SUPPLEMENTARY INFORMATION

Novel Morpholino-Based Phosphorothioate Analogs via Oxathiaphospholane Chemistry: Synthesis, Structural Insights and Stability

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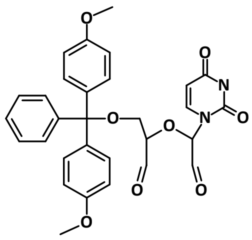
Table of Contents

Figure S1. Structures of the compounds studied, along with their calculated molecular masses:



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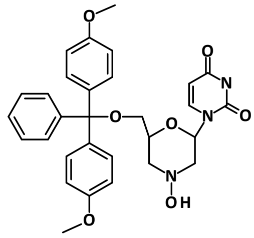

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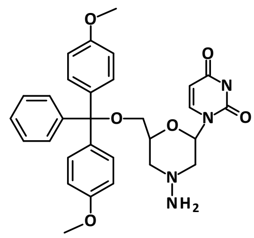

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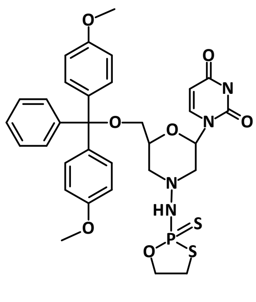

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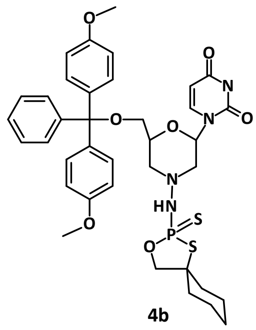

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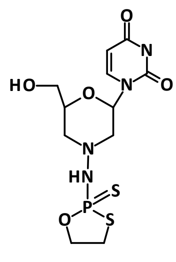

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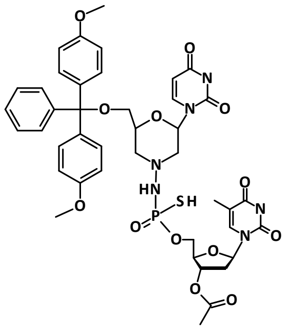

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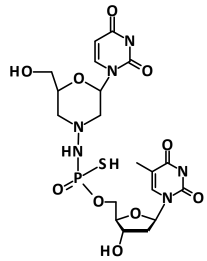

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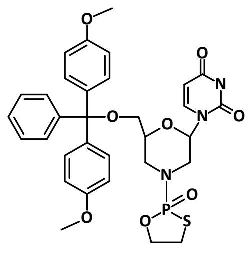

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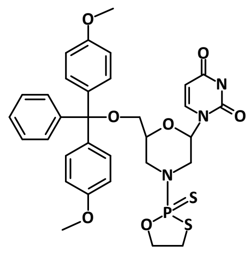

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1. Materials

Silica gel chromatography media were supplied by MERCK. TLC silica gel 60 plates were used for routine analyses, while HPTLC silica gel 60 plates were used for the assessment of the chromatographic separability of P-diastereomers; all plates were with a UV F254 indicator. Silica gel 60, 230–400 mesh, was used for routine open column chromatographic purification. Unless otherwise stated, solvents of analytical grade were used. The cartridges packed with silica gel (FP ECOFLEX Si 25g) was used for flash chromatographic purification. Anhydrous acetonitrile, DBU (1,4-diazabicyclo[5.4.0]undec-7-ene), TBD (1,5,7-Triazabicyclo[4.4.0]dec-5-ene) and Verkade base (Vb, 2,8,9-Trimethyl-2,5,8,9-tetraaza-1-phosphabicyclo[3.3.3]undecane were supplied by Merck. For chromatographic separation of OTP monomers, HPLC grade ethyl acetate, hexane were supplied by Witko (Lodz, Poland; solvents of a ChemSolve class). 5'-DMT-protected ribonucleosides of uridine were purchased from ChemGenes Corporation (Wilmington, MA). The 2-Chloro-1,3,2-oxathiaphospholane and 2-Chloro-"spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane (the phosphitylating reagents) were synthesized according to the published protocols[1].

2. Methods

2.1. Synthesis of dialdehyde (**1**)

5'-dimethoxytrityl-protected ribonucleoside (2g, 1 equiv.) was dissolved in 500 mL of anhydrous methanol, followed by the addition of 1.2 equiv. of sodium periodate. The mixture was stirred until no unreacted ribonucleoside could be detected by TLC (~6h). The reaction mixture was filtered, and the solvent was evaporated. The residue was extracted with methylene chloride (3×70 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure, yielding compound **1** as a white solid in quantitative yield.

2.2. Synthesis of DMT-protected *N*-hydroxymorpholino-uridine nucleoside, *m*U-^{OH} (**2**)

The dialdehyde **1** (1g, 1 equiv.) was dissolved in anhydrous methanol (250 mL). $NaHCO_3$ (2.0 equiv.) and hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$, 2.0 equiv.) were added. The reaction mixture was stirred for 1 hour at room temperature, after which Et_3N (2.5 equiv.) and $NaCNBH_3$ (2.0 equiv.) were introduced. The mixture was stirred for an 15 minutes at room temperature, then trifluoroacetic acid (3.0 equiv.) was added and the reaction was stirred for a further 1 hour. The reaction progress was monitored by TLC using a 9:1 (v/v) $CHCl_3$:MeOH mixture as an eluent (Rf of the substrate **1**: 0.68; Rf of the product **2**: 0.54). The reaction mixture was filtered, and the solvent was evaporated. The residue was extracted with methylene chloride (3×70 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography (hexane:ethyl acetate, 20:80→0:100, affording compound **2** as a white solid in 58% yield. HRMS (ESI) found: m/z 544.2086; calculated $[M+H]^+$ 545.2162. HR MS is given in Figure S3.

2.3. Synthesis of DMT-protected *N*-aminomorpholino-uridine nucleoside, *m*U-^{NH2} (**3**)

The dialdehyde **1** (1g, 1 equiv.) was dissolved in anhydrous methylene chloride (250 mL). Hydrazine (1.5 equiv.) and boric acid (1.5 equiv.) were added at 0°C. After 15 minutes, sodium cyanoborohydride $NaCNBH_3$ (1.3 equiv.) and trifluoroacetic acid (TFA, 1 equiv.) were added, and the mixture was stirred at room temperature. The reaction progress was monitored by TLC using a 9:1 (v/v) EtOAc:MeOH mixture as an eluent (Rf of the substrate **1**: 0.68; Rf of the product **3**: 0.52). After 10 minutes, the reaction was quenched. The crude product was purified by extraction and flash chromatography (hexane:ethyl acetate, 20:80→0:100, affording compound **3** as a white solid in 62% yield. HRMS (ESI) found: m/z 543.2251; calculated $[M+H]^+$ 544.2322. HR MS is given in Figure S4.

2.4. Attempt at the synthesis of oxathiaphospholane monomer mU-^{O}OTP

*m*U-^{OH} (**2**, 100 mg, 1 equiv.) in 15 mL round-bottom flask and elemental sulfur were dried overnight at a high vacuum. To the flask anhydrous pyridine (water content < 20ppm) was added, followed by 1.2 equiv. of 2-chloro-1,3,2-oxathiaphospholane. After ca. 10 minutes, dry elemental sulfur (2 equiv.) was added and the stirring was continued for ca. 20 minutes at room temperature. TLC analysis showed no remaining substrate but TLC analysis showed no remaining substrate but did not allow for clear identification of the product. ^{31}P NMR analysis indicated that the phosphitylation of 4-hydroxymorpholino nucleoside uridine (mU-^{OH}, **2**) resulted in a mixture of 2-thio-1,3,2-oxathiaphospholane and its oxidized analogue, 2-oxo-1,3,2-oxathiaphospholane. Excess sulfur was filtered off, and the solvent was evaporated. The crude mixture was purified by silica gel flash chromatography (Reveleris system) using ethyl acetate as the eluent. The appropriate fractions were collected and concentrated under reduced pressure, yielding a first fraction containing the 2-thio-1,3,2-oxathiaphospholane and a second fraction containing the 2-oxo-1,3,2-oxathiaphospholane. HR MS for 2-thio-1,3,2-oxathiaphospholane (**2.2**): HRMS (ESI) found: m/z 666.1483; calculated $[M+H]^+$ 667.1575. HR MS for 2-oxo-1,3,2-oxathiaphospholane (**2.1**): HRMS (ESI) found: m/z 650.1717; calculated $[M+H]^+$ 651.2804. HR MS and ^{31}P NMR spectra are given in Figures S5-9.

2.5. Synthesis of oxathiaphospholane monomer mU-^{N}OTP (**4a**^{MIX})

*m*U-^{NH2} (**3**) (500 mg) in 15 mL round-bottom flask and elemental sulfur were dried overnight at a high vacuum. To the flask anhydrous pyridine (water content < 20ppm) was added, followed by 1.2 equiv. of 2-chloro-1,3,2-oxathiaphospholane. After ca. 10 minutes, dry elemental sulfur (2 equiv.) was added and the stirring was continued for ca. 20 minutes at room temperature. TLC analysis showed no substrate remaining (Rf 0.71; $CHCl_3$: MeOH, 9:1). Excess sulfur was filtered off and the solvent was evaporated. The crude mixture was isolated by means of silica gel chromatography using a flash chromatography system (Reveleris) eluted with ethyl acetate. The appropriate fractions were combined and concentrated under reduced pressure to give **4a^{MIX}** (as a mixture of P-diastereomers) in 65% yield. HRMS (ESI) found: m/z 681.1602; calculated $[M+H]^+$ 682.1685. HR MS and ^{31}P NMR spectra are given in Figures S13 and S10-11.

2.6. Synthesis of oxathiaphospholane monomer mU-^{N}OTP (4b^{MIX})

*m*U-^{NH2} (**3**) (500 mg) in 15 mL round-bottom flask and elemental sulfur were dried overnight at a high vacuum. To the flask anhydrous pyridine (water content < 20ppm) was added, followed by 1.2 equiv. of 2-chloro-"spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane. After ca. 10 minutes, dry elemental sulfur (2 equiv.) was added and the stirring was continued for ca. 20 minutes at room temperature. TLC analysis showed no substrate remaining (Rf 0.74; $CHCl_3$: MeOH, 9:1). Excess sulfur was filtered off and the solvent was evaporated. The crude mixture was isolated by means of silica gel chromatography using a flash chromatography system (Reveleris) eluted with ethyl acetate. The appropriate fractions were combined and concentrated under reduced pressure to give **4b^{MIX}** (as a mixture of P-diastereomers) in 72% yield. HRMS (ESI) found: m/z 749.2220; calculated $[M+H]^+$ 750.2211. HR MS spectrum is given in Figure S14.

2.7. Separation of *m*U-^{N}OTP 4a into P-diastereomers

Approximately 1.5 mL of an ethyl acetate solution containing 200 mg of **4a^{MIX}** monomer was filtered through an organic solvent-resistant membrane filter (0.2 µm), and the filtrate was applied to a flash chromatography system. The cartridge packed with silica gel was eluted at a flow rate of 4 mL/min using the eluents specified in Table 1. Under these conditions, baseline separation was achieved and after evaporation of the solvents pure P-diastereomers were obtained at ca. 90% recovery. Figure S15 shows the flash chromatogram of the semi-preparative separation of monomer **4a^{MIX}** into its P-diastereomers. ^{31}P NMR, ^{1}H NMR, and ^{13}C NMR spectra for the *fast*- (***4a^{Fast}***) and *slow*-eluting (***4a^{Slow}***) P-diastereomers are given in Figures S17-22.

2.8. Separation of *m*U-^{N}OTP 4b into P-diastereomers

Approximately 1.5 mL of an ethyl acetate solution containing 100 mg of **4b^{MIX}** monomer was filtered through an organic solvent-resistant membrane filter (0.2 µm), and the filtrate was applied to an HPLC system. The column (Pursuit XRs 10 µm, 250×21,2 mm) was eluted at a flow rate of 5 mL/min using the eluents specified in Table 1. Under these conditions, baseline separation was achieved and after evaporation of the solvents pure P-diastereomers were obtained at ca. 90% recovery. Figure S16 shows the HPLC chromatogram of the semi-preparative separation of monomer **4b^{MIX}** into its P-diastereomers. ^{31}P NMR, ^{1}H NMR, and ^{13}C NMR spectra for the *fast*- (***4b^{Fast}***) and *slow*-eluting (***4b^{Slow}***) P-diastereomers are given in Figures S23-28.

2.9. Detritylation of 4a^{Fast}

The dinucleotides **4a^{Fast}** (100 mg, 0.15 mmol) were dissolved in 1.5% DCA in CH_2Cl_2 (10 mL), with stirring at room temperature. The reaction progress was monitored by TLC using a 8:2 (v/v) $CHCl_3$:MeOH mixture as an eluent (Rf of the substrate 0.70; Rf of the product **5a^{Fast}** 0.44). After 10 min, the reaction mixture was concentrated under vacuum. The crude product was purified with silica gel of Reveleris Flash Chromatography and was eluted with of CH_2Cl_2 → MeOH a linear 0→80% gradient. The product was isolated in 63% yield (36 mg). HR MS for **5a^{Fast}**: HRMS (ESI) found: m/z 379.0304; calculated $[M+H]^+$ 380.0378. HR MS for **5a^{Fast}** is given in Figure S29.

2.10. Single crystal X-ray analysis of **5a*Fast***

Suitable crystal of **5a*Fast*** was selected, transferred to mineral oil, and mounted on cryo loops. The crystal was then flash-cooled directly in a stream of N2. Diffraction intensities were recorded using a Rigaku XtaLAB Synergy-S diffractometer (Rigaku Europe SE, Neu-Isenburg, Germany) equipped with a Cu Kα radiation source (λ = 1.5418 Å) and a HyPix-6000HE hybrid photon counting detector (Rigaku Europe SE, Neu-Isenburg, Germany). The total number of runs and images was based on the strategy calculation of the CrysAlisPro program (Rigaku Oxford Diffraction, v 1.171.43.122a, 2024). The molecular models of the structures were created with the structure solution program SHELXT 2018/2[2] using intrinsic phasing with Olex2 v.1.5[3] as the graphical interface and refined by least squares with the 2018/3 version of SHELXL[4]. All non-hydrogen atoms were refined anisotropically. The positions of the hydrogen atoms were calculated geometrically and refined using the riding model. The structures were validated with CheckCif (http://checkcif.iucr.org, accessed: July 2025) and deposited in the Cambridge Crystallographic Data Centre (CCDC) under the accession number **2470809** (Figure S38, Table S2, ESI†).

2.11. The solid-phase synthesis of dinucleotides **7*M***

Loading of the support with the respective nucleoside as determined by trityl assay: CPG-sarcosinyl-dT: 35.0 µmol/g. The coupling reactions were performed at the 1 µmol scale (in all experiments thymidine was attached to the support) using ~25-fold molar excess of each OTP monomer and 50-, 150- and 100-fold excess of DBU (1M), TBD (3M) and Verkade (2M), respectively.
In the column 5'-O-DMT nucleoside unit (1 mmol load) anchored to the LCA CPG support was detritylated with a 3% solution of DCA (dichloroacetic acid) in methylene chloride, washed with 5 mL of dry acetonitrile followed by 5 mL of dry methylene chloride and dried under high vacuum. For the coupling step, a solution of the oxathiaphospholane monomer **4a*MIX*** (as a mixture of P-diastereomers, 20 mg) and activator (50 µmol) in dry acetonitrile (150 µL) was freshly prepared and immediately introduced into the column[5].

Detailed procedure			
Solution (stock)	1 M DBU	3 M TBD	2M Verkade
	40µL DBU + 210µL CH_3CN	104mg TBD + 250µL CH_3CN	108mg Verkade + 250µL CH_3CN
One condensation: 20mg of monomer **4a** were dissolved in:	50µL + 100µL CH_3CN (50µmols stock of DBU were used)	50µL + 100µL CH_3CN (150µmols stock of TBD were used)	50µL + 100µL CH_3CN (100µmols stock of Verkade were used)

After 15 minutes of vigorous swirling, the column was washed sequentially with dry acetonitrile (5 mL) and dry methylene chloride (5 mL). Subsequently, the column containing the dimer was detritylated using a 3% solution of DCA in methylene chloride.

Figure S2. Coupling efficiency was monitored by measuring the absorbance at 504 nm

Coupling efficiency was monitored by measuring the absorbance at 504 nm, corresponding to the released DMT^+ cation (Figure S2). The dimer **7*M*** was then cleaved from the support under standard conditions (25% NH_4OH, 2h), and the resulting solution was concentrated under reduced pressure using a Speed-Vac concentrator. The resulting dinucleotide was isolated using semi-preparative RP-HPLC. Conditions: Varian Dynamax Pursuit XRs C18 column (250 × 21.2 mm, 10 µm); flow rate: 3 mL min^{-1}; elution with 0.1 M TEAB (pH 7.3) and 40% CH_3CN in 0.1 M TEAB. Gradient: 0–50% Buffer B over 30 min. Purified $\mathbf{7^{M}}$ was analyzed by analytical RP-HPLC (Figure S33). Conditions: a C18 column, 250 ×4.6 mm, 5 µm; elution at 0.5 mL min^{-1} with a gradient of 0.1 M TEAB, pH 7.3 to 40% CH_3CN in 0.1 M TEAB over 32 minutes.

2.12. The solid-phase synthesis of dinucleotides $\mathbf{7^{S}}$

In the column 5'-O-DMT nucleoside unit (1 mmol load) anchored to the LCA CPG support was detritylated with a 3% solution of DCA (dichloroacetic acid) in methylene chloride, washed with 5 mL of dry acetonitrile followed by 5 mL of dry methylene chloride and dried under high vacuum. For the coupling step, a solution of the oxathiaphospholane monomer $\mathbf{4a^{Slow}}$ (*slow*-eluting diastereomer, 20 mg) and DBU (50 µmol) in dry acetonitrile (150 µL) was freshly prepared and immediately introduced into the column. After 15 minutes of vigorous swirling, the column was washed sequentially with dry acetonitrile (5 mL) and dry methylene chloride (5 mL). Subsequently, the column containing the dimer was detritylated using a 3% solution of DCA in methylene chloride. The dimer $\mathbf{7^{S}}$ was then cleaved from the solid support and purified under the same conditions as described above (Figures S30-31). HRMS (ESI) found: m/z 561.1138; calculated $[M+H]^+$ 562. HR MS for $\mathbf{7^{t}}$ is given in Figure S32.

2.13. Stability Assessment of $\mathbf{7^{M}}$ in FBS

Dinucleotide $\mathbf{7^{M}}$ (from mixture of P-diastereomers, 0.5 OD) was dissolved in 10% FBS. Following incubation at 37 °C for 24 h, the protein was denatured. The mixture was then filtered and subjected to RP-HPLC analysis (Figure S37). Conditions: a C18 column, 250 ×4.6 mm, 5 µm; elution at 0.5 mL min^{-1} with a gradient of 0.1 M TEAB, pH 7.3 to 40% CH_3CN in 0.1 M TEAB over 32 minutes.

2.14. Digestion of $\mathbf{7^{M}}$ with *svPDE*

Dinucleotide $\mathbf{7^{M}}$ (from mixture of P-diastereomers, 0.5 OD) was dissolved in a 25 mM Tris-Cl (pH 8.5), 5 mM $MgCl_2$ buffer (20 µL) containing 1 µL of the svPDE suspension. Following incubation at 37 °C for 24 h, the protein was denatured. The solution was then filtered and analyzed by RP-HPLC under the same conditions as above.

2.15. Digestion of $\mathbf{7^{M}}$ with *nP1*

Dinucleotide $\mathbf{7^{M}}$ (from mixture of P-diastereomers, 0.5 OD) was dissolved in a 100 mM Tris-Cl (pH 7.2), 1 mM $ZnCl_2$ buffer (20 µL) containing 1 µL of the svPDE suspension. Following incubation at 37 °C for 24 h, the protein was denatured. The solution was then filtered and analyzed by RP-HPLC under the same conditions as above.

2.16. Stability Assessment of $\mathbf{7^{M}}$ in Buffers Across a Broad pH Range

Dinucleotide $\mathbf{7^{M}}$ (from mixture of P-diastereomers, 0.5 OD) were incubated for 24 hours at 37 °C in the following media (Table S1): acetate buffer (pH 5), phosphate-buffered saline (PBS, pH 7.4), Tris-HCl buffer (pH 9). The solutions were then filtered and analyzed by RP-HPLC under the same conditions as above.

Table S1. Conditions for chemical stability studies of dinucleotide $mU_{PS}T$ at different pH values. Samples were incubated for 24 h at 37°C and analyzed by RP-HPLC to assess potential degradation.

Buffer	pH	Incubation conditions
100 mM sodium acetate	5	24 h, 37°C
Commercial PBS solution (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) PBS	7,4	
100 mM Tris-HCl	9	

3. Equipment

^{1}H NMR, ^{13}C NMR and ^{31}P NMR spectra were recorded using Bruker instruments (Avance Neo 400; operating for ^{1}H at 400 MHz). Chemical shift (σ) values are in ppm, referenced to internal tetramethylsilane (TMS) or residual solvent protons for ^{1}H NMR and external 85% H_3PO_4 for ^{31}P NMR. High-resolution mass spectra (HR MS) were acquired using a Synapt G2 Si mass spectrometer (Waters Corporation, Milford, MA) consisting of ESI source and a quadrupole time-of-flight mass analyzer. Measurements were performed in negative ion mode with the capillary and sampling cone voltages set at 2.7 kV and 20 V, respectively. The source temperature was 110^{o}C. To ensure satisfactory accuracy, data were obtained in centroid mode and readings were corrected during acquisition with using leucine-enkephalin as an external reference (Lock-SprayTM), which generates reference ions at m/z 554.2615 Da ($[M–H]^{-}$) in negative ESI mode. Data sets were processed with the use of MassLynx 4.1 software (Waters).
Purification of compounds and separation of **4a**^{MIX} were performed using a flash chromatography system equipped with UV and ELSD detectors (Reveleris, Fluka).
Preparative HPLC separation of **4b**^{MIX} was performed using a binary Varian HPLC system, consisting of two PrepStar 210 pumps (equipped with 25 mL pump heads) and a ProStar 320 UV/VIS detector set at 275 nm. A silica gel column Pursuit XRs (10 µm, 250 × 21.2 mm) was eluted at a flow rate of 5 mL min^{-1}.
Gradient-grade HPLC solvents from Baker, or ChemPur were used.
UV absorption measurements were performed in a 1 cm path-length cell, using a double beam spectrophotometer (CINTRA 10e, GBC, Dandenong, Australia), equipped with a silicon photo-diode detector.
The crystallographic data were acquired using Rigaku XtaLAB Synergy-S diffractometer equipped with a HyPix-6000HE Hybrid Photon Counting (HPC) detector and Cu microfocus sealed X-ray source as well as a low-temperature Oxford Cryostream 800 liquid nitrogen cooling system at 100 K.

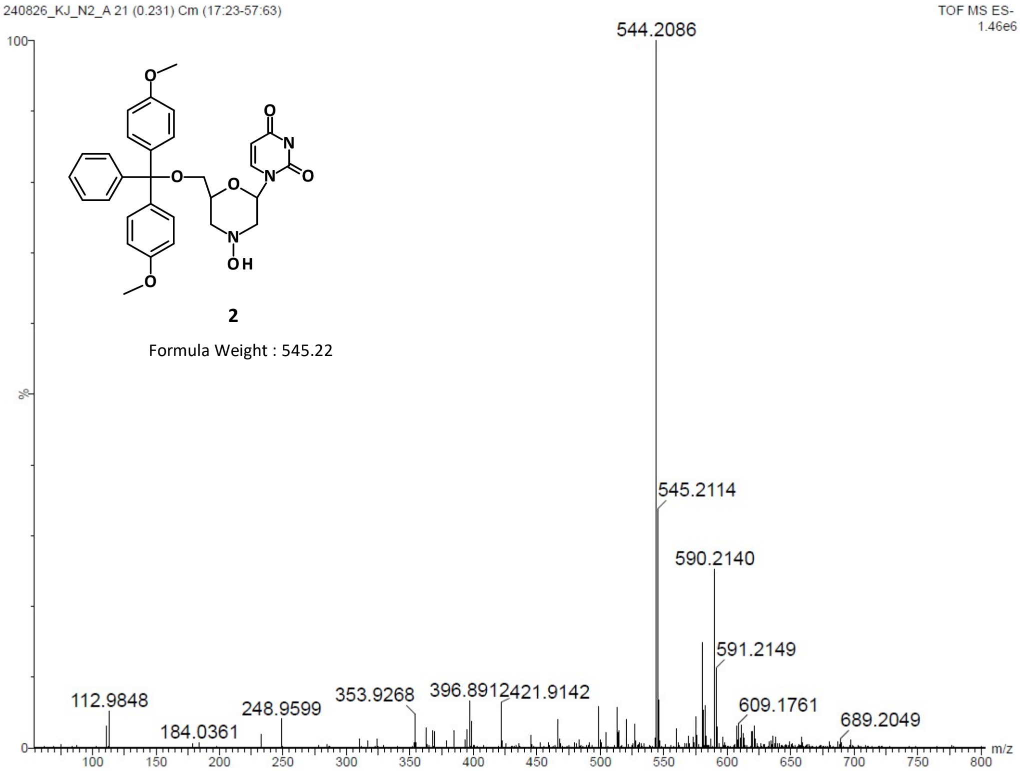


Figure S3. HR MS spectra for *mU-*^{OH}(**2**)

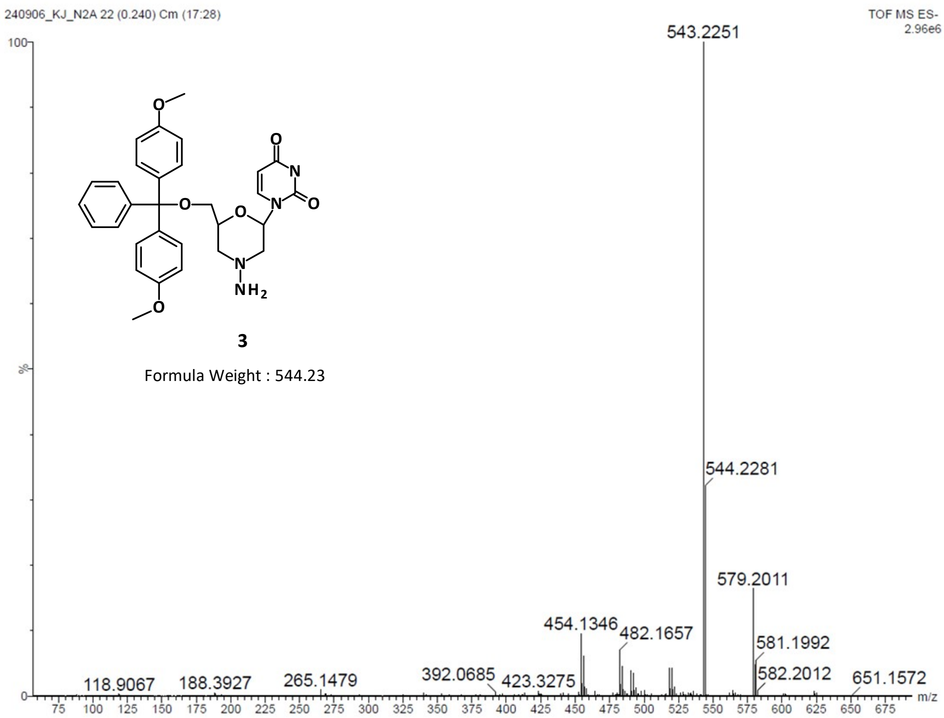


Figure S4. HR MS spectra for *mU-*^{NH2} (**3**)

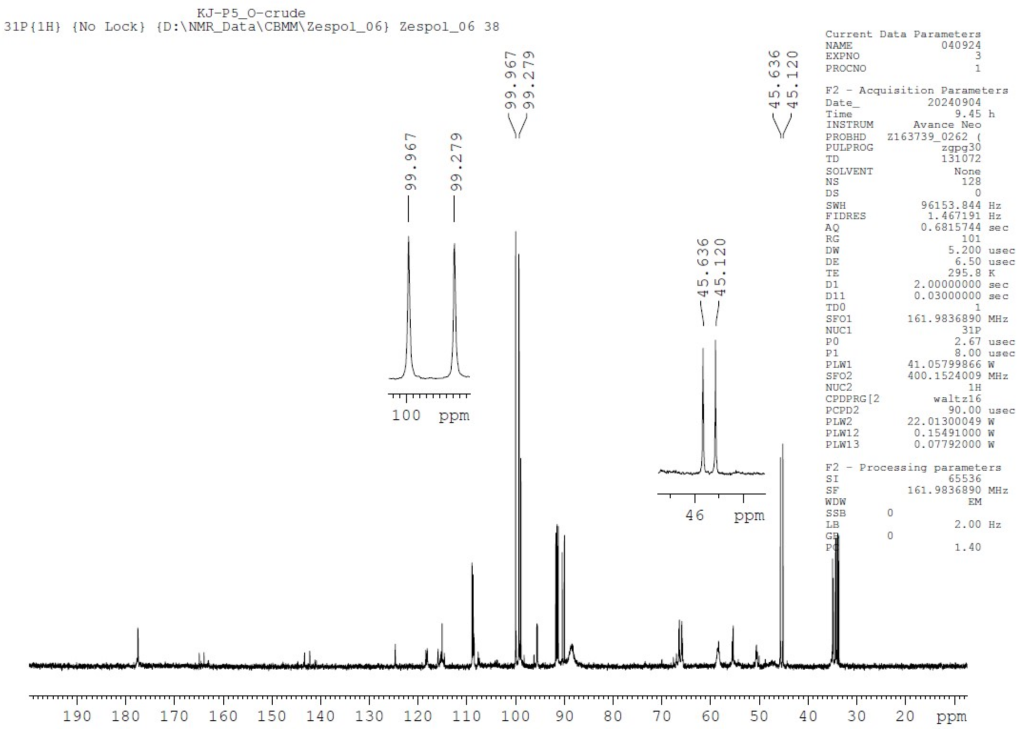


Figure S5. ^{31}P NMR spectrum of the crude reaction mixture from the attempted synthesis of morpholino mU-^{O}OTP; analysis was performed in a non-deuterated solvent.

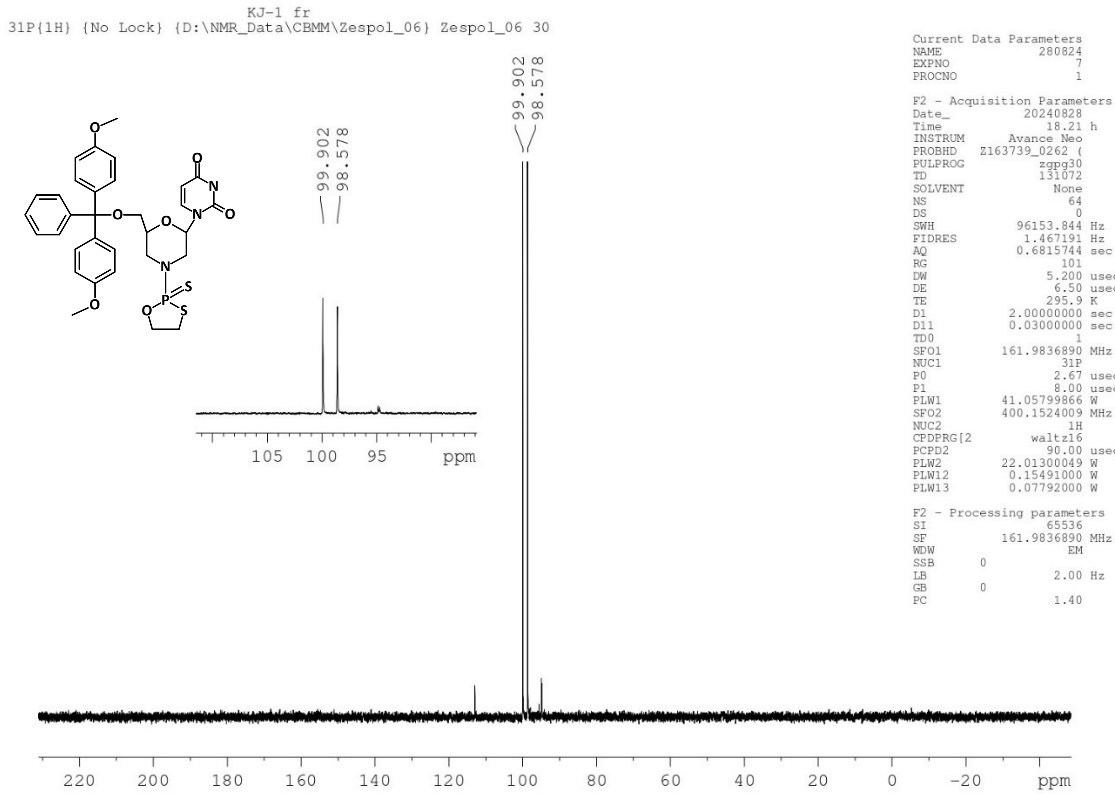


Figure S6. ^{31}P NMR spectrum of the first fraction (compound **2.1**) obtained after purification of the crude reaction mixture from the attempted synthesis of morpholino mU-^{O}OTP; analysis was performed in a non-deuterated solvent.

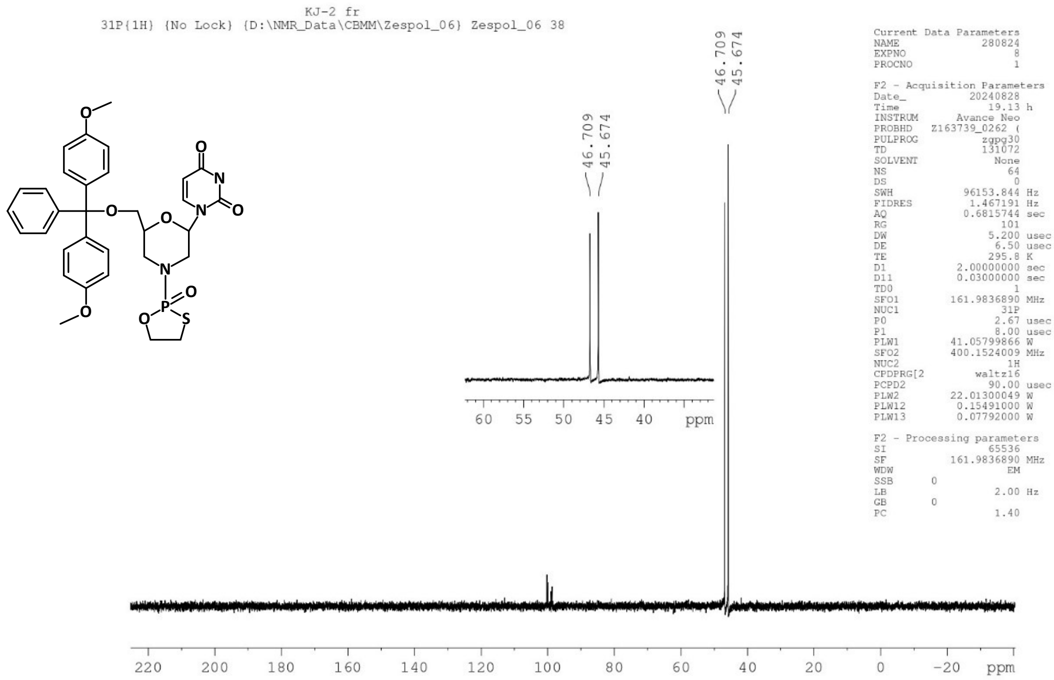


Figure S7. ^{31}P NMR spectrum of the second fraction (compound **2.2**) obtained after purification of the crude reaction mixture from the attempted synthesis of morpholino mU-^{O}OTP; analysis was performed in a non-deuterated solvent.

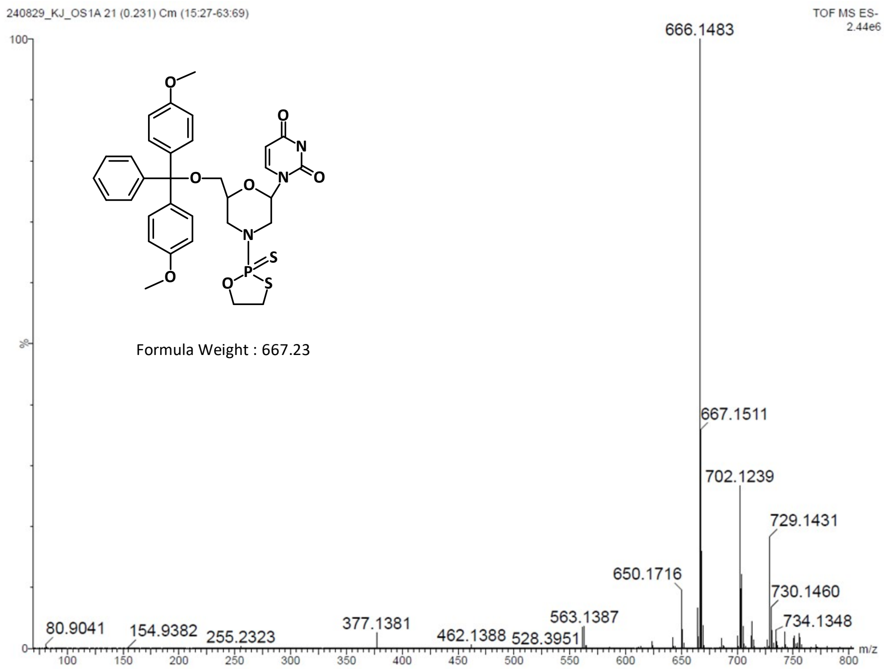


Figure S8. HR MS spectra for the first fraction obtained after purification of the crude reaction mixture from the attempted synthesis of morpholino mU-^{O}OTP

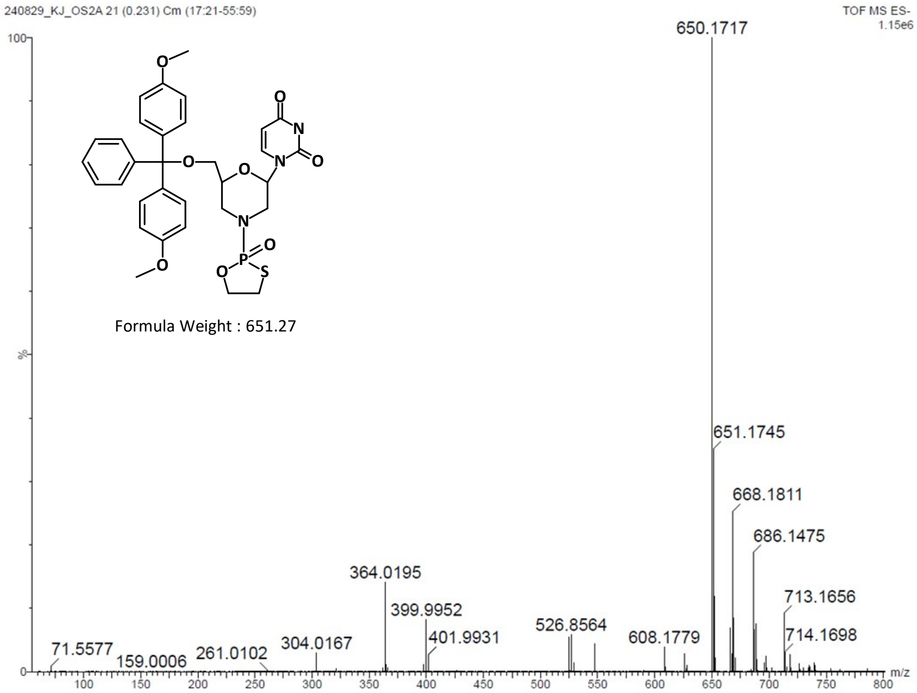


Figure S9. HR MS spectra for the second fraction obtained after purification of the crude reaction mixture from the attempted synthesis of morpholino mU-^{O}OTP

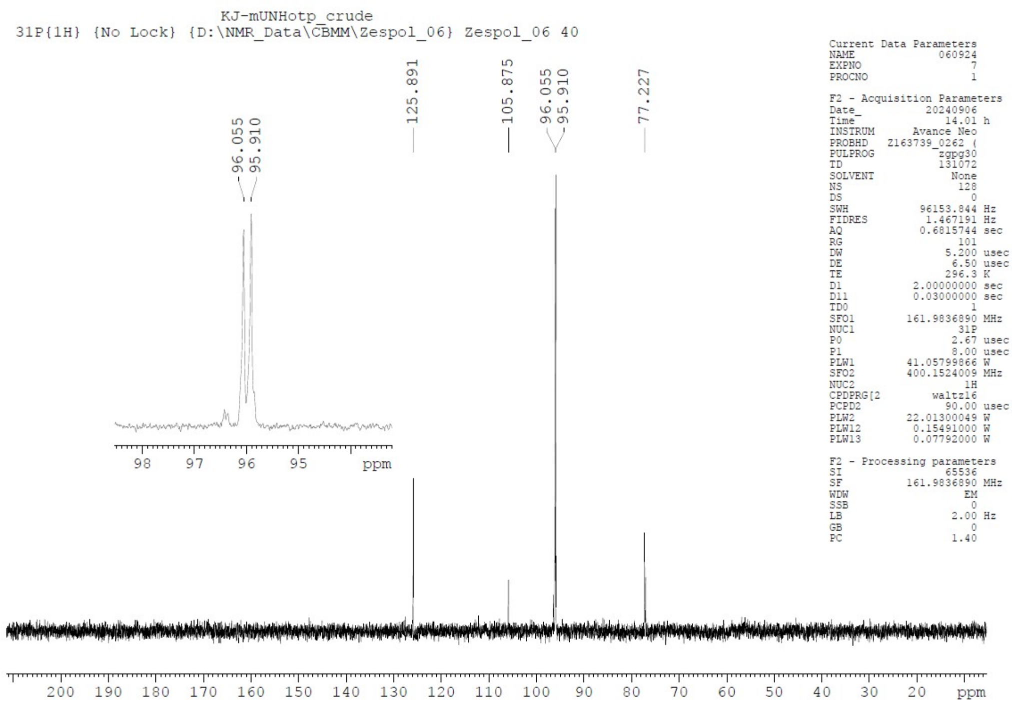


Figure S10. ^{31}P NMR spectrum of crude reaction mixture of *m*U-^{N}OTP formation (**4a^{MIX}**); analysis performed in non-deuterium solvent.

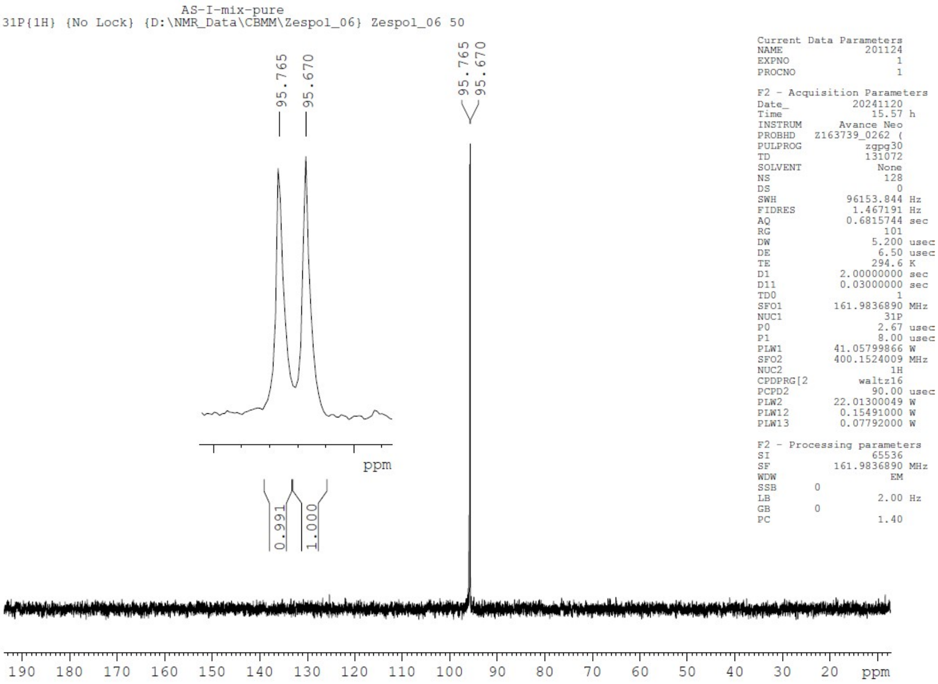


Figure S11. ^{31}P NMR spectrum of purified *m*U-^{N}OTP **4a^{MIX}**; analysis performed in non-deuterium solvent.

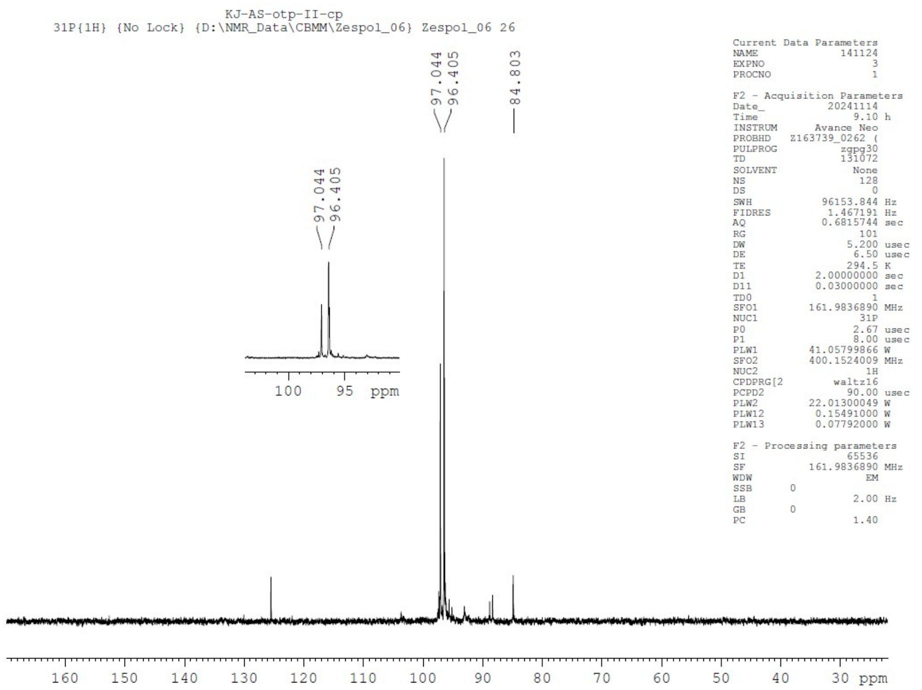


Figure S12. ^{31}P NMR spectrum of crude reaction mixture of *m*U-^{N}OTP formation (**4b^{MIX}**); analysis performed in non-deuterium solvent.

Figures S13-14. HR MS spectra for *mU-^{N}*OTPs (**4a^{MIX}** and **4b^{MIX}**)
HR MS spectra recorded with a SYNAPT G2-Si High Definition Mass Spectrometer (qTOF, Electro Spray Ionization; Waters)

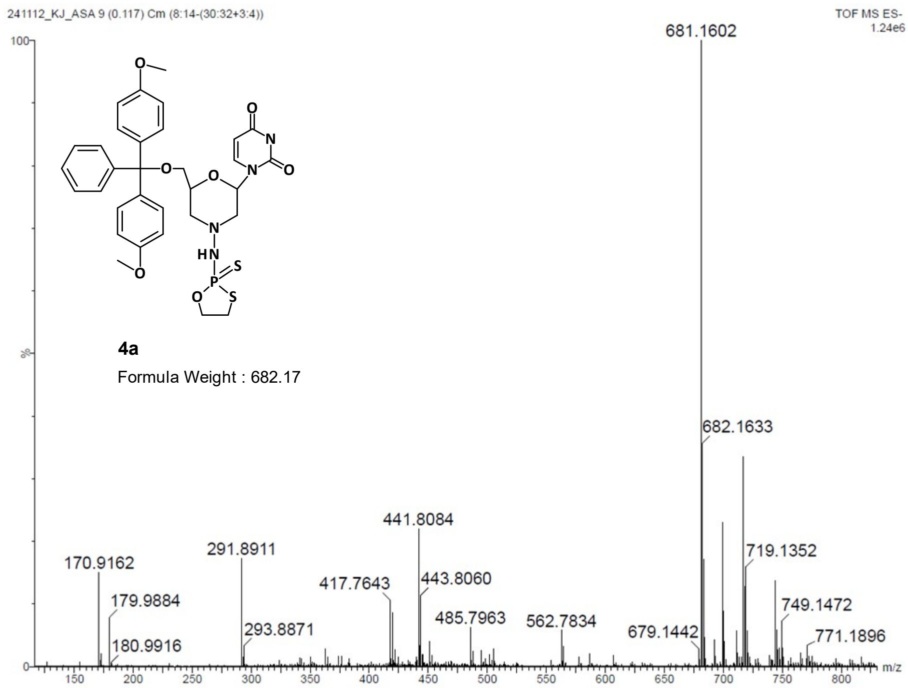


Figure S13. HR MS spectra for *mU-^{N}*OTP (**4a^{MIX}**)

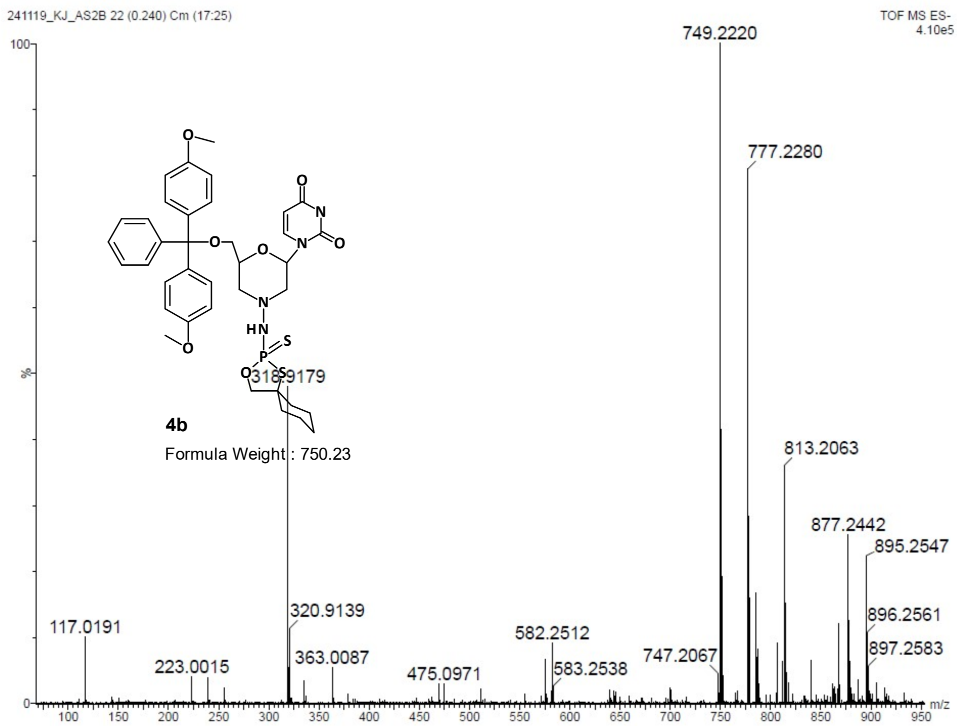


Figure S14. HR MS spectra for *mU-^{N}*OTP (**4b^{MIX}**)

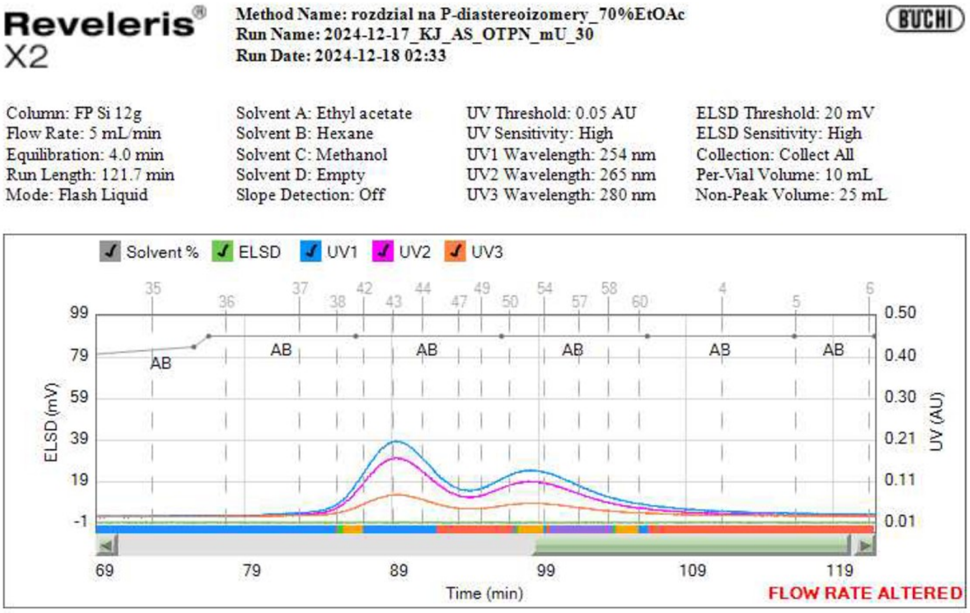


Figure S15. Semi-preparative HPLC profile for the separation of P-diastereomers of **4a**^{MIX}. Conditions: Column: cartridge packed with silica gel (FP ECOFLEX Si 12g) of Reveleris Flash Chromatography system; flow rate: 5 mL min^{-1}; isocratic elution with a hexane:ethyl acetate mixture (70:30); approximately 400 mg of **4a**^{MIX} was loaded onto the column.

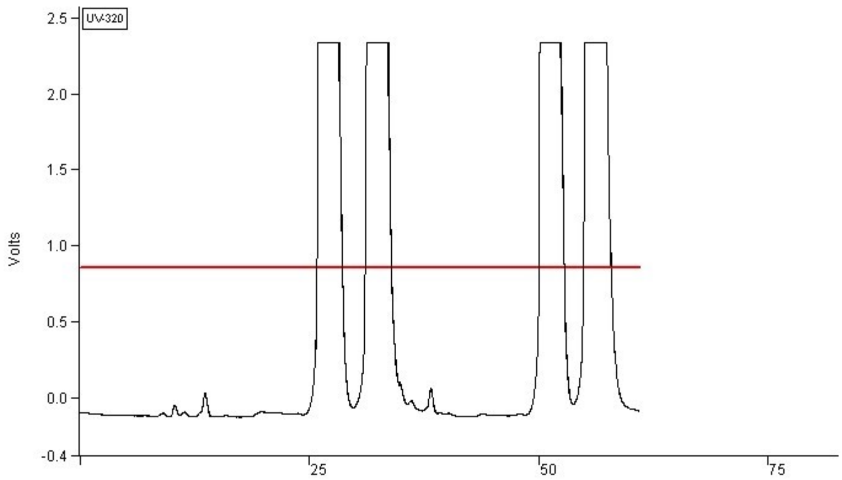


Figure S16. Semi-preparative HPLC profile for the separation of P-diastereomers of **4b**^{MIX}. Conditions: Pursuit XRs 10 µm silica gel HPLC column (250 × 21.2 mm); flow rate: 5 mL min^{-1}; isocratic elution with a hexane:ethyl acetate mixture (60:40); approximately 80 mg of **4b**^{MIX} was loaded onto the column (two injections).

Figures S17-28. NMR spectra for separated P-diastereomers of 4a and 4b
^{1}H NMR, ^{31}P NMR and ^{13}C NMR spectra recorded with a Bruker AV-400 spectrometer (400 MHz)

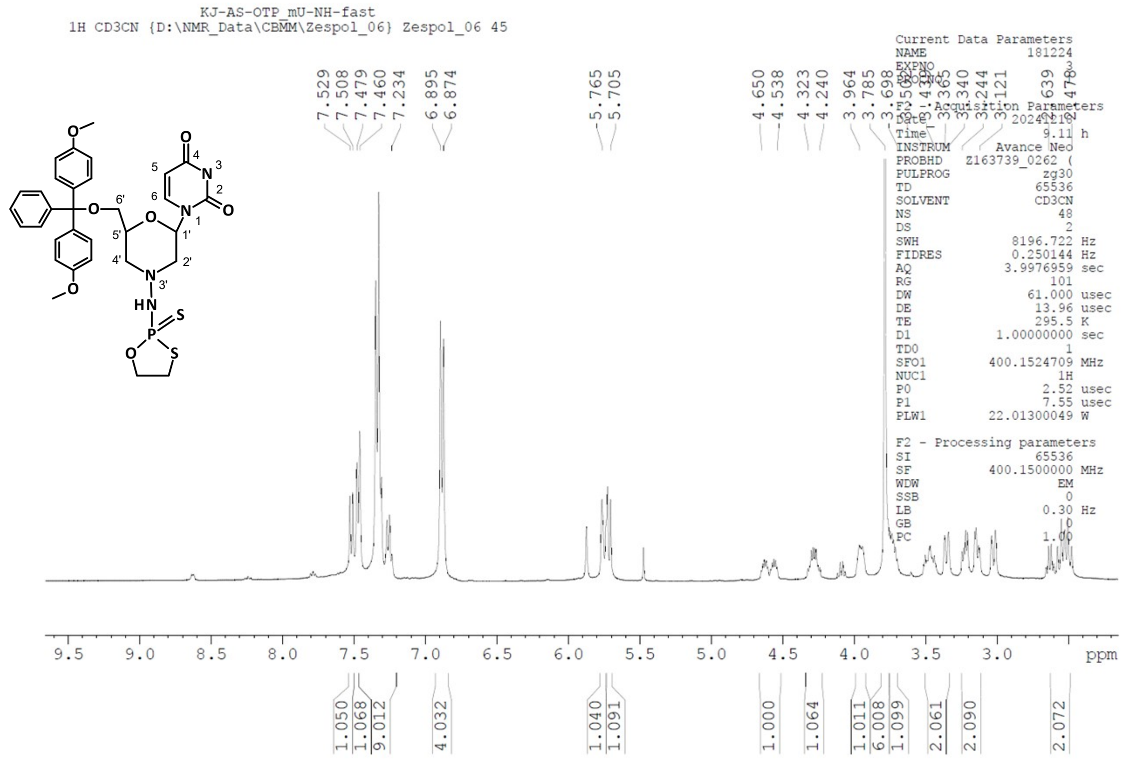


Figure S17. ^{1}H NMR *for fast*-eluting ***4a^{Fast}***; δ (ppm, CD_3CN) 7,53-7.51 (1H, C6-H), 7,48-7,46 (1H, C5-H), 7,23-6,87 (13H, DMT), 5,76 (1H, C1’-H), 5.71 (1H, C5’-H), 4,65-4.24 (2H, C2’-H), 3,96-3.70 (2H, C4’-H), 3,79 (6H, 2xOCH_3), 3.50-3.44 (2H, 6’CH_2), 3,24-2,48 (4H, P-O-CH_2CH_2-S)

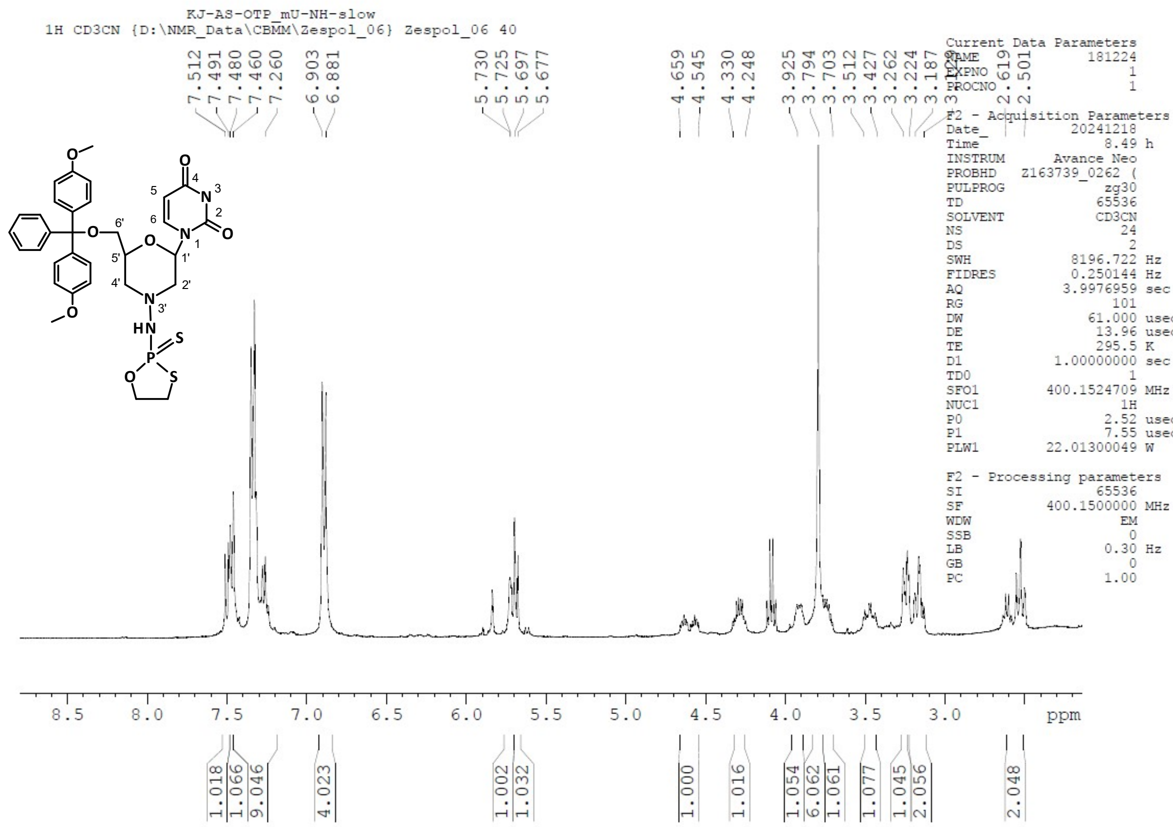


Figure S18. [1]H NMR *for slow*-eluting ***4a**^{Slow}*; δ (ppm, CD_3CN) 7,51-7.49 (1H, C6-H), 7,48-7,46 (1H, C5-H), 7,26-6,88 (13H, DMT), 5,73-5,72 (1H, C1'-H), 5.70-5,68 (1H, C5'-H), 4,66-4.25 (2H, C2'-H), 3,93-3.70 (2H, C4'-H), 3,79 (6H, $2xOCH_3$), 3.51-3.26 (2H, 6'CH_2), 3,22-2,50 (4H, P-O-CH_2CH_2-S)

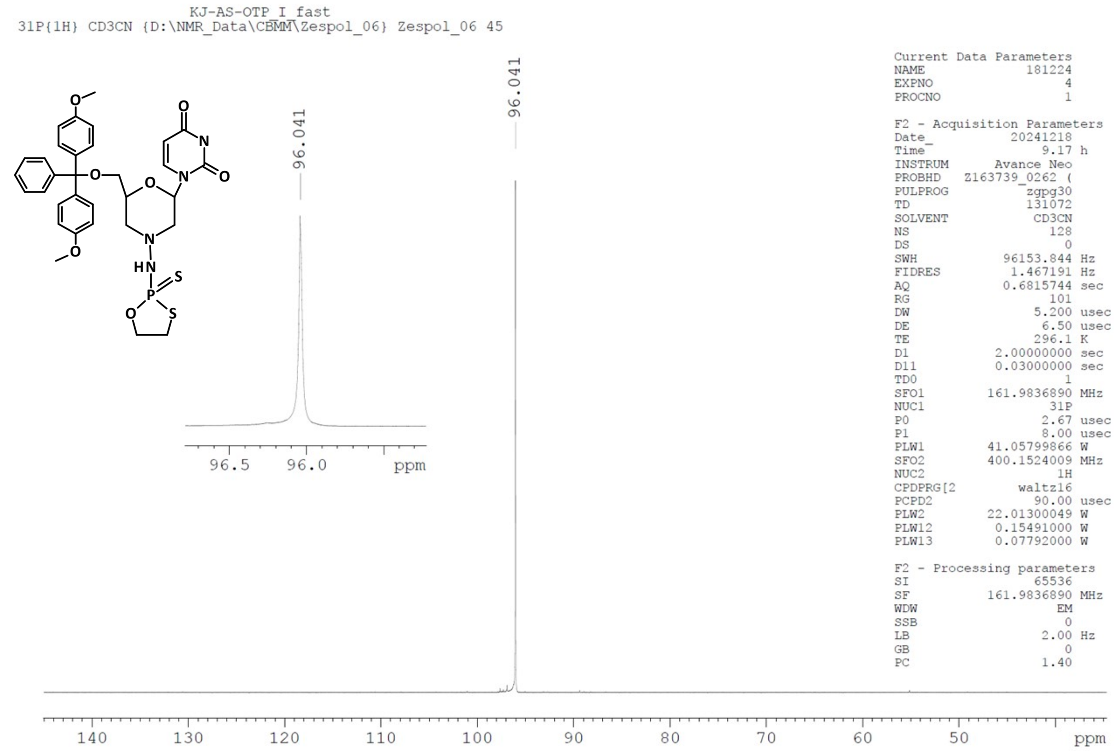


Figure S19. ^{31}P NMR *for fast*-eluting ***4a**$^{\boldsymbol{Fast}}$*; δ (ppm, CD_3CN)

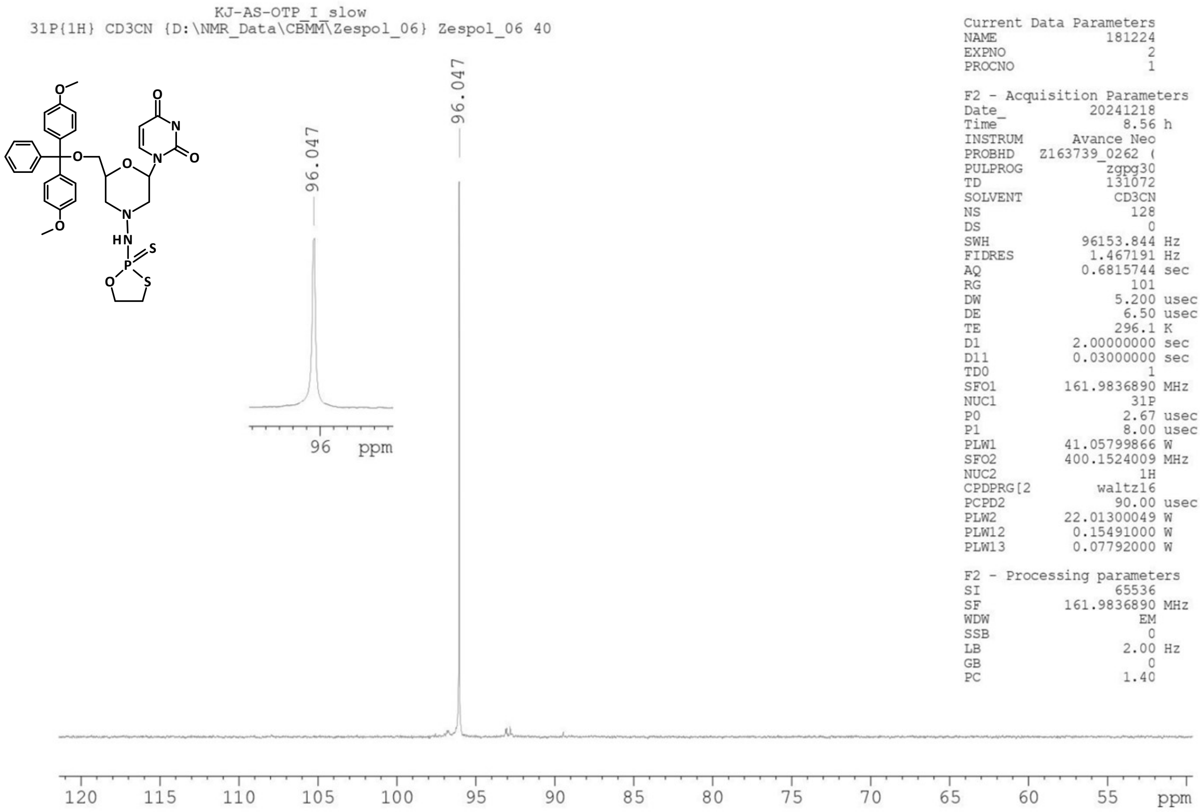


Figure S20. ^{31}P NMR *for slow*-eluting ***4a***$^{\textbf{\textit{Slow}}}$; δ (ppm, CD_3CN)

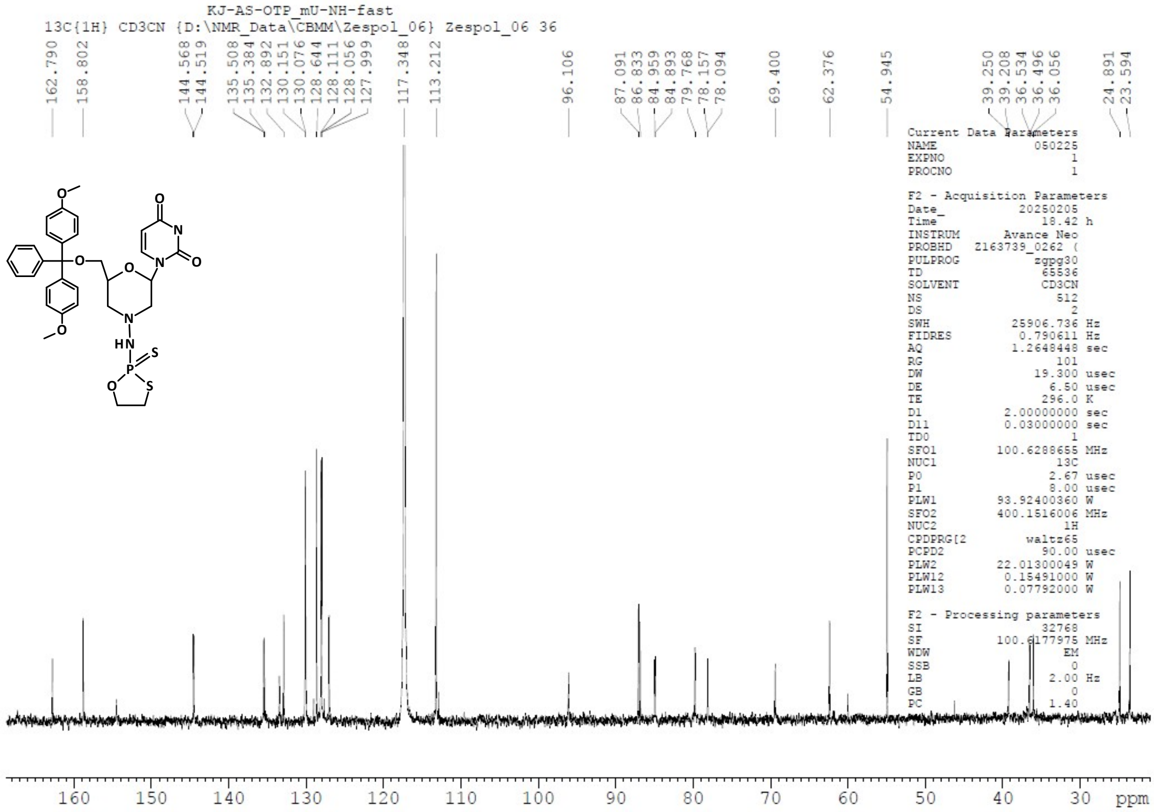


Figure S21. ^{13}C NMR *for fast*-eluting ***4a^{Fast}***; δ (ppm, CD_3CN)

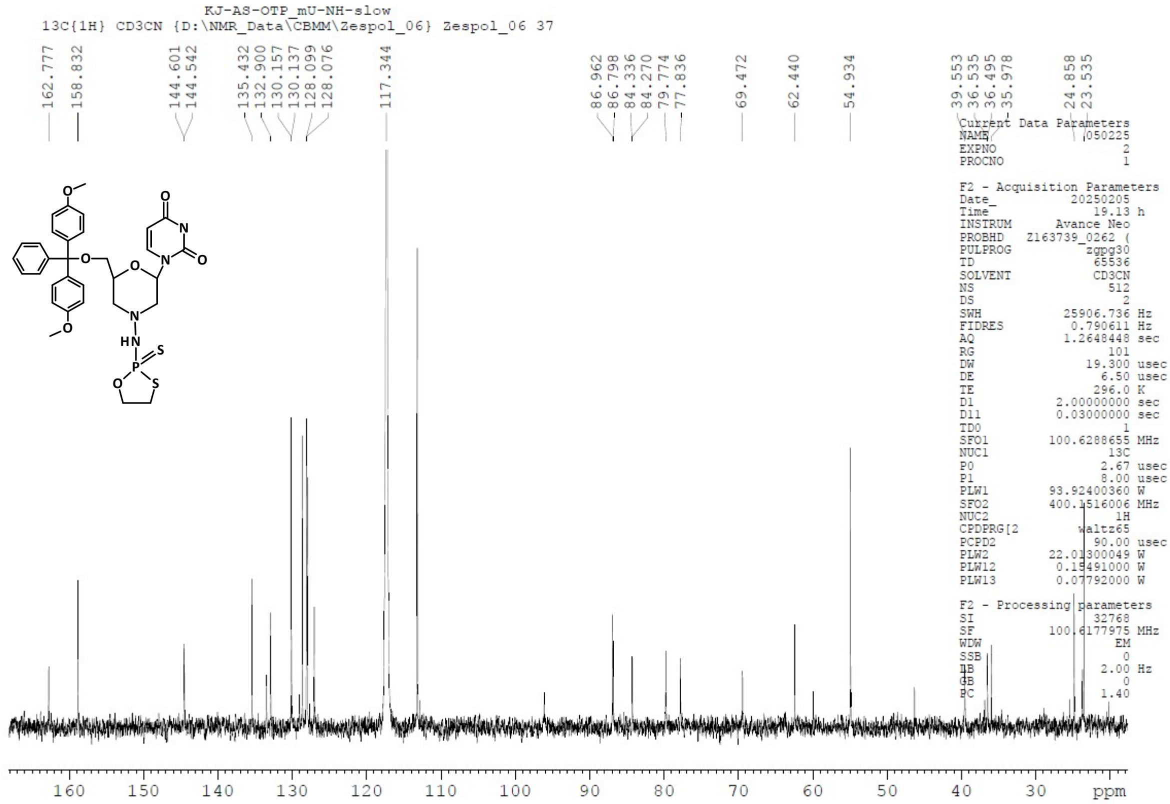


Figure S22. ^{13}C NMR *for slow*-eluting ***4a^{Slow}***; δ (ppm, CD_3CN)

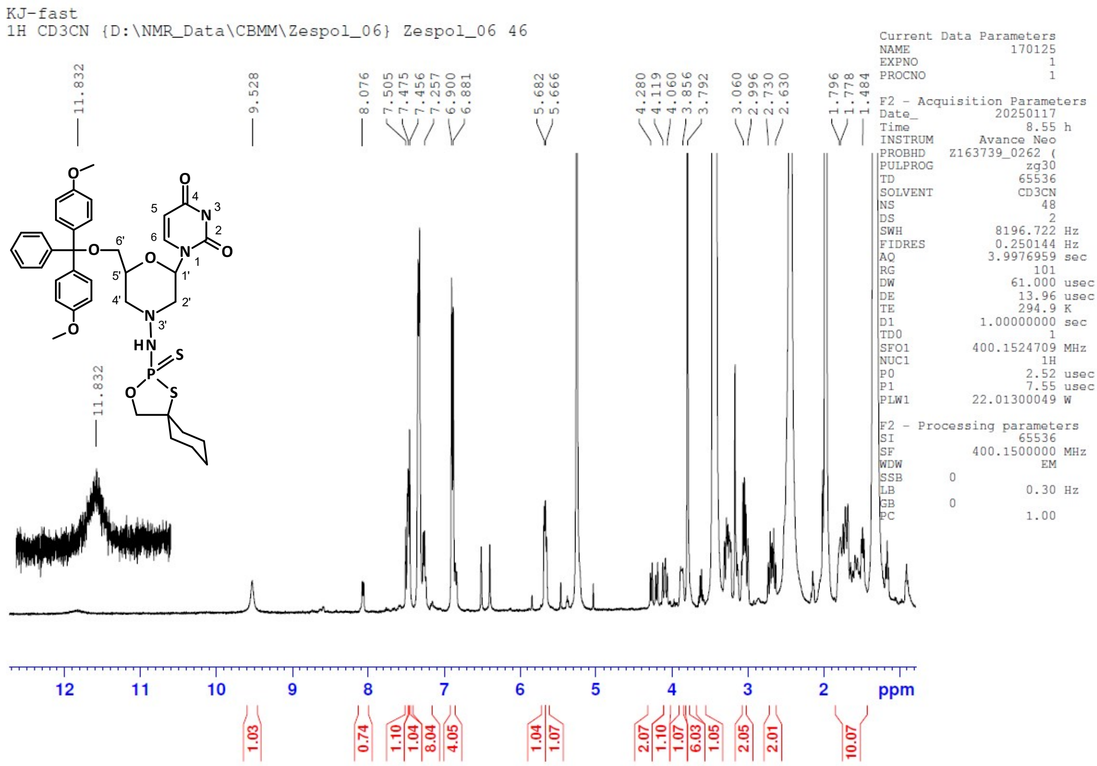


Figure S23. ^{1}H NMR *for fast*-eluting ***4b^{Fast}***; δ (ppm, CD_3CN) 11.83 (1H, P-NH-N), 9.53 (1H, N3-H), 8.08 (1H, C6-H), 7,50-7,47 (1H, C5-H), 7,46-6,88 (13H, DMT), 5,68 (1H, C1'-H), 5.67 (1H, C5'-H), 4,28-4.12 (2H, C2'-H), 4.06-3.86 (2H, C4'-H), 3,79 (6H, $2xOCH_3$), 3.06-3.00 (2H, 6'CH_2), 2.73-2.63 (2H, P-O-CH_2C-S), 1.80-1.48 (10H, -$(CH_2)_5$-„*spiro*")

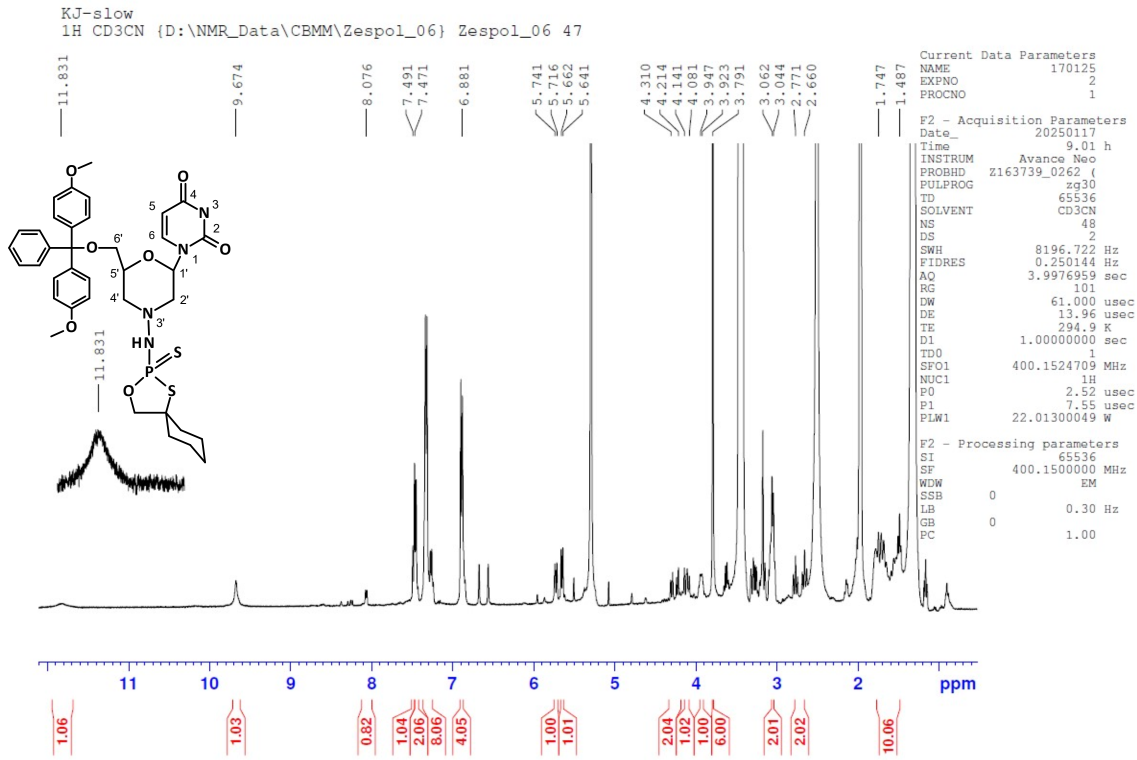


Figure S24. ^{1}H NMR *for slow*-eluting ***4b^{Slow}***; δ (ppm, CD_3CN) 11.83 (1H, P-NH-N), 9.67 (1H, N3-H), 8.08 (1H, C6-H), 7.49 (1H, C5-H), 7.47-6.88 (13H, DMT), 5.74-5.72 (1H, C1'-H), 5.66-5.64 (1H, C5'-H), 4.31-4.21 (2H, C2'-H), 4.14-3.92 (2H, C4'-H), 3,79 (6H, 2xOCH_3), 3.06-3.04 (2H, 6'CH_2), 2.77-2.66 (2H, P-O-CH_2C-S), 1.74-1.49 (10H, -$(CH_2)_5$-*„spiro"*)

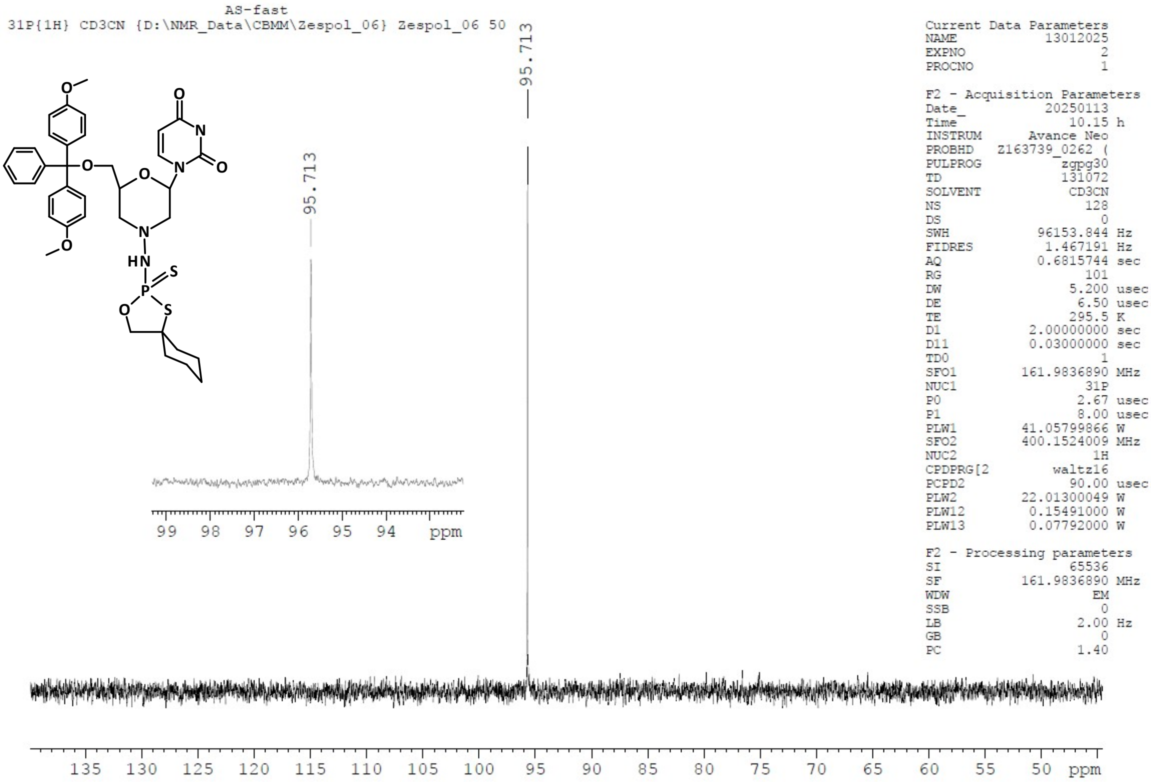


Figure S25. ^{31}P NMR *for fast*-eluting ***4b^{Fast}***; δ (ppm, CD_3CN)

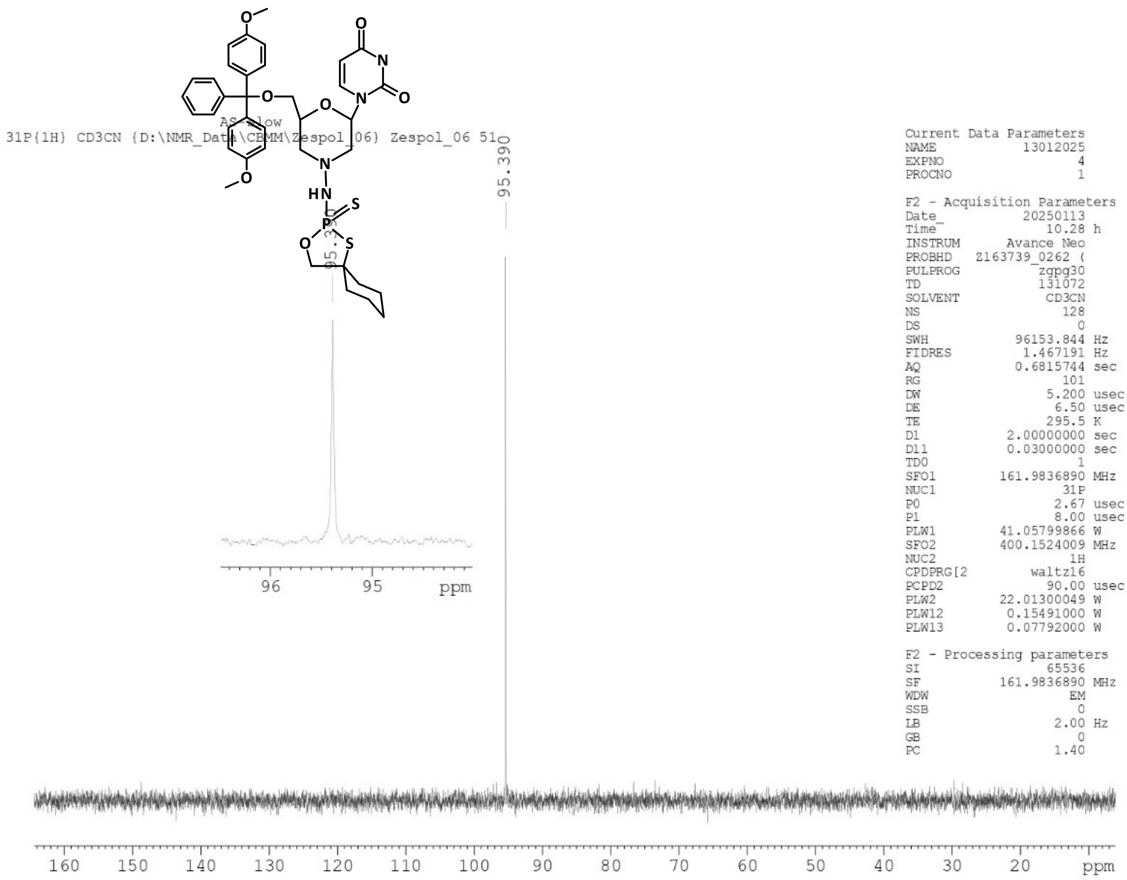


Figure S26. ^{31}P NMR *for slow*-eluting ***4b^{Slow}***; δ (ppm, CD_3CN)

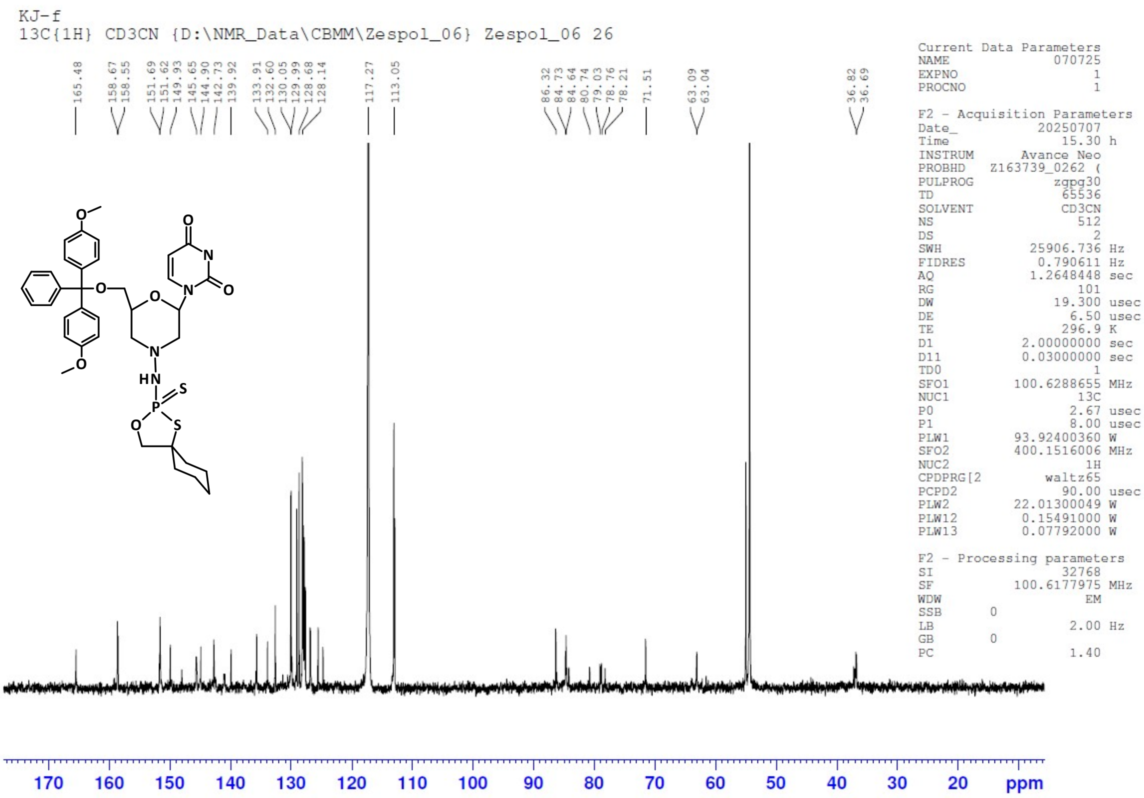


Figure S27. ^{13}C NMR *for fast*-eluting ***4b^{Fast}***; δ (ppm, CD_3CN)

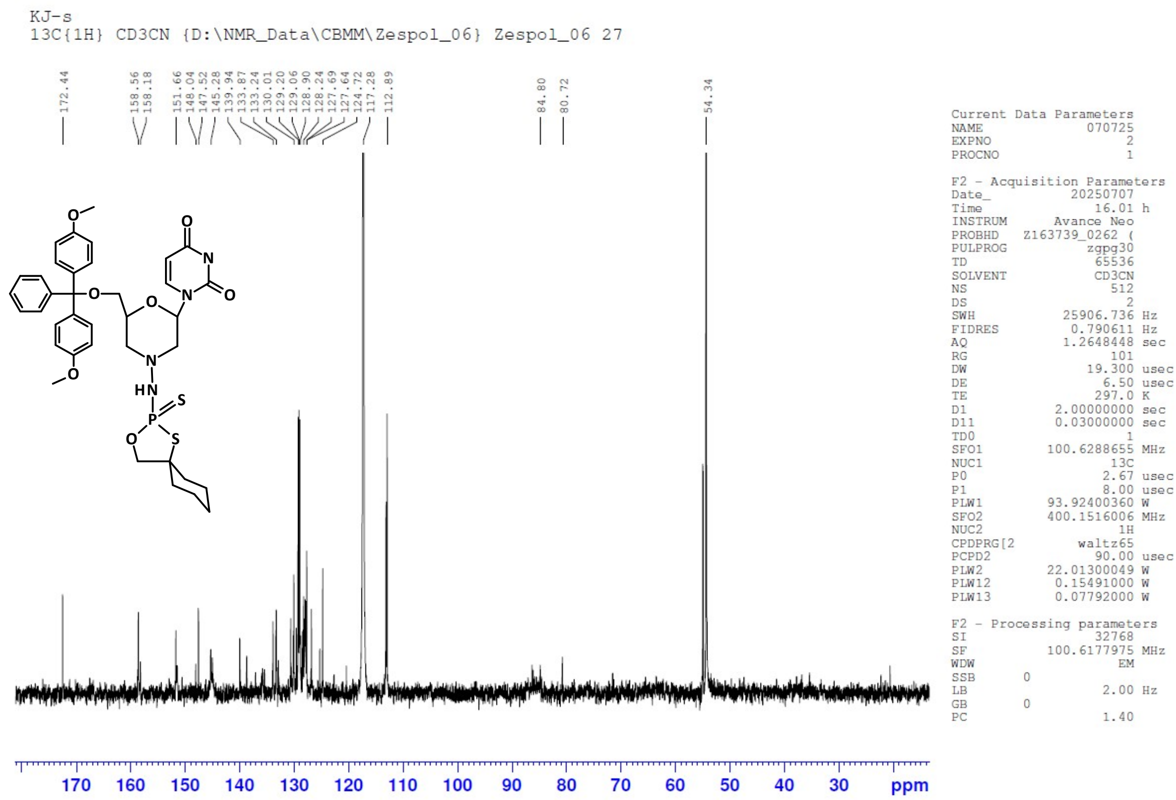


Figure S28. ^{13}C NMR *for slow*-eluting ***4b^{Slow}***; δ (ppm, CD_3CN)

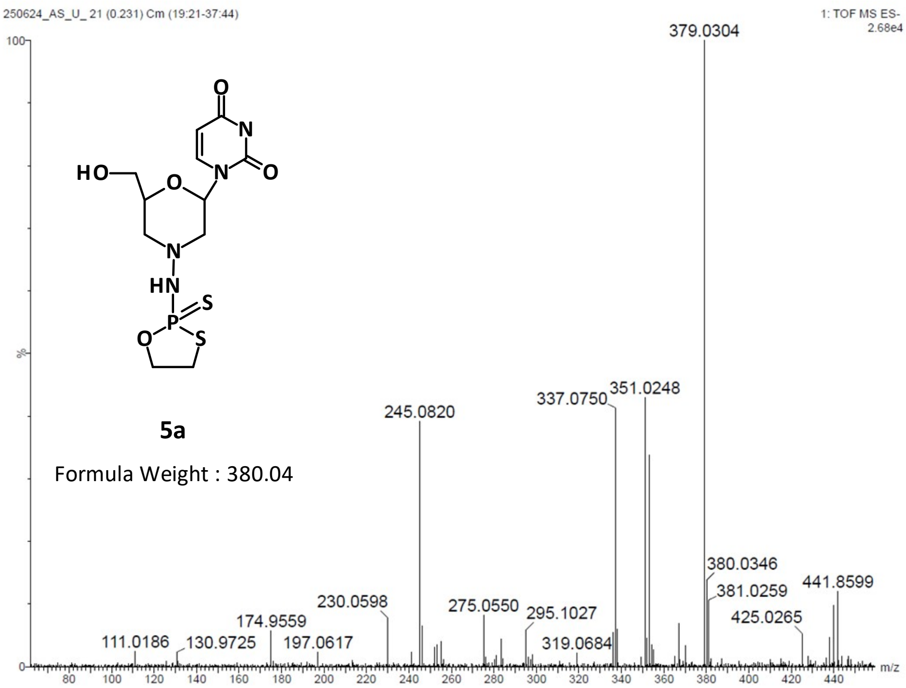


Figure S29. HR MS spectra for *fast*-eluting ***4a***$^{\mathit{Fast}}$ after detritylation (**5a**$^{\mathit{Fast}}$).

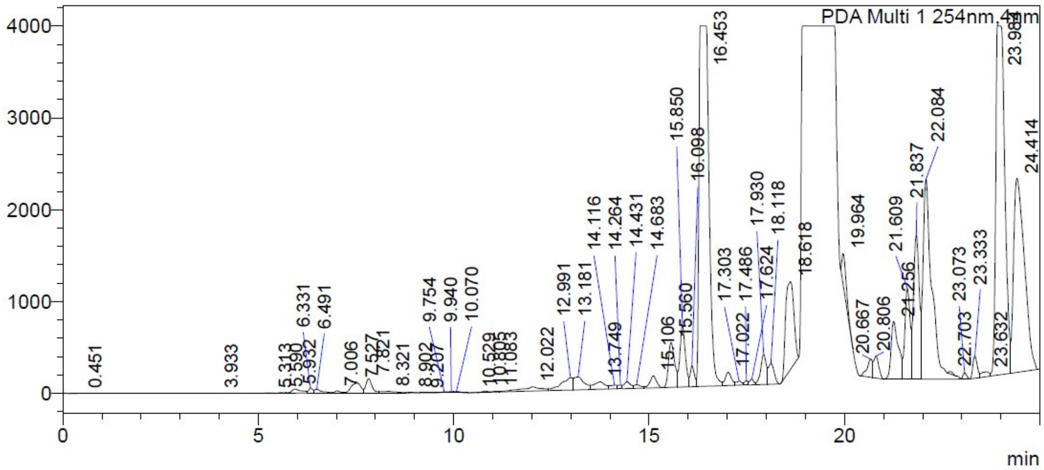


Figure S30. Semi-preparative HPLC profile from the purification of the P-stereodefined dinucleotide ***7***^{S}, obtained from the *slow*-eluting P-diastereomer ***4a***^{Slow}. Conditions: Varian Dynamax Pursuit XRs C18 column (250 × 21.2 mm, 10 µm); flow rate: 3 mL min^{-1}; elution with 0.1 M TEAB (pH 7.3) and 40% CH_3CN in 0.1 M TEAB. Gradient: 0–50% Buffer B over 30 min.

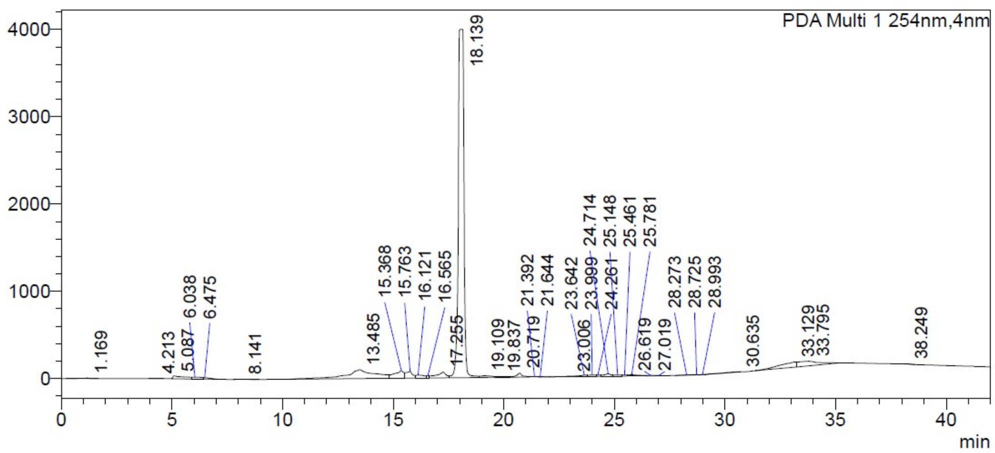


Figure S31. Analytical HPLC profile of the P-stereodefined dinucleotide **7**^{S}, obtained from the *slow*-eluting P-diastereomer ***4a***^{Slow}. Conditions: Alltima HP C18 column (250 × 4.6 mm, 5µ); flow rate: 0.5 mL min^{-1}; elution with 0.1 M TEAB (pH 7.3) and 40% CH_3CN in 0.1 M TEAB. Gradient: 0–50% Buffer B over 40 min.

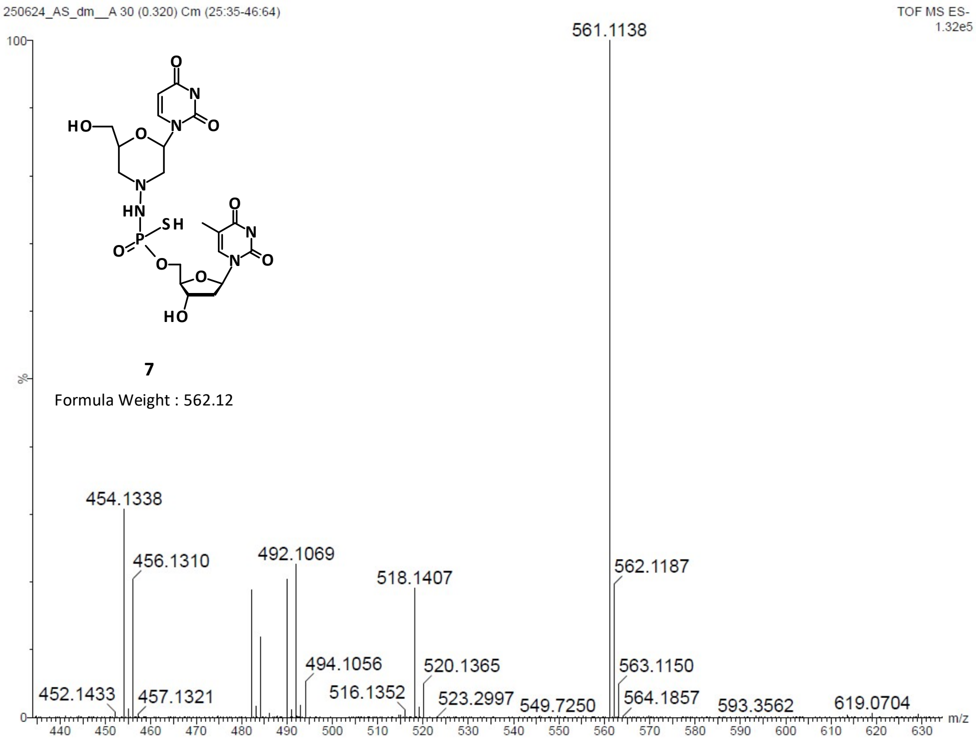


Figure S32. HR MS spectra for dinucleotide **7**^{S} obtained from *slow*-eluting P-diastereomer ***4a***^{Slow}

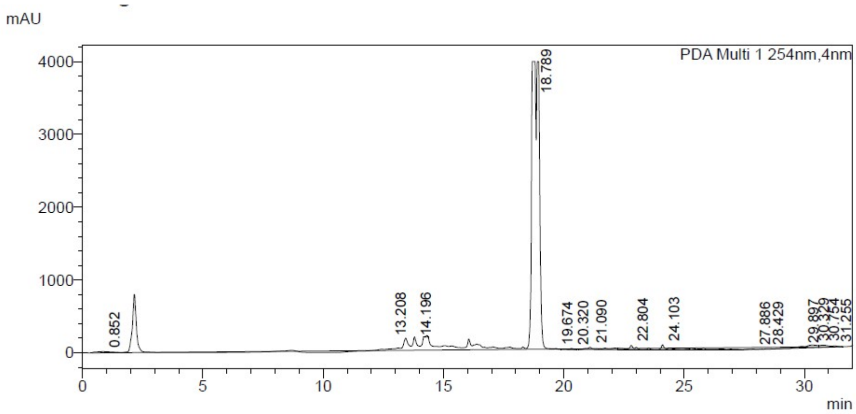
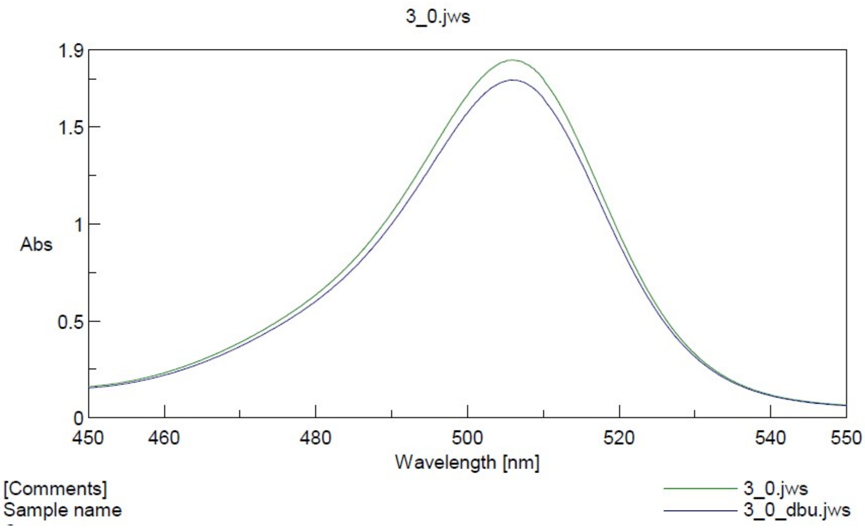


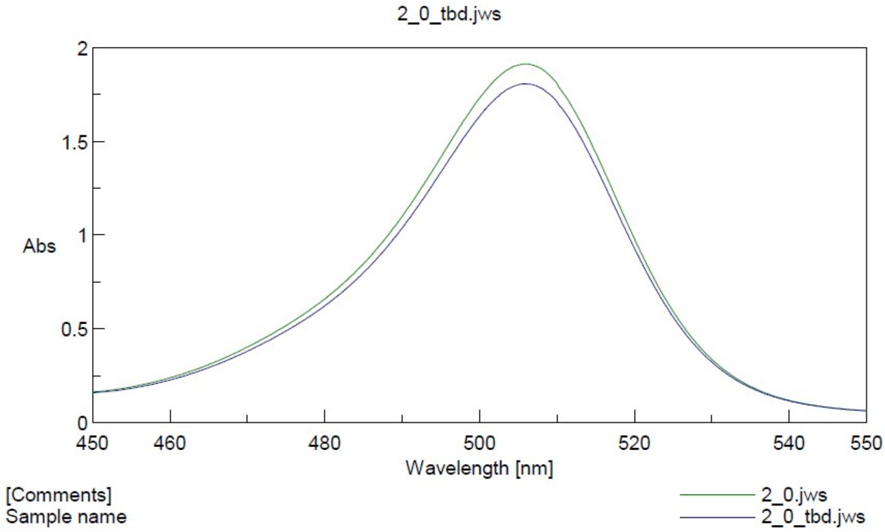
Figure S33. Analytical HPLC profile of dinucleotide **7**^{M}, obtained from the *mix* P-diastereomers ***4a***^{Mix}. Conditions: Alltima HP C18 column (250 × 4.6 mm, 5µ); flow rate: 0.5 mL min^{-1}; elution with 0.1 M TEAB (pH 7.3) and 40% CH_3CN in 0.1 M TEAB. Gradient: 0–50% Buffer B over 40 min.



No.	File Name	Sample Name	Comment	Date	Peak 1
1	3_0.jws			2025/02/14 16:2	505.8
2	3_0_dbu.jws			2025/02/14 16:2	505.6

No.	Peak Vakue 1
1	1.84515
2	1.74288

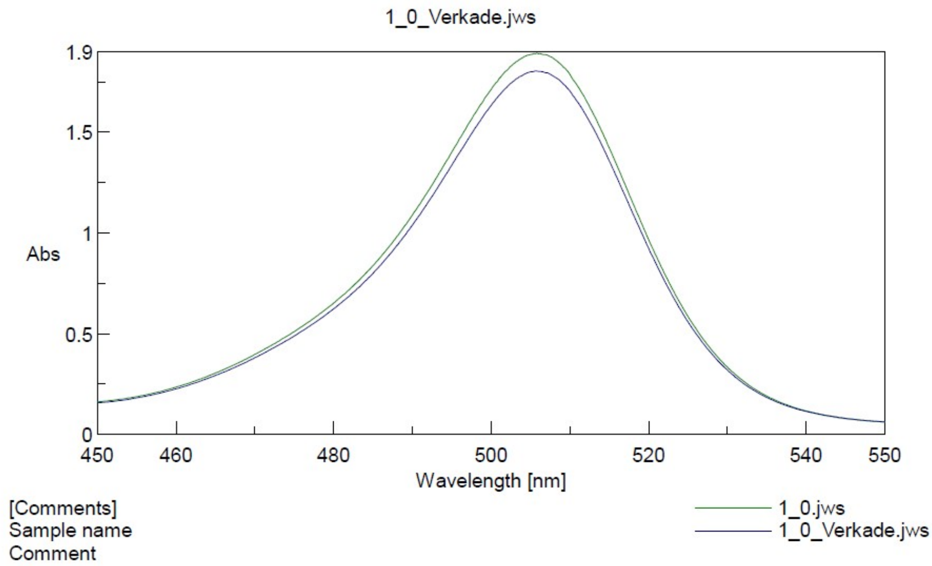
Figure S34. Overlaid VIS spectra for measurement of the DMT$^+$ cation absorption (λmax=504 nm) after consecutive detritylation steps during the coupling of **4a***^{MIX}* in the presence of 1M DBU (‘solid phase’ at 1 µmole scale)



No.	File Name	Sample Name	Comment	Date	Peak 1
1	2_0.jws			2025/02/14 16:1	505.6
2	2_0_tbd.jws			2025/02/14 16:1	506

No.	Peak Vakue 1
1	1.91207
2	1.8065

Figure S35. Overlaid VIS spectra for measurement of the DMT$^+$ cation absorption (λmax=504 nm) after consecutive detritylation steps during the coupling of **4a**^{MIX} in the presence of 3M TBD ('solid phase' at 1 µmole scale)



No.	File Name	Sample Name	Comment	Date	Peak 1
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2	1_0_Verkade.jw			2025/02/14 16:0	505.6

No.	Peak Vakue 1
1	1.89123
2	1.80263

Figure S36. Overlaid VIS spectra for measurement of the DMT⁺ cation absorption (λmax=504 nm) after consecutive detritylation steps during the coupling of **4a*MIX*** in the presence of 2M Verkade ('solid phase' at 1 µmole scale)

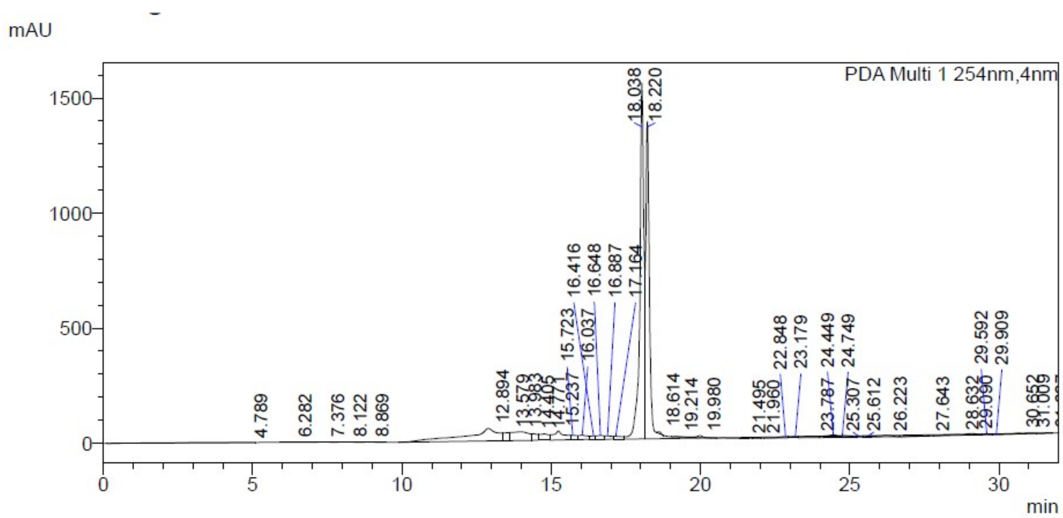

Figure S37. Analytical HPLC profile of dinucleotide **7*M*** (obtained from a mixture of P-diastereomers) after 24 hours of incubation in 10% fetal bovine serum (FBS) at 37 °C. Conditions: Alltima HP C18 column (250 × 4.6 mm, 5µ); flow rate: 0.5 mL min⁻¹; elution with 0.1 M TEAB (pH 7.3) and 40% CH_3CN in 0.1 M TEAB. Gradient: 0–50% Buffer B over 35 min.

Table S2. The data-collection, processing and refinement statistics for ***4a***^{Fast}.

Compound	***4a***^{Fast}
Crystal data	
CCDC	2470809
Chemical formula	$2(C_{11}H_{17}N_4O_5PS_2)$, $0.35(CH_3CN)$, $0.35(CHCl_3)$
Formula weight	436.52
Crystal system	monoclinic
Space group	$P2_1$
Temperature (K)	99.99(10)
a [Å]	17.0836(3)
b [Å]	7.2277(1)
c [Å]	17.8604(3)
β (°)	112.621(2)
V [Å^3]	2035.66(6)
Z	4
d_{calc} [g/cm^3]	1.424
Crystal dimensions [mm]	0.25 × 0.15 × 0.05
Radiation type	CuK_{α}
Wavelength (Å)	1.54184
μ [mm^{-1}]	4.665
Data collection	
Reflections measured	38701
Range/indices (h, k, l)	-21, 21; -8, 9; -22, 22
θ (max, min) [°]	76.114, 3.084
Total no. of unique data	8524
No. of observed data, I > 2σ(I)	7961
R_{int}	0.051
Refinement	
R [$F^2 > 2\sigma(F^2)$]	0.056
$wR(F^2)$	0.164
S	1.05
No. of reflections	8524
No. of parameters	485
No. of restraints	1
H-atom treatment	H-atoms treated by a mixture of independent and constrained refinement
$\Delta\rho$ (min, max), e/Å^3	-0.53, 1.62
Absolute structure parameter	-0.017(9)

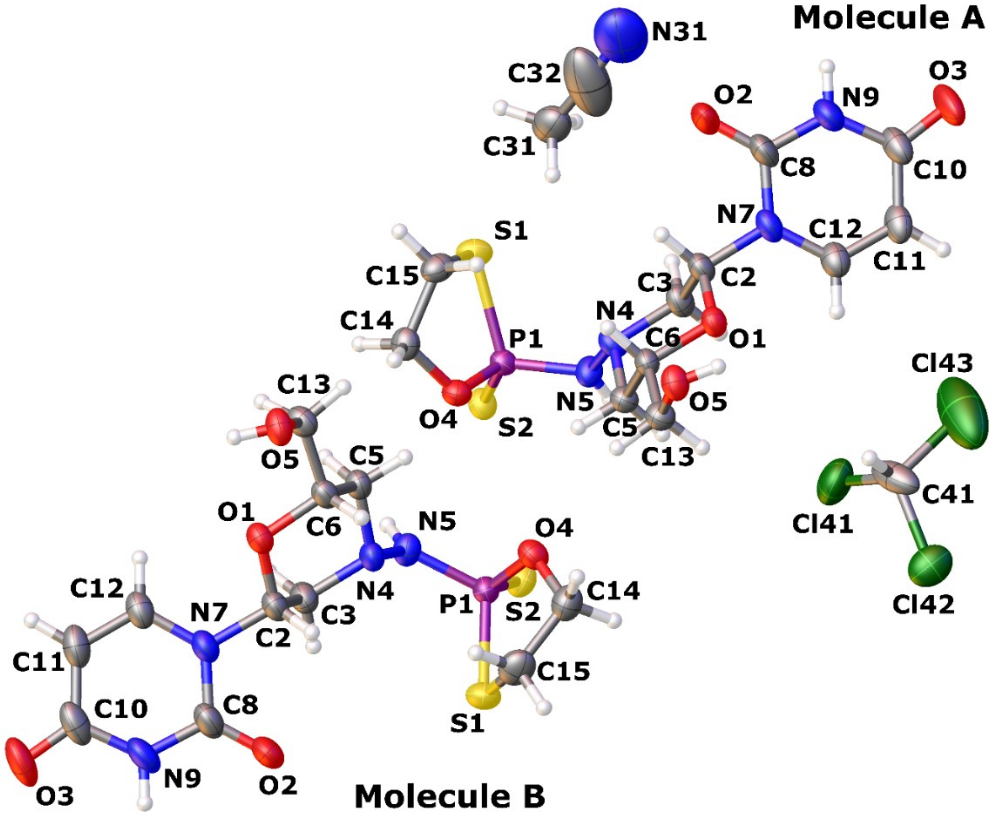

Figure S38. Unit-cell content of the ***4a**^{Fast}* crystal.

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1 Guga P., Stec W.J., In Current Protocols in Nucleic Acid Chemistry, ed. S. L. Beaucage, D. E. Bergstrom, G. D. Glick and R. A. Jones, John Wiley and Sons, Hoboken, N.J., 2003, p. 4.17.1.

2 G. M. Sheldrick, SHELXT - Integrated space-group and crystal-structure determination. *Acta Crystallogr A Found Adv.,* 2015, **71**, 3–8.

3 L. J. Bourhis, O. V. Dolomanov, R. J. Gildea, J. A. K. Howard, H. Puschmann, The anatomy of a comprehensive constrained, restrained refinement program for the modern computing environment - Olex2 dissected, *Acta Crystallogr A Found Adv.,* 2015, **71**, 59–75.

4 G. M. Sheldrick, Crystal structure refinement with *SHELXL*, *Acta Crystallogr C Struct Chem*., 2015, **71**, 3–8.

5 Jastrzębska K., An efficient alternative to DBU in the oxathiaphospholane (OTP) method for the solid phase synthesis of P-stereodefined phosphorothioate analogs, *RSC Adv.*, 2024, **14,** 21174 – 21179.