Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry

Thermal Stabilisation of RNA•RNA duplexes and G-quadruplexes by phosphorothiolate linkages

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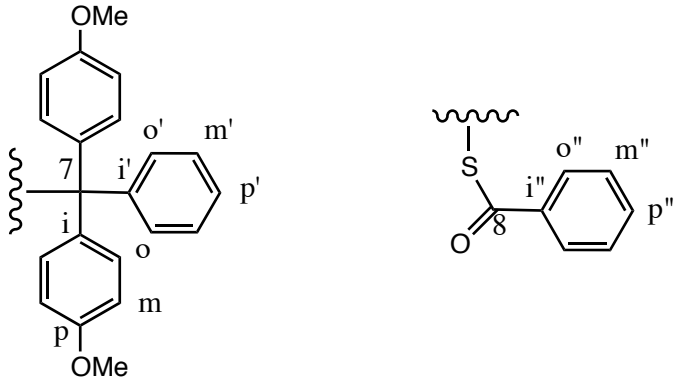
Electronic Supplementary Information

Synthesis of 5′-*O*-dimethoxytrityl-2′-deoxy-3′-thiouridine phosphoramidite (5) for incorporation into RNA

Numbering system for NMR spectra



5′-*O*-(4,4′-Dimethoxytrityl)-2,3′-anhydro-2′-deoxyuridine (2)

Diisopropylazodicarboxylate (2.56 mL, 12.99 mmol) was dissolved in ethyl acetate and added dropwise to a stirred solution of 5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyuridine[1] (4.59 g, 8.66 mmol) and PPh_3 (3.41 g, 12.99 mmol) in EtOAc (40 mL) over a 10 minute period. The reaction mixture was subsequently left to stir overnight, after which the solvents were removed under reduced pressure. Purification by flash silica chromatography, eluting with EtOAc followed by 1:4 MeOH:EtOAc, afforded the title compound as a white amorphous solid (3.85 g, 7.51 mmol, 86.7 %).

R_F (EtOAc:MeOH [9:1]): 0.15. δ_H = 2.35(1H, m, H2′); 2.59(1H, d [$^3J_{H2''\text{-}H2'}$ = 12.9], H2″); 3.31(1H, dd [$^3J_{H5'\text{-}H5''}$ = 10.2, $^3J_{H5'\text{-}H4'}$ = 6.1], H5′); 3.36(1H, dd [$^3J_{H5''\text{-}H5'}$ = 10.3, $^3J_{H5''\text{-}H4'}$ = 7.2], H5″); 3.77(6H, s, OMe); 4.24(1H, m, H4′); 5.13(1H, s [broad], H3′); 5.49(1H, d [$^3J_{H1'\text{-}H2'}$ = 3.8], H1′); 5.87(1H, d [$^3J_{H5\text{-}H6}$ = 7.2], H5); 6.80(4H, m, *m*-H); 7.05(1H, d [$J_{H6\text{-}H5}$ = 7.4], H6); 7.18(1H, m, *p*′-H); 7.27(6H, m, *o*-H & *m*′-H); 7.40(2H, m, *o*′-H). δ_C = 34.0(C2′); 55.6(OMe); 62.4(C5′); 77.2(C3′); 84.9(C4′); 87.1(C1′); 88.1(C7); 109.8(C5); 113.6(*m*-C); 127.3(*p*′-C); 128.29, 128.33 & 130.4 (*o*-C, *o*′-C & *m*′-C); 135.8(*i*-C); 139.7(C6); 144.8(*i*′-C); 154.1(C2); 159.0 (*p*-C); 171.5(C4). ESMS: m/z = 535 $(M+Na)^+$, 100%; 513 $(M+H)^+$, 4%; 1048 $(2M+Na)^+$, 45%. HRMS; $C_{30}H_{28}N_2NaO_6$ requires 535.1849; $[M+Na]^+$ = 535.1845 (+0.7 ppm).

5′-*O*-(4,4′-Dimethoxytrityl)-3′-thio-3′-*S*-benzoyl-2′-deoxyuridine (3)

Method 1: The anhydronucleoside **2** (3.78 g, 7.29 mmol) was dissolved in anhydrous dimethylacetamide, placed under N_2 and heated to 110 ^oC. Sodium thiobenzoate (11.68 g, 73.0 mmol) was added in four portions over 2 hr. 30 min after the addition of the final portion, EtOAc was added and the reaction mixture washed with saturated $NaHCO_3$ solution (x 3) and water. The organic layers were dried over $MgSO_4$ filtered and concentrated under reduced pressure. The residue was purified by flash silica chromatography, eluting with 2:1 EtOAc:n-hexane, afforded the title compound as a pale yellow amorphous solid. (Yields between 35% (1.66 g, 2.55 mmol) and 74 % (3.51 g, 5.39 mmol,).

*Method 2:*The anhydronucleoside **2** (166 mg, 0.33 mmol) was dissolved in anhydrous pyridine (2 mL), placed under N_2 and heated to 110 ^oC. Thiobenzoic acid (57 µL, 0.49 mmol) was added and heating continued for 4 hours. The solvent was removed under reduced pressure, the residue redissolved in EtOAc (25 mL) and washed with saturated $NaHCO_3$ solution (3 x 20 mL), water (2 x 20 mL) and brine (2 x 20 mL). The organic layers were dried over $MgSO_4$ filtered and concentrated under reduced pressure to afford the impure product. Purification by flash silica chromatography, eluting with 2:1 EtOAc:n-hexane, afforded the title compound as a pale brown amorphous solid (101 mg, 0.16 mmol, 48.0 %).
R_F (EtOAc:MeOH [9:1]): 0.67. δ_H = 2.55(1H, ddd [$^2J_{H2'-H2''}$ = 14.1, $^3J_{H2'-H3'}$ = 8.7, $^3J_{H2'-H1'}$ = 6.8], H2′); 2.71(1H, ddd [$^2J_{H2''-H2'}$ = 14.0, $^3J_{H2''-H3'}$ = 8.0, $^3J_{H2''-H1'}$ = 3.7], H2″); 3.49(1H, dd [$^2J_{H5'-H5''}$ = 11.0, $^3J_{H5'-H4'}$ = 2.9], H5′); 3.54(1H, dd [$^2J_{H5''-H5'}$ = 11.0, $^3J_{H5''-H4'}$ = 2.4], H5″); 3.73(6H, s, OMe); 4.12(1H, m, H4′); 4.5(1H, m, H3′); 5.38 (1H, d [J_{H5-H6} = 8.1], H5); 6.25(1H, m [$^3J_{H1'-H2'}$ = 6.8, $^3J_{H1'-H2''}$ = 3.7], H1′); 6.80(4H, m, *m*-H); 7.18-7.32 (7H, m, *o*-H, *m*′-H & *p*′-H); 7.42-7.47(4H, m, *m*″-H & *o*′-H); 7.61(1H, m, *p*″-H); 7.9(2H, m, *o*″-H); 8.06(1H, d [J_{H6-H5} = 8.1], H6); 9.01(1H, s [broad], NH). δ_C = 39.5 (C3′); 40.6(C2′); 55.6(OMe); 62.1(C5′); 84.7(C4′); 85.4(C1′); 87.5(C7); 102.6(C5); 113.7(*m*-C); 127.6 (p′-C); 127.8, 128.4, 128.6 & 129.2(o′-C, m′-C, o″-C & m″-C); 130.6 (o-C); 134.3 (*p*″-C); 135.6(*i*-C); 136.7(*i*″-C); 140.6(C6); 144.7(*i*′-C); 150.6(C2); 159.1(*p*-C); 163.6(C4); 190.3(C8). ESMS: m/z = 673 $(M+Na)^+$, 100%. HRMS; $C_{37}H_{34}N_2NaO_7$ requires 673.1969; $[M+Na]^+$ = 673.1984 (-2.3 ppm).

5′-*O*-(4,4′-Dimethoxytrityl)-2′,3′-dideoxy-3′-thiouridine (4)

N_2 was bubbled through a stirred solution of ethanol (25 mL) and aqueous NaOH (10 M, 2.3 mL, 22.6 mmol) for a 30 min period. The thiolate ester **3** (3.403 g, 5.23 mmol) was added and the mixture stirred under N_2 for a further hour. A solution of potassium dihydrogen phosphate (1 M, 93 mL) was then added dropwise over a 15 min period and left for a further hour. The resulting cream precipitate formed was filtered, washed with water (3 x 90 mL) and dried over P_2O_5 for 2 days to yield the title compound as a cream solid (2.64 g, 4.83 mmol, 92 %).
R_F (EtOAc): 0.45; R_F (EtOAc: Hexane [7:3]): 0.23; R_F (EtOAc:CH_2Cl_2:NEt_3 [9:9:2]): 0.21. δ_H = 1.49(1H, d [$^3J_{SH3'-H3'}$ = 7.4], SH3′); 2.35(1H, ddd [$^2J_{H2'-H2''}$ = 13.9, $^3J_{H2'-H3'}$ = 11.3, $^3J_{H2'-H1'}$ = 6.9], H2′); 2.71(1H, ddd [$^2J_{H2''-H2'}$ = 13.9, $^3J_{H2''-H3'}$ = 7.3, $^3J_{H2''-H1'}$ = 1.8], H2″); 3.48-3.65 (2H, m, H3′& H5′); 3.76-3.84(8H, m, OMe, H4′ & H5″); 5.27(1H, d, J_{H5-H6} = 8.3], H5); 6.12(1H, dd [$^2J_{H1'-H2'}$ = 6.9, $^3J_{H1'-H2''}$ = 1.8], H1′); 6.84(4H, m, *m*-H); 7.27(7H, m, *o*-H, *m*′-H & *p*′-H); 7.40(2H, m, *o*′-H); 7.06(1H, d [$^3J_{H6-H5}$ = 8.12], H6); 9.23(1H, s, NH). δ_C = 33.9(C3′); 43.96(C2′); 55.7(OMe); 60.4(C5′); 85.1(C1′); 87.5(C4′); 88.8(C7); 102.3(C5); 113.8(*m*-C); 127.5(*p*′-C); 128.4, 128.5 & 130.5 (*o*-C, *o*′-C & *m*′-C); 135.6(*i*-C); 140.6(C6); 144.7(*i*′-C); 150.6(C2); 159.2 (*p*-C); 163.8(C4). ESMS: m/z = 569 $(M+Na)^+$, 100%. HRMS; $C_{30}H_{30}N_2O_6NaS$ requires 569.1718; $[M+Na]^+$ = 569.1722 (-0.4ppm).

5′-O-(4,4′-dimethoxytrityl)-2′,3′-dideoxy-3′-thiouridine-3′-S-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphorothioamidite (5)

2-Cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphordiamidite (1.03 mL, 3.24 mmol) was added to a solution of the thionucleoside **4** (1.18 g, 2.16 mmol) and tetrazole (used as a 0.5 M

solution in acetonitrile, 3.24 mL, 1.62 mmol) in anhydrous MeCN (20 mL). The reaction mixture was then stirred under N_2 overnight after which it was quenched with H_2O. The mixture was taken up in CH_2Cl_2 and washed with saturated $NaHCO_3$ solution (2 washes). The organic layers were dried over sodium sulphate, filtered and concentrated under reduced pressure to afford the impure product as a off-white amorphous solid. Purification by flash silica chromatography, eluting with 1:1 EtOAc:CH_2Cl_2, resulted in the isolation of the two diastereoisomers of the title compound off-white amorphous solid (0.353 g, 0.47 mmol, 72 %). Samples of the slow running diastereoisomer obtained by this procedure could not be obtained without contamination from the faster-running diastreoisomer, but the enrichment was sufficient to enable the complete assignment of the NMR spectra.

R_F (EtOAc: Hexane [7:3]): 0.61/0.51; R_F (EtOAc:CH_2Cl_2:NEt_3 [9:9:2]): 0.61/0.51. ESMS: m/z = 769 $(M+Na)^+$, 100%; 785 $(M+k)^+$, 8%. HRMS; $C_{39}H_{47}N_4NaO_7PS$ requires 769.2798; $[M+Na]^+$ = 769.2801 (-0.4ppm). CHN: Calculated for $C_{39}H_{47}N_4O_7PS$: C, 62.72%; H, 6.34%; N, 7.50%. Found: C, 62.73%; H, 6.38%; N, 7.44%.

Faster-running diastereoisomer; δ_P = 166.4. δ_H = 1.2(12H, m, H12); 2.42-2.55(4H, m, H2′, H2″ & H9); 3.56-3.68(7H, m, H3′, H5′, H5″, H8 & H11); 3.8(6H, s, OMe); 4.01(1H, m, H4′); 5.20(1H, d [$^3J_{H5-H6}$ = 8.2], H5); 6.14(1H, m, H1′); 6.84(4H, m, *m*-H); 7.29-7.42 (9H, m, *o*-H, *o*′-H, *m*′-H & *p*′-H); 8.11(1H, d [$^3J_{H6-H5}$ = 8.3], H6); 9.01(1H, s, NH). δ_C = 20.4(C9); 24.5(C12); 38.1(C3′); 43.2(C2′); 47.0(C11); 55.6(OMe); 60.8(C5′ & C8); 85.3(C1′); 87.1(C4′); 87.4(C7); 102.1(C5); 113.6(*m*-C); 117.5(C10); 127.6(*p*′-C); 128.3, 128.8 & 130.8 (*o*-C, *o*′-C & *m*′-C); 135.6(*i*-C); 140.7(C6); 144.7(*i*′-C); 150.6(C2); 159.1 (*p*-C); 163.7(C4).

Slower-running diastereoisomer; δ_P = 161.35. δ_H = 1.15(12H, m, C12); 2.6-2.8(4H, m, H2′, H2″ & H9); 3.46-3.68(7H, m, H3′, H5′, H5″, H8 & H11); 3.79(6H, s, OMe); 4.03(1H, m, H4′); 5.23(1H, d [$^3J_{H5-H6}$ = 8.1], H5); 6.14(1H, m, H1′); 6.84(4H, m, *m*-H); 7.27-7.41 (9H, m, *o*-H, *o*′-H, *m*′-H & *p*′-H); 8.02(1H, d [$^3J_{H6-H5}$ = 8.1], H6); 8.95(1H, s, NH). δ_C = 20.7(C9); 24.6(C12); 38.7(C3′); 43.4(C2′); 47.0(C11); 55.6(OMe); 61.5(C5′ & C8); 85.5(C1′); 86.8(C4′); 87.3(C7); 102.2(C5); 113.6(*m*-C); 117.6(C10); 127.5(*p*′-C); 128.3, 128.7 & 130.6 (*o*-C, *o*′-C & *m*′-C); 135.6(*i*-C); 140.8(C6); 144.8(*i*′-C); 150.6(C2); 159.1 (*p*-C); 163.7(C4).

HPLC chromatography

HPLC was performed on an automated Gilson HPLC system equipped with an autoinjector, a photodiode array detector and a dual hydraulic pump. Chromatographic data was handled using UniPoint softwareVersion 3.0.

Ion-Exchange HPLC (IE-HPLC) was performed on a 250 mm x 4 mm, DNA Pac® PA-100 ion exchange column supplied by Dionex. Reverse-Phase HPLC (RP-HPLC) was performed on a 250 mm x 4.6 mm, Gemini 5µ C18, 110 Å reverse-phase column supplied from Phenomonex®. Elution gradients are as described in the text and figure legends.

Solid-phase oligonucleotide synthesis

Solid-Phase oligonucleotide synthesis was carried out on a BioAutomation MerMade 4® synthesiser equipped with MerMade 12© v2.3.2 software. All syntheses were undertaken

using 1.0 μmol, 500 Å controlled-pore glass (CPG) columns purchased from Link Technologies. For the synthesis of oligodeoxynucleotides, commercially supplied standard 5′-*O*-dimethoxytrityl-2′-deoxynucleoside phosphoramidite derivatives were diluted with anhydrous acetonitrile and used at the recommended concentration of 0.1 M. For the synthesis of RNA oligomers, commercially supplied 5′-*O*-dimethoxytrityl-2′-*O*-TBDMS protected ribonucleoside phosphoramidite derivatives were also used at the recommended concentration of 0.1 M. In both cases ETT (0.25 M) as the activator using the instrument-recommended coupling times for both the RNA and DNA monomers. For the introduction of phosphorothiolate linkages into either DNA or RNA, the phosphorothioamidite monomers (**5**, **7** or **12**), were diluted with anhydrous acetonitrile (0.15 M) and coupled using ETT (1 M) as the activator. A double coupling (2 X 6 min) was used with a wash in between the coupling steps. The iodine solution used for the oxidation was 0.02 M.

Deprotection and purification of oligodeoxynucleotides (ODNs): These were synthesized with DMT group retained. Once synthesis was complete the reaction column was removed and dried by passage of nitrogen. The crude DMT-protected ODNs were obtained by treating the solid support with concentrated aqueous ammonia (1 mL) for 48-64 hrs at room temperature in a sealed Wheaton vial. DMT-protected ODNs were concentrated in a vacuum centrifuge, redissolved in triethylammonium bicarbonate (TEAB, 0.1 M, pH 7.6) and purified by reverse-phase HPLC using a gradient of 0-40% acetonitrile in TEAB (0.1 M) over 25 min. The DMT group was removed as previously described.[2] ODNs that were less than 98% pure, as determined by HPLC, were repurified by reverse-phase HPLC using a gradient of 0-20% acetonitrile in TEAB (0.1 M) over 35 min.

Deprotection and purification of oligoribonucleotides (ORNs): These were synthesized with DMT group removed. Once synthesis was complete the reaction column was removed and dried by passage of nitrogen. Aqueous ammonia solution (0.5 mL) was introduced onto the CPG column and left for 45 min. The ammonia was then transferred into a Wheaton V-Vial® and the procedure repeated with the second aliquot of ammonia combined with the first. *Milli-Q* water (0.5 mL) was subsequently washed through the column, added to the same vial and the mixture left to stand at rt for 64 hours. Following removal of the aqueous ammonia using the Speed Vac® the ORN residue was dissolved in anhydrous DMSO (0.3 mL) and heated to 50 °C to aid dissolution if necessary. An aliquot of $NEt_3.3HF$ (0.3 mL) was added and solution wrapped in aluminium foil and incubated at 30 °C for 24 hours. The reaction mixture was cooled to -25 °C and a solution of chilled 1 M NH_4HCO_3 (~1.5 mL) was added slowly until effervescence ceased. The total solution (~2.1 mL) was then desalted using a Sep-Pak column, previously conditioned by washing with acetonitrile (10 mL), 1:1:1 *Milli-Q* water/methanol/acetonitrile (10 mL) and *Milli-Q* water (20 mL). The ORN solution was loaded onto the SepPak column and all salt removed with *Milli-Q* water (10 mL). The ORN was eluted with 1:1:1 *Milli-Q* water/methanol/acetonitrile (10 mL) and concentrated using the Speed Vac®. The sample was redissolved in *Milli-Q* water (1 mL). IE-HPLC was performed on a ~200 μL aliquots using a gradient of 0.0 - 0.8 M ammonium chloride. The fractions containing the desired ORN strands were collected, combined, concentrated *in vacuo*, redissolved in *Milli-Q* water (1 mL) and finally desalted as described above.

Thermal melting studies

Thermal melting experiments for the RNA.RNA,[3] the DNA.DNA duplexes[4] and the quadruplexes[4] were recorded as previously described. Oligonucleotide solutions for these experiments were prepared as outlined in the figure legends.

References

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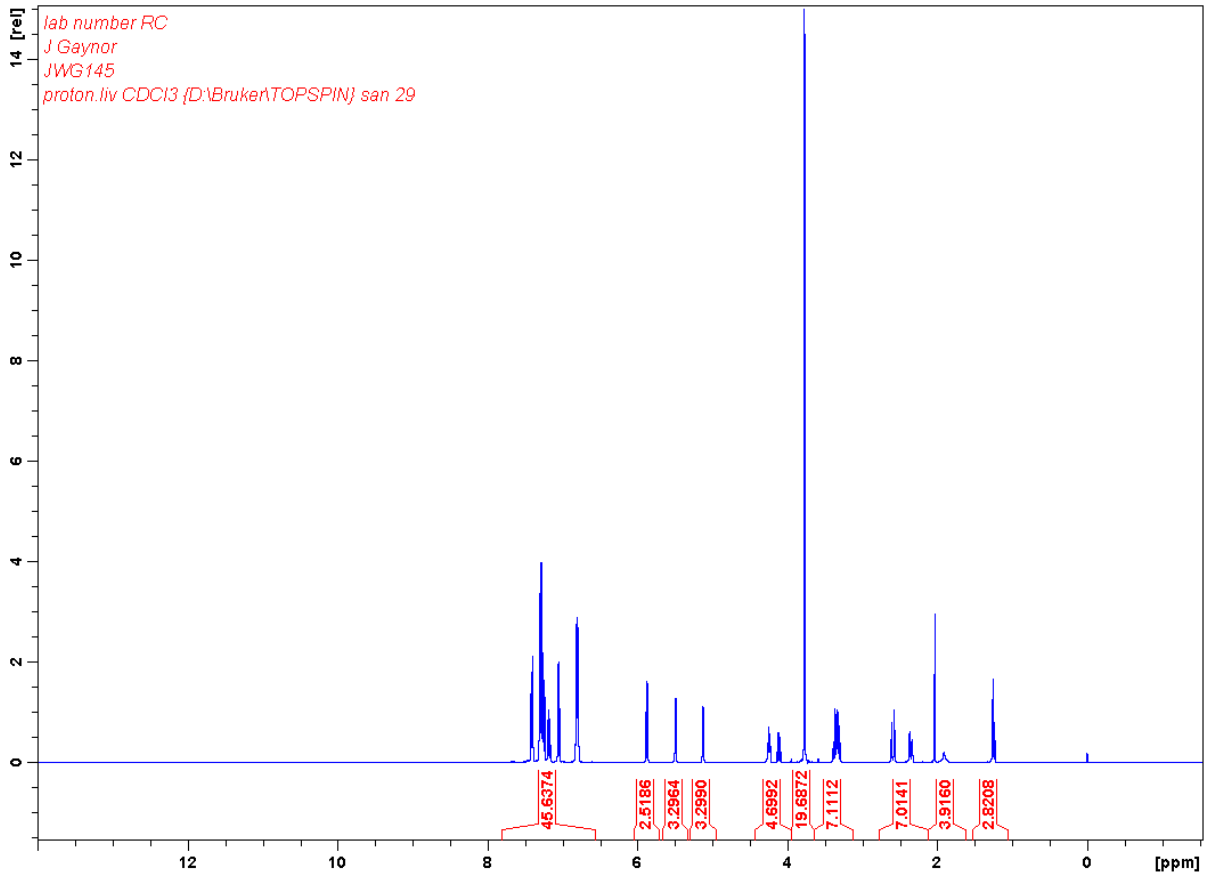
5′-*O*-(4,4′-Dimethoxytrityl)-2,3′-anhydro-2′-deoxyuridine (2)



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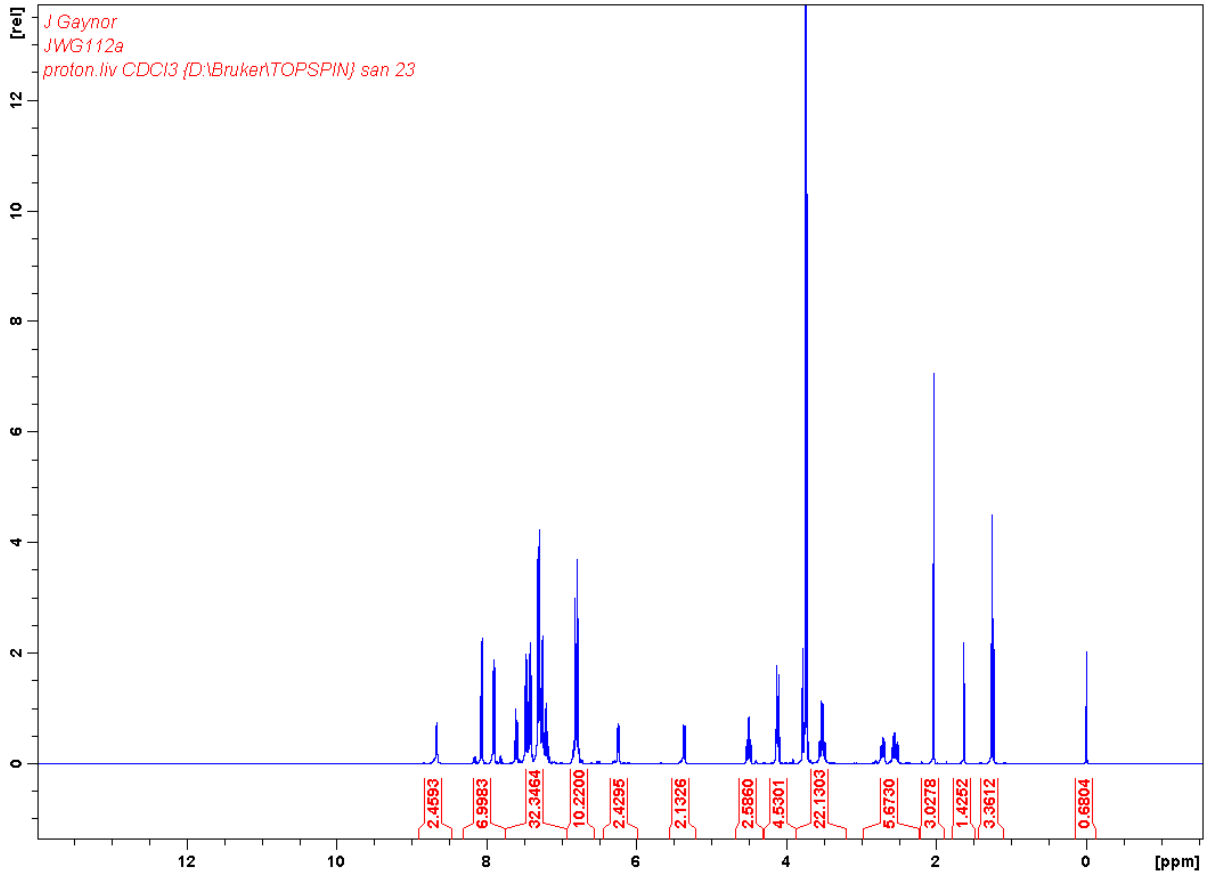

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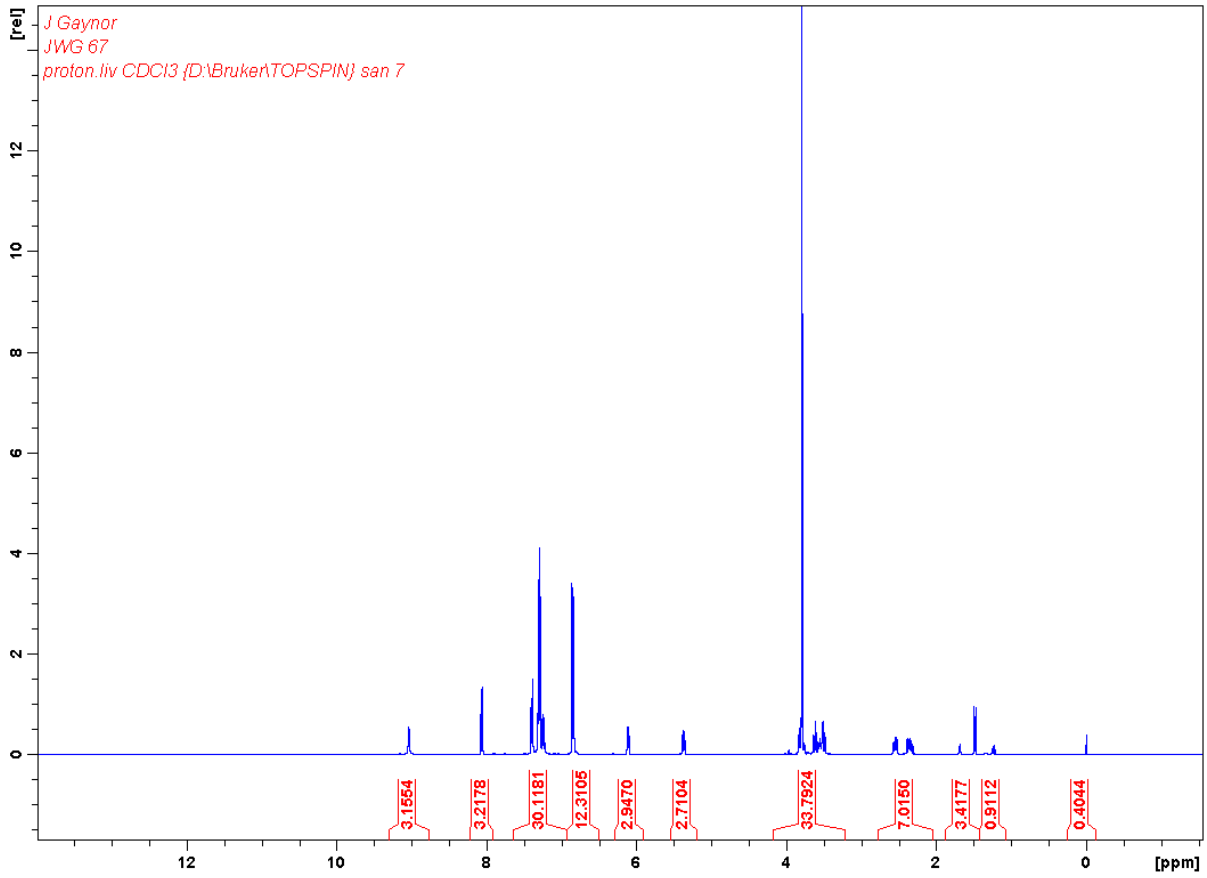
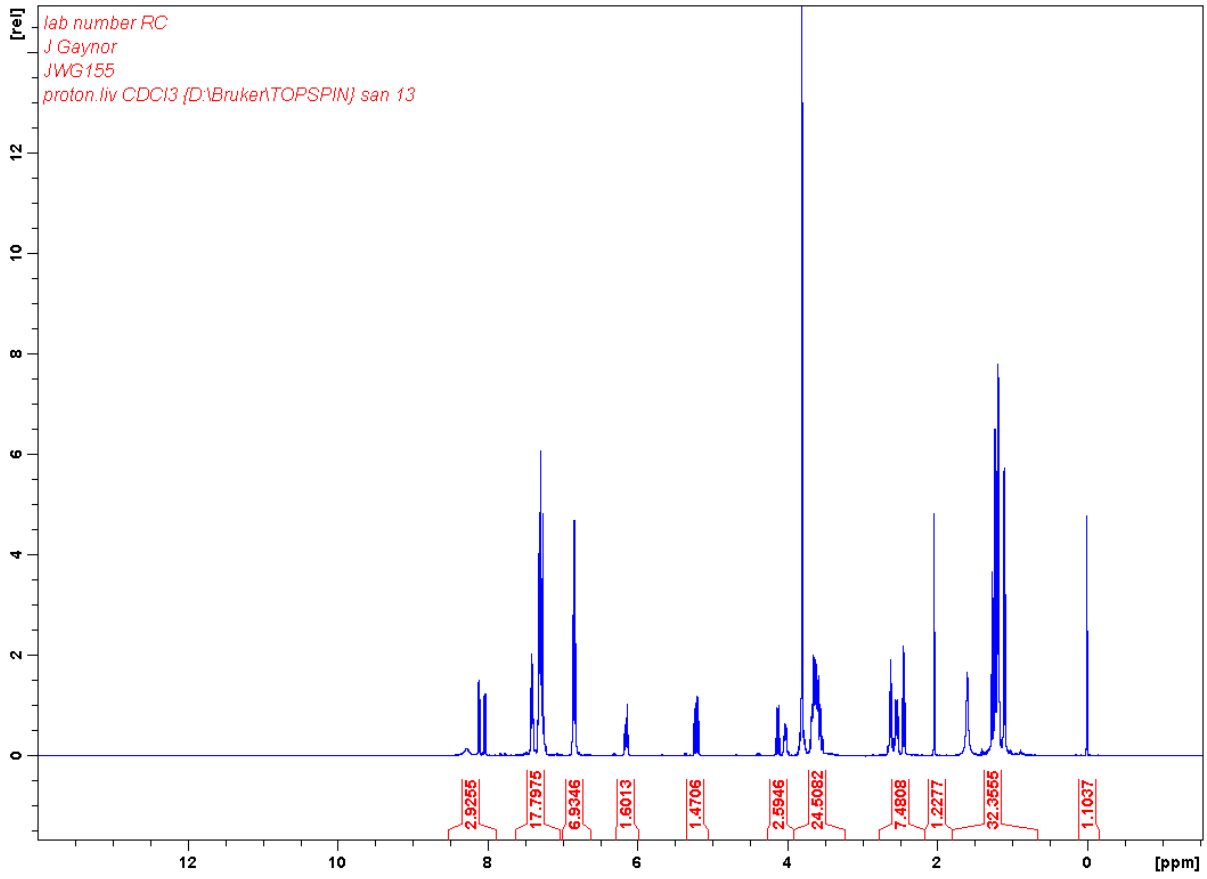
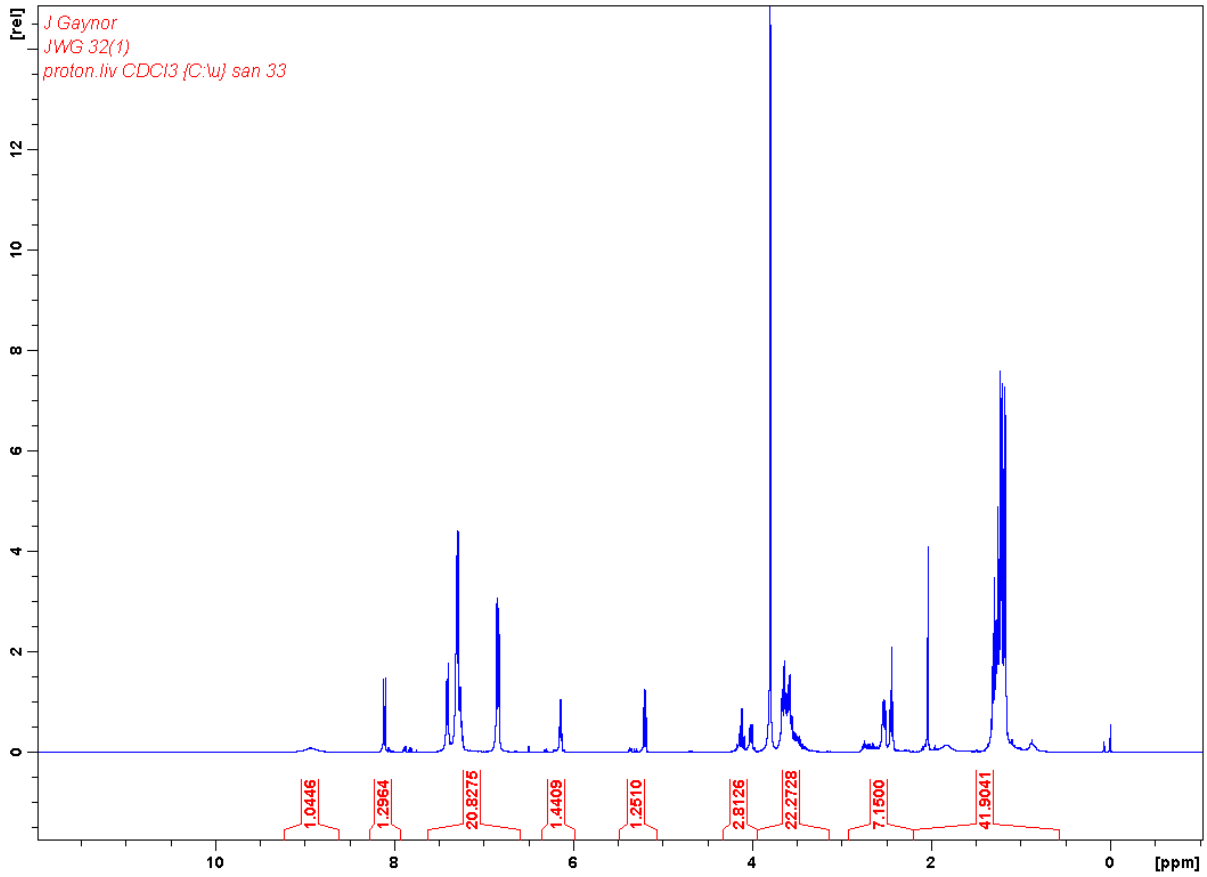


5′-O-(4,4′-dimethoxytrityl)-2′,3′-dideoxy-3′-thiouridine-3′-S-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphorothioamidite (5 both diastereoisomers)



5′-O-(4,4′-dimethoxytrityl)-2′,3′-dideoxy-3′-thiouridine-3′-S-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphorothioamidite (5 fast diastereoisomer)



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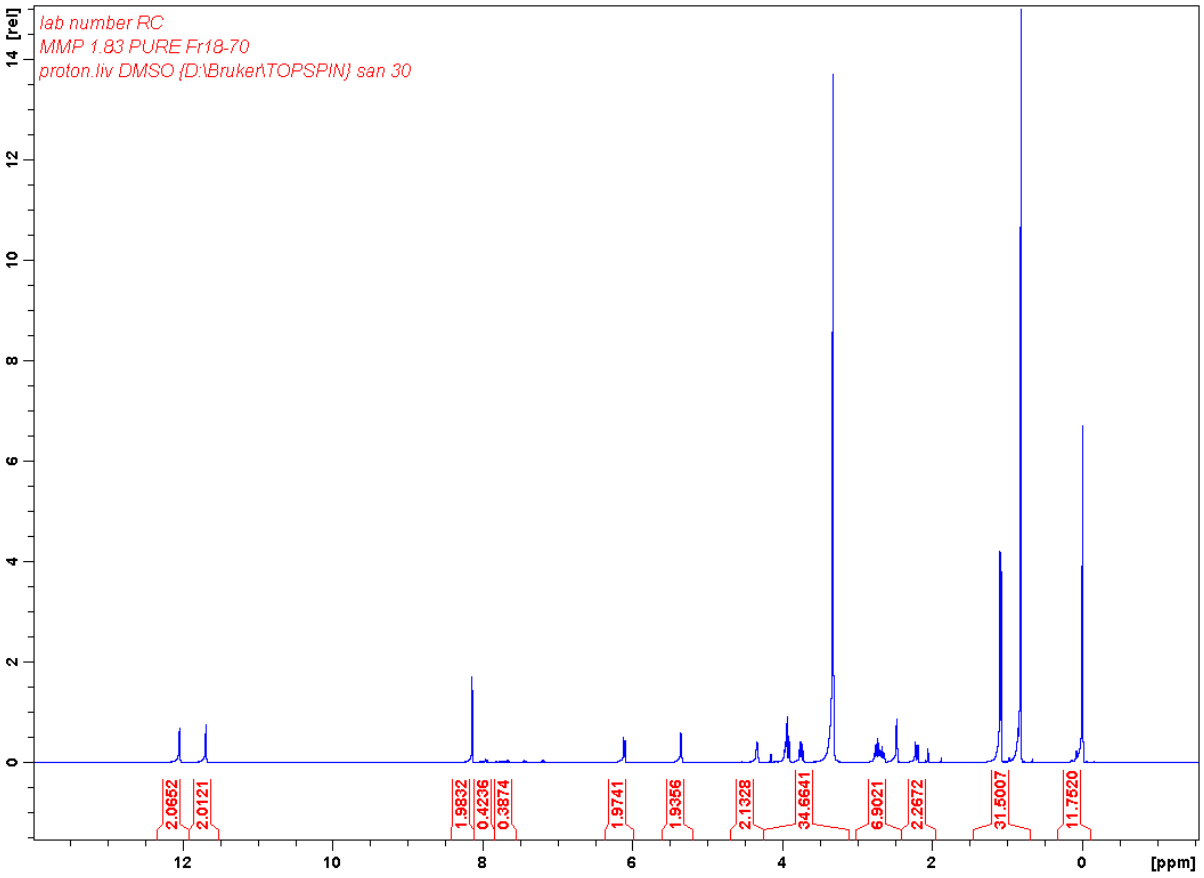

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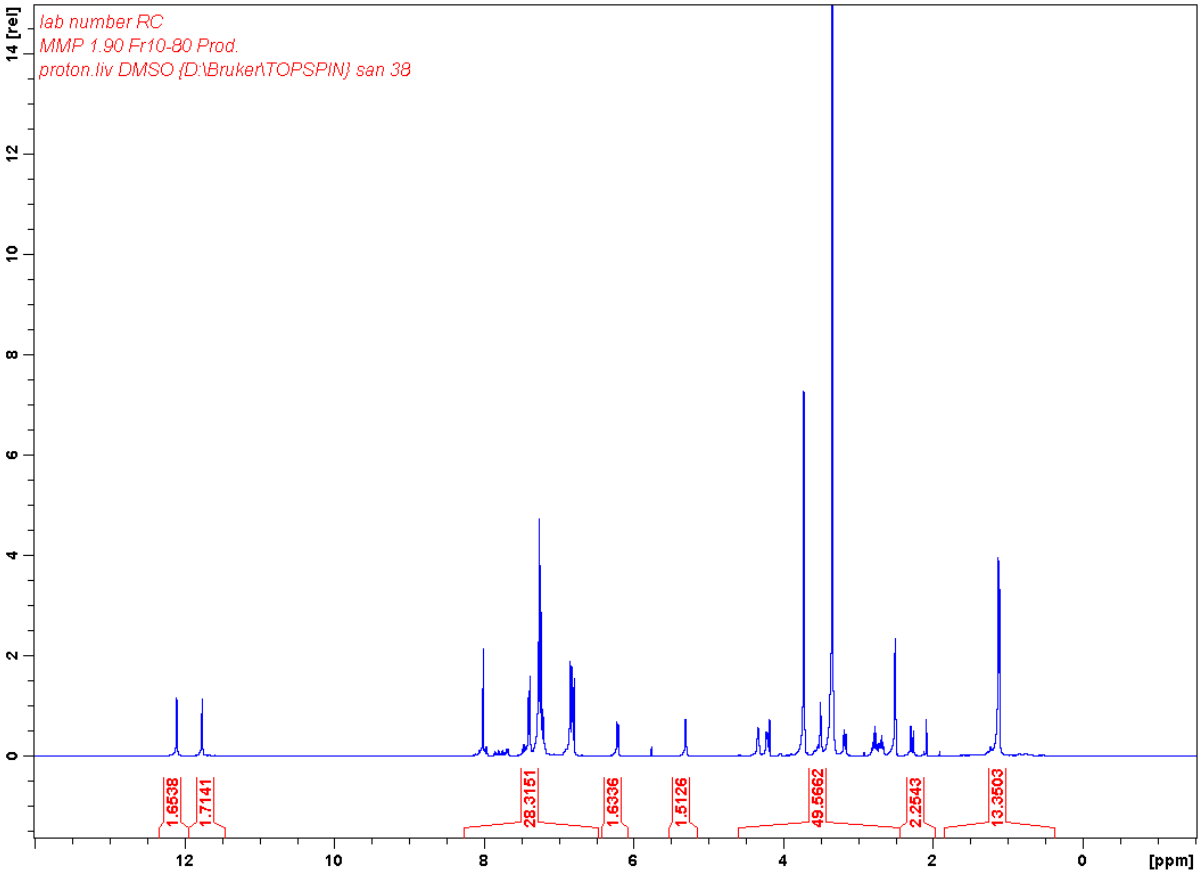

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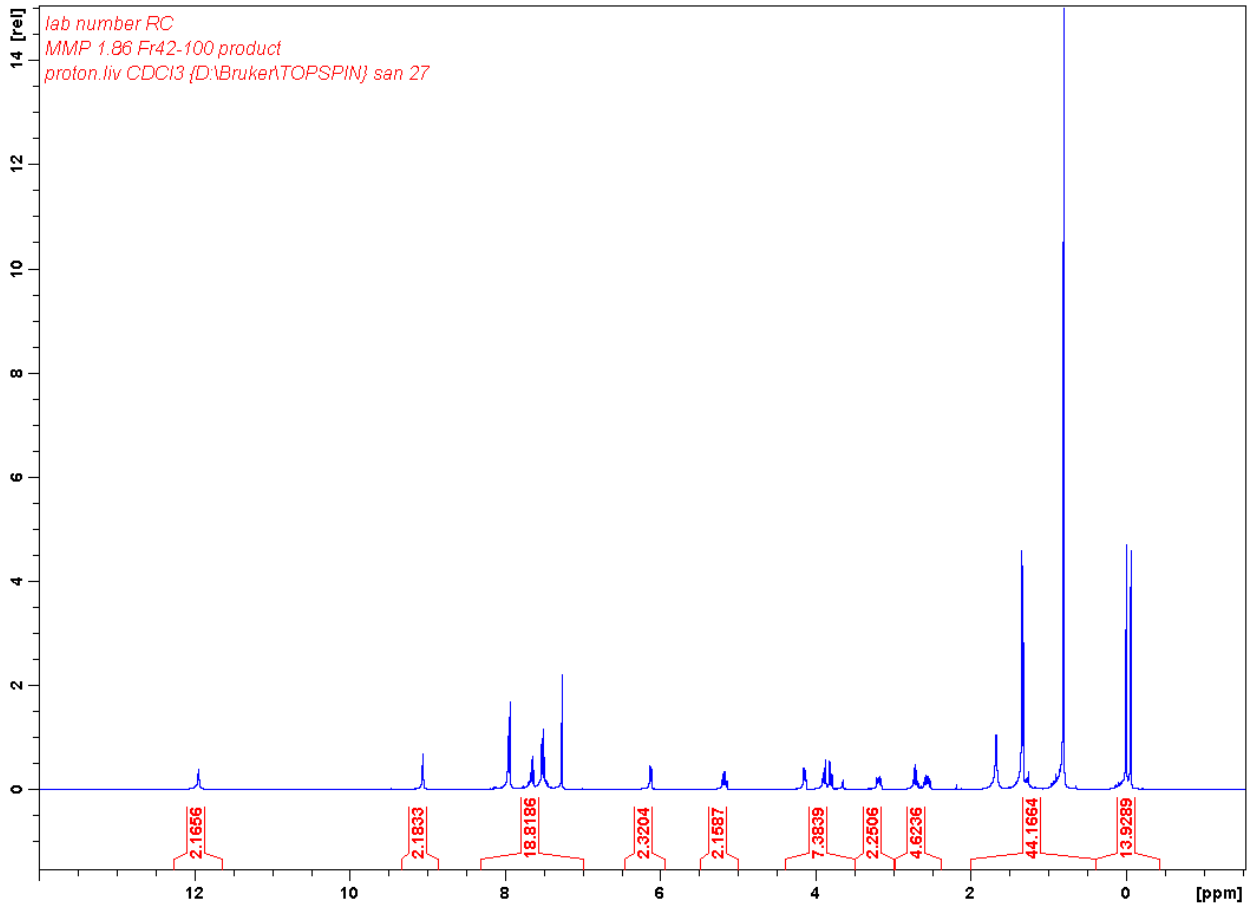

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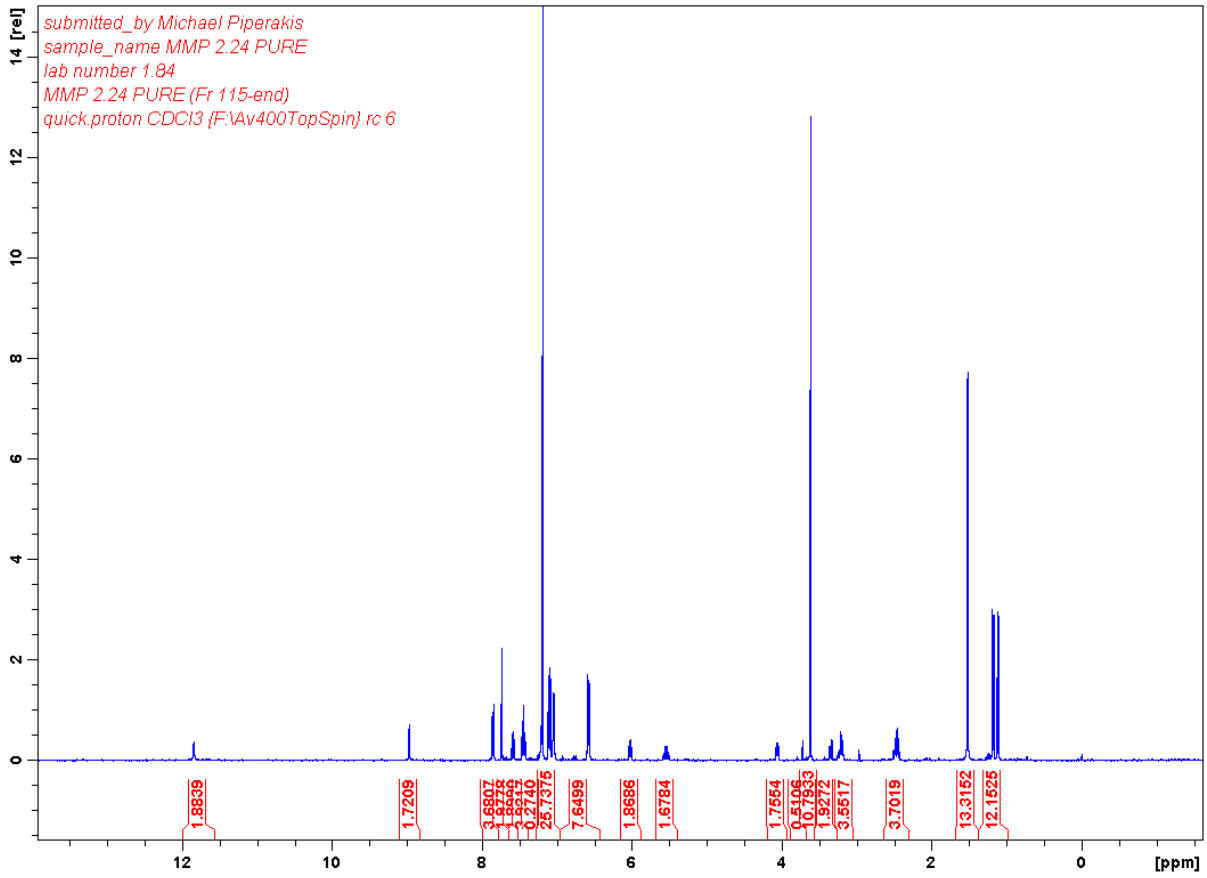

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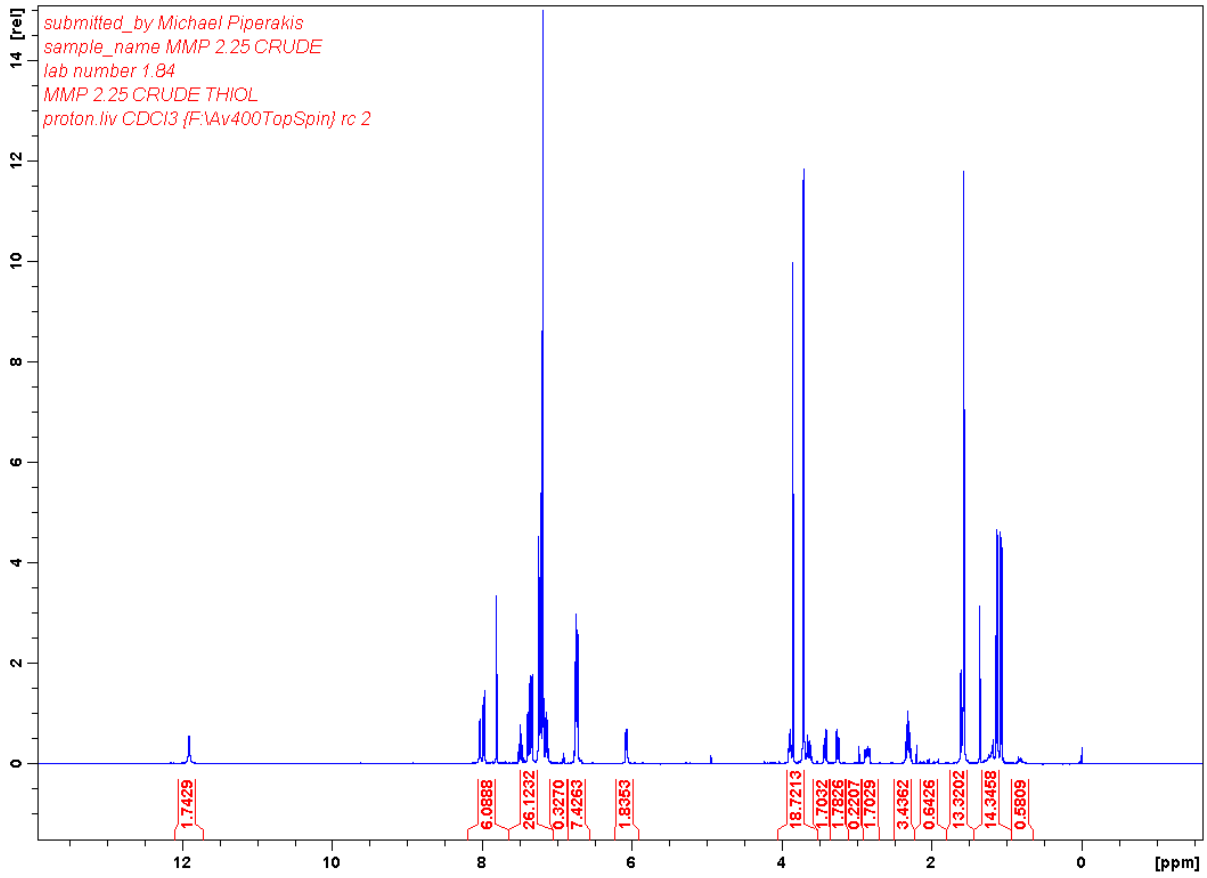
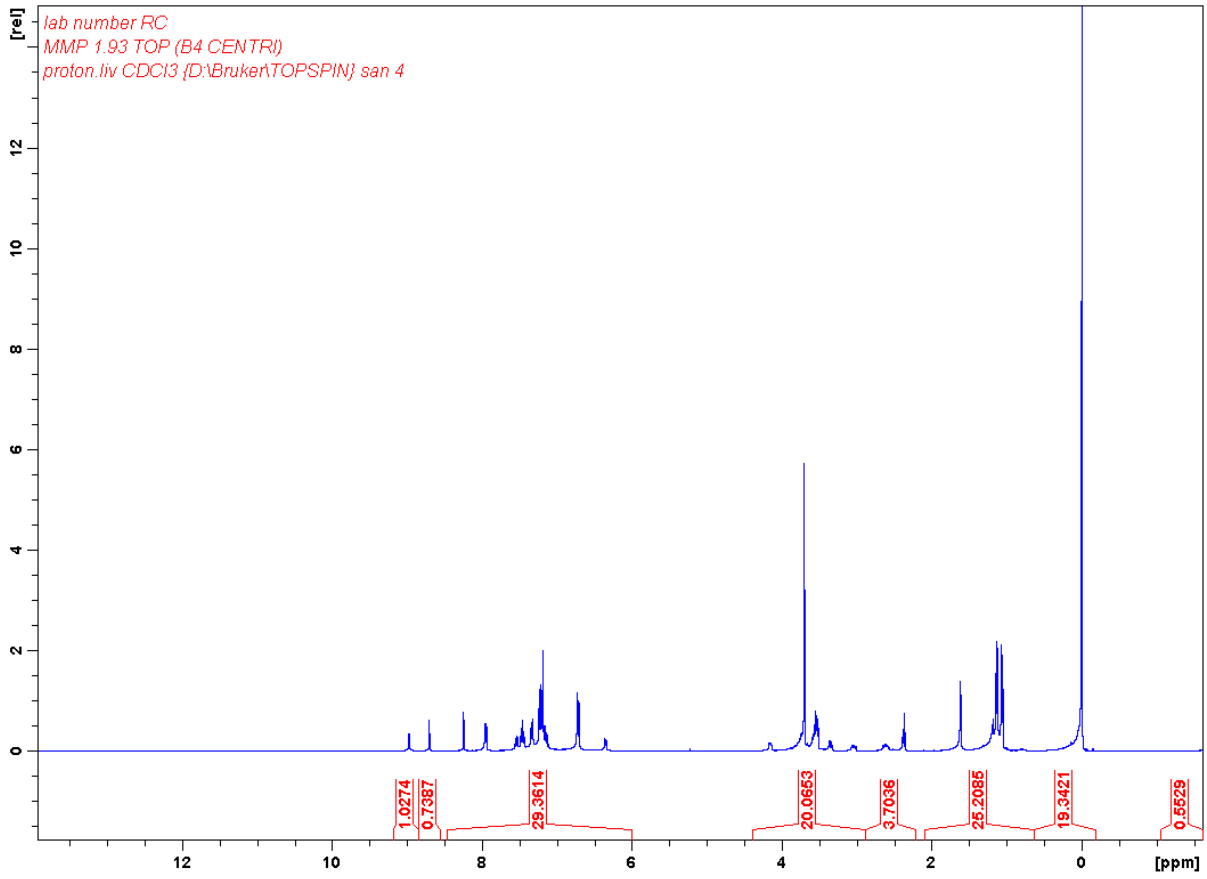
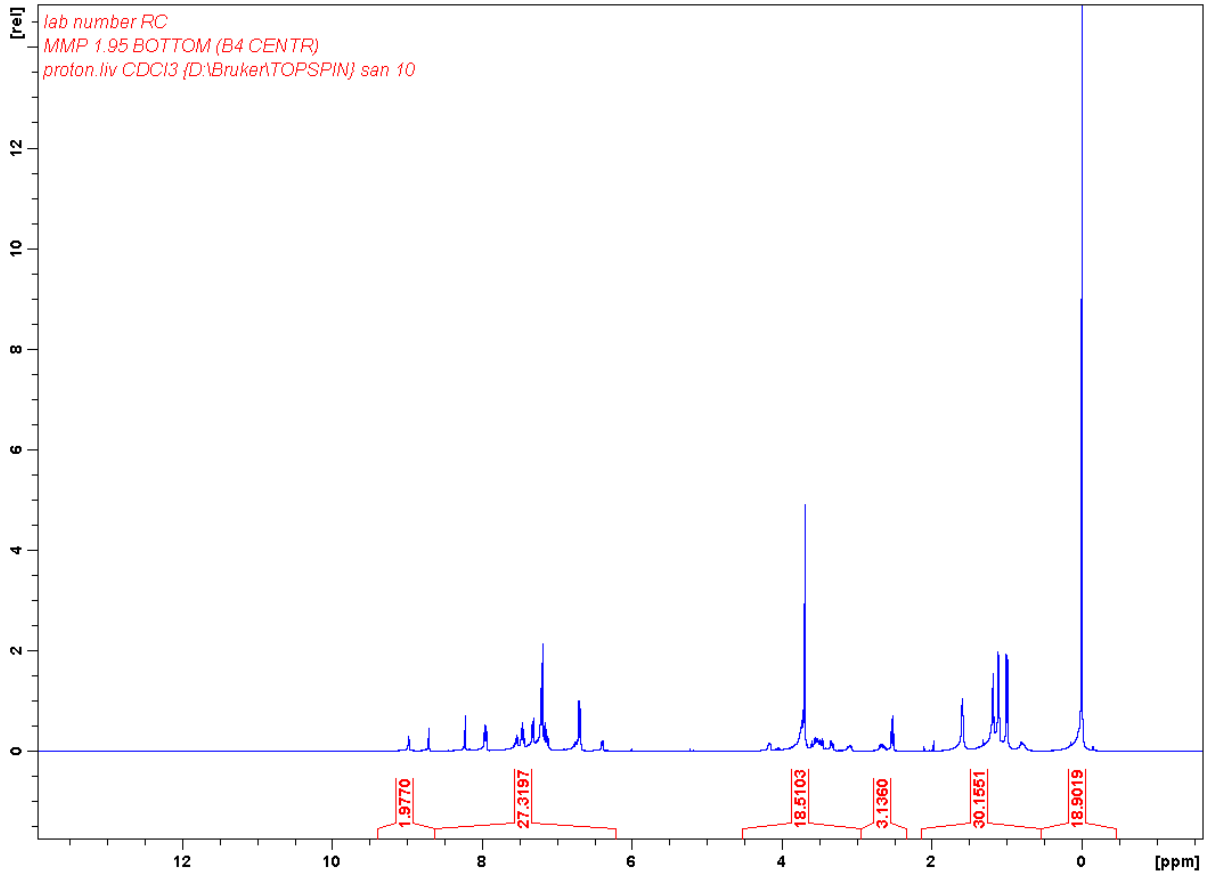


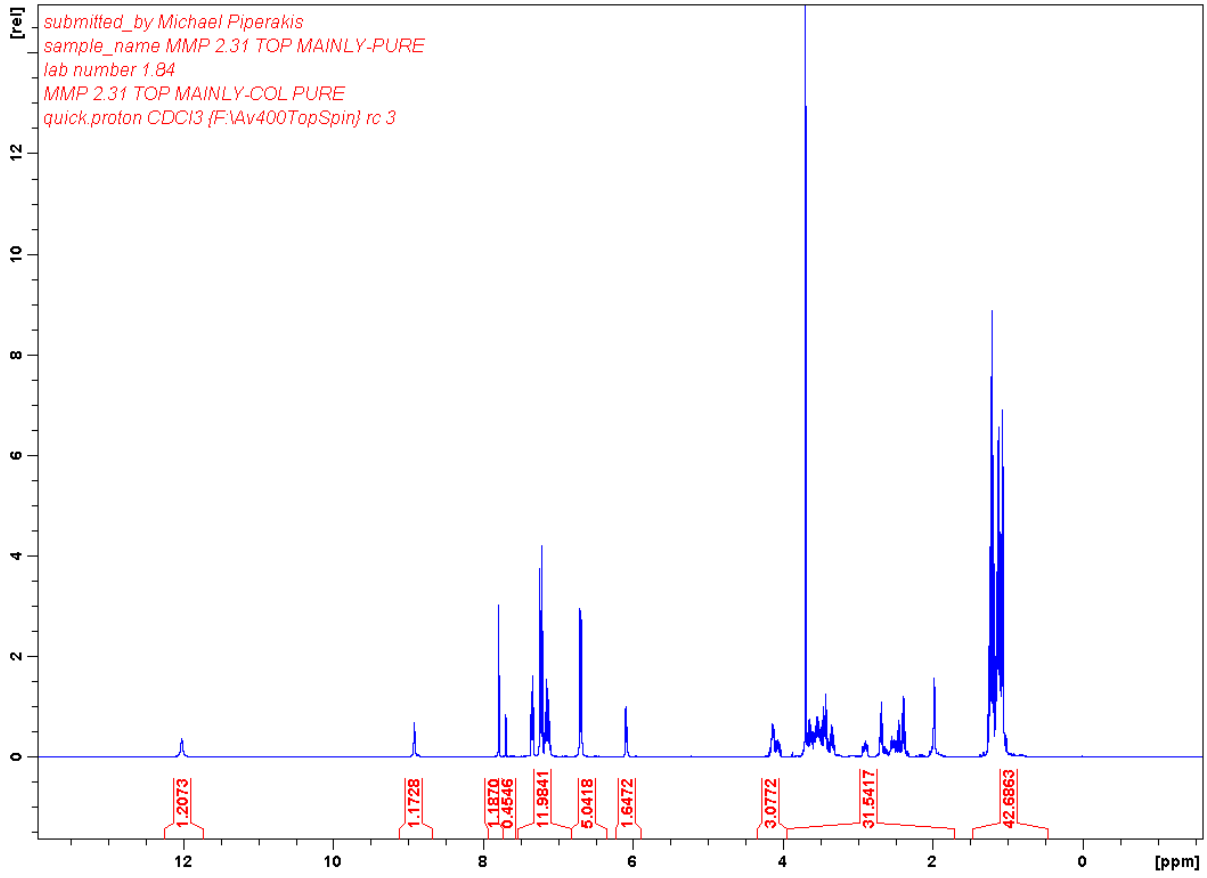
_N_6-benzoyl-5′-_O_-dimethoxytrityl-2′-deoxy-3′-thioadenosine-3′-_S_-[(2-cyanoethyl)-(_N_,_N_-diisopropyl)]-phosphoramidite (7, Fast diastereoisomer)



_N_6-benzoyl-5′-_O_-dimethoxytrityl-2′-deoxy-3′-thioadenosine-3′-_S_-[(2-cyanoethyl)-(_N_,_N_-diisopropyl)]-phosphoramidite (7, Slow diastereoisomer)



_N_2-isobutyryl-5′-_O_-dimethoxytrityl-2′-deoxy-3′-thio-guanosine-3′-_S_-[(2-cyanoethyl)-(_N_,_N_-diisopropyl)]-phosphoramidite (12, Fast diastereoisomer)



_N_2-isobutyryl-5′-_O_-dimethoxytrityl-2′-deoxy-3′-thio-guanosine-3′-_S_-[(2-cyanoethyl)-(_N_,_N_-diisopropyl)]-phosphoramidite (12, Slow diastereoisomer)