.e.*,^{32}pCp) (Figure 5). This also proved to be advantageous for monitoring debranching efficacy by DBR, as it allows one to follow the release of both 2′, 5′- and 3′, 5′-linked phosphodiester (or phosphorothioate) hydrolysis fragments generated by the enzyme. However, the small size of the oligonucleotides, and consequently greater steric hindrance of 2′ and 3′ end termini, caused them to be labeled with low efficiency. As shown in Figure 5, the all-phosphodiester V-trimer was more efficiently labeled by T4 RNA ligase relative to the corresponding Rp and Sp thioated isomers.

Debranching Studies Using His-Tagged yDBR

Previous studies on DBR hydrolytic efficacy have only examined as test substrates lariats with an isomerically pure R$_p$-configured 2′, 5′-phosphorothioate linkage, which were not cleaved by the enzyme.[15] To examine the effect of linkage chirality at the branchpoint on this reaction, we therefore evaluated the ability of the newly synthesized Y-shaped compound with S$_p$-configuration to undergo debranching by DBR (Figure 6). We also determined the susceptibility of both V-shaped R$_p$- and S$_p$ compounds

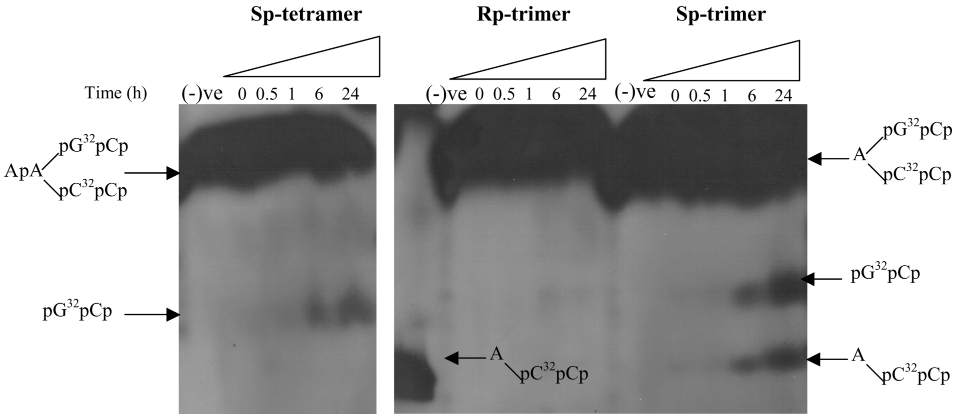


FIGURE 6 Debranching of branched compounds by yDBR. The (−)ve lane contains only the oligomer (no yDBR).

to debranching, in order to demonstrate the necessity of a residue at the $5'$-position of the adenosine branchpoint for substrate recognition by the enzyme.

Debranching assays were initially conducted using HeLa cell extracts, however, significant degradation appeared to accompany the observed debranching of all oligonucleotide test substrates as verified using a linear control. We subsequently switched to a purified source of enzyme to eliminate the occurrence of competing nuclease degradation reactions, and thereby simplify both the characterization and quantitation of debranching efficiency in these substrates. His-tagged purified yDBR was thus used to carry out debranching assays. When this enzyme was used to debranch a Y-shaped 18-mer (*i.e.*, $5'$-UACUAA(pGUAUGU)pGUAUGU), over half of the starting material was debranched at the earliest time point (30 min; data not shown). Conversely, linear oligomers were not hydrolyzed by the pure enzyme.

In principle, scission of the branchpoint $2'$, $5'$-bond of the $3'$ end labeled V-shaped compounds should yield two products, consisting of the linearized $3'$-tail trimer (*i.e.*, ApC^{32}pCp) and the $2'$-tail fragment (*e.g.*, p$_s$G^{32}pCp). By comparison, scission of the $2'$, $5'$-bond of the phosphorothioate Y-shaped compound should yield similar products, which differ only in the nature of the linear $3'$-tail (*e.g.*, ApApC^{32}pCp and p$_s$G^{32}pCp). Indeed, debranching of the labeled S$_P$-V-shaped oligomer yielded two bands that migrated faster than the starting material, one of which corresponded to the linear control trimer ApC^{32}pCp (Figure 6). The new species were evident after only 6 h of incubation with the enzyme. The labeled S$_P$-Y-shaped oligomer was similarly hydrolyzed by yDBR, as evidenced by the presence of a debranching product co-migrating with one of the products of the V-shaped substrate, suggesting this to be the linear $2'$-tail dimer (p$_s$G^{32}pCp; Figure 6). However, the presence of a second product band could not clearly be visualized, owing to the low amount of radioactivity used in the assay. The R$_P$-V-shaped compound, on the other hand, showed no signs of debranching even after 24 h (Figure 6) as expected from previous work.[15]

Debranching studies of the control phosphodiester A(pG^{32}pCp)G^{32}pCp revealed partial debranching at 6 h, as indicated by the appearance of a new faster moving band migrating close to the linear ApC^{32}pCp control (data not shown). Although the extent of debranching increased at 24 h, there were significant amounts of branched molecule still intact, indicating that it was not recognized as efficiently by the enzyme as with larger branched molecules (*e.g.*, Y-shaped 18mer).

Stereospecificity of yDBR-Rp Versus Sp

yDBR is a member of the metallophosphoesterase superfamily that includes bacteriophage phosphatase (a phosphomonoesterase) and *Pyrococcus furiosus* Mre11 (a phosphodiesterase).[37–42] Structural studies of these enzymes showed that conserved His, Asp, and Asn side chains are involved in binding of the two manganese ions, whereas conserved His and Asn side chains contact the phosphate in the active site.[41,42] Recent structure-function analysis of yDBR by Schwer and co workers revealed that the enzyme requires a similar group of conserved amino acids for activity.[37] Specifically, they found that six of the seven metal-binding amino acids in Mre11 (His10, Asp49, Asn84, His173, His206, His208) are conserved in yDBR (as His13, Asp40, Asn85, His179, His231, His233) and essential for debranching *in vivo*. Their work strongly suggested that yDBR likely contains a binuclear manganese cluster similar to that seen in Mre11.[37]

Figure 7 shows *Pfu*Mre11 structure with 5′-dAMP bound in the active site (adapted from reference 37). As discussed by Schwer and coworkers,[37] the bridging water between the two manganese ions is a good candidate for the attacking nucleophile, because it is located 3.1Å from the phosphorus atom and is almost perfectly apical to the phosphate 2′-oxygen atom of the leaving group, which is a nucleoside 3′-O in the case of Mre11. The bridging water ligand between the manganese ions is activated by the metals for attack on the phosphate, which, in Mre11, is coordinated by Asn84 and His85 side chains.[41] Since the corresponding Asn85 and His86 side chains of yDBR are critical for debranching, Schwer and co-workers proposed that Asn85 and His86 of yDBR contact the scissile 2′-5′ phosphodiester of the branched RNA in a manner analogous to that seen in the Mre11-dAMP complex.[37] According to this model, it can be appreciated that replacement of the prochiral R_p-oxygen with the larger sulfur atom would lead to steric interactions with both Asn85 and a manganese ion. Therefore binding of R_p-bRNA, if it occurs, will likely result in non-optimal yDBR-bRNA interactions for catalysis. Such steric effects are less evident for the S_p-bRNA isomer, where only binding to the second metal ion would be perturbed (Figure 7). This may explain the inability of yDBR to debranch 2′, 5′-linked R_p-phosphorothioate linkages.

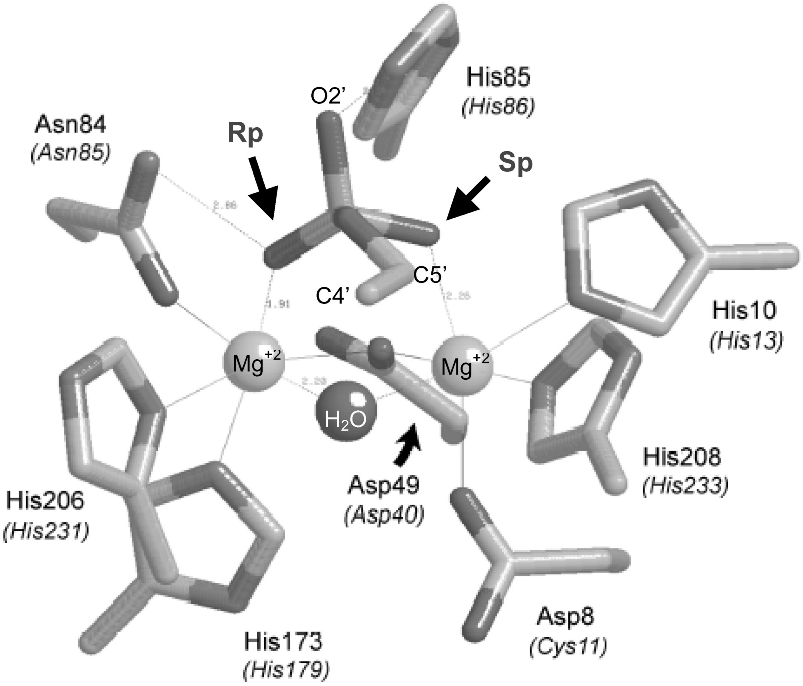


FIGURE 7 The figure, adapted from the work of Schwer and coworkers,[37] depicts the active site of the manganese- and 5′-dAMP-bound *P. furiosus* phosphodiesterase Mre11 from the crystal structure (PDB 1II7).[42] The amino acid side chains coordinating the binuclear metal cluster and the nucleotide 5′ phosphate are shown. The corresponding amino acids of yDBR enzyme are indicated in parentheses. The manganese ions and water nucleophile are also shown. For simplicity, only the phosphate and the ribose C4′ and C5′ atoms of the nucleotide are shown. The departing O2′ atom (O3′ in the case of dAMP) is shown apical to the water nucleophile.

It is intriguing to note that whereas yDBR carries out stereoselective hydrolysis of the S_p isomer (branched tri and tetranucleotides), SVPDE prefers the R_p configuration of the substrate tested (2′,5′-linked dinucleotides). The apparent biochemical differences between the two enzymes appear to extend to a mechanistic level, e.g., hydrolysis by SVPDE proceeds via a covalent nucleotide enzyme intermediate (2-step process),[43] whereas that of yDBR is likely one step (e.g., in-line attack at phosphorus by a water molecule; Figure 7).

CONCLUSIONS

Our results indicate that the size of the substrate plays a significant role in efficient debranching, since the small substrates were not cleaved as efficiently as the large branched 18-mer. Furthermore, the 2′, 5′-internucleotide linkage of a branched R_p oligonucleotide was not cleaved by the yDBR enzyme, suggesting that the yDBR enzyme is sensitive to changes carried out on the pro-R_p oxygen. The S_p phosphorothioate isomers, along with all phosphodiester branched compounds [V-A(pGpCp)pGpCp and Y-shaped 18-mer], were all substrates of the yDBR enzyme, albeit with diverse efficacies.

Employing capillary electrophoresis as an alternative method to study the debranching of these small branched molecules by yDBR[14] could prove a good method to verify and corroborate the results obtained here in addition to avoiding the problems that arise from radiolabeling these small branched and sterically hindered molecules.

Future studies involving subjecting the S_p-branched RNA substrate to the yDBR and studying the stereochemistry of the debranching products could provide further insight into the mechanism of specific $2'$, $5'$-phosphodiester linkage hydrolysis by the yDBR enzyme. Moreover, competitive inhibition studies can be carried out in order to ascertain if a R_p phosphorothioate branched RNA (the substrate which is not hydrolyzed) is nonetheless recognized by the enzyme and binds to its active site. Given that this isomer (or other analogues under study in our laboratory) may potentially be an inhibitor of debranching, it may find use in co-crystallization of yDBR:bRNA complexes, thus possibly allowing for the attainment of a much desired X-ray crystal structure.

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