Pre-synthetic conjugation of DNA and RNA: Combining on-column conjugation and tandem oligonucleotide synthesis

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Note: all sequences are read as 5'--->3' (moving from left to right).

Synthesis and characterization of small molecules

Flash column chromatography was performed using Davisil silica gel 60 (35-70 micron) purchased from ThermoScientific (Fisher ChemicalTM) (Waltham MA). Automated flash chromatography was done using a Teledyne ISCO CombiFlash NextGen 100 (Lincoln NE) system. Thin layer chromatography (TLC) was carried out with precoated TLC plates (Merck, Kieselgel 60 F254, 0.25 mm) purchased from Sigma-Aldrich (Burlington, MA).

The following chemicals were purchased from Sigma-Aldrich (Burlington, MA): dichloromethane, dimethylsulfoxide-d_6, dimethylformamide, methylamine (40% solution in water), ammonium hydroxide solution (28.0-30.0%), acetone and Fmoc-Gly-OH. Hexanes was purchased from Caledon Laboratories Chemicals (Georgetown, ON). Ethyl Acetate was also purchased from ThermoScientific (Fisher Chemical™).

The following chemicals were purchased from ThermoScientific (Fisher Chemical™): triethylamine, 1,3-Diamino-2-propanol, anhydrous sodium sulfate, 4-bromo benzoic acid, N-methylimidazole, sodium chloride, sodium bicarbonate, ammonium chloride and triethylamine trihydrofluoride ca 37% HF.

The following chemicals were purchased from Combi-Blocks (CA, USA): 1,8-Diazabicyclo(5.4.0)undec-7-ene, Boc-L-valine hydroxysuccinimide ester, 2,5-Dioxopyrrolidin-1-yl 2-(prop-2-yn-1-yloxy) acetate, Lauric acid-OSu, *N*-(*tert*-Butoxycarbonyloxy)succinimide, N-(Benzyloxycarbonyloxy)succinimide, cystamine dihydrochloride, ((1R,8S,9S)-Bicyclo[6.1.0]non-4-yn-9-yl) methanol (HB-8604), 4-Methoxytrityl chloride, Amino-Peg7-alcohol. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC•HCl), N-acetoxysuccinimide, N-(Allyloxycarbonyloxy)succinimide were purchased from AK Scientific (Union City, CA). Acetonitrile was purchased from VWR Avantor (Radnor, PA). Amine-Peg4-Azide was purchased from Lumiprobe (MD, USA).

NMR spectra were recorded on a Bruker 300 MHz NMR spectrometer at room temperature. 1H NMR spectra were recorded at a frequency of 300.0 MHz and chemical shifts were reported in parts per million (ppm) downfield from tetramethylsilane. ^{13}C NMR spectra (1H decoupled) were recorded at a frequency of 75 MHz and chemical shifts were reported in ppm with tetramethylsilane as a reference. Data were reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, dd = doublet of doublets, t = triplet, m = multiplet), integration, and coupling constants.

High resolution mass spectrometry of small molecules was obtained using an LC-UHD Q-Tof (Aligent; Santa Clara, CA) at the University of Guelph's Advanced Analysis Centre's (AAC) Mass Spectrometry Facility. Low resolution mass spectrometry of oligonucleotides was also done using ThermoFisher Scientific LCQ Fleet Ion Trap with Ion Max-S Source Housing and Ion Max ESI Source. The mass spectrometer was operated in negative ion detection mode from 400-2000 m/z. The oligonucleotide samples were solubilized in 1:1 (v/v) acetonitrile/Milli Q water, before

direct injection into the instrument. The scans were recorded for 0.5 min and averaged to give the results. MALDI-TOF/TOF MS was also done Bruker Autoflex maX MALDI-TOF/TOF at the University of Guelph's Advanced Analysis Centre's (AAC) Mass Spectrometry Facility. We utilized the 3-HPA dried droplet protocol (ground steel targets) as per Bruker's Guide to MALDI Sample Preparation. The oligonucleotides samples were prepared at 10 µM (in Milli Q) for analysis.

Oligonucleotide synthesis, purification and characterization

DNA and RNA oligonucleotides (ONs) were synthesized on using an ABI-394 DNA synthesizer with DNA ONs using *N*-benzoyl-dA, *N*-isobutyryl-dG, *N*-acetyl-dC, and dT phosphoramidites purchased from ChemGenes (Wilmington, MA), while RNA ONs used 2'-*O*-TBDMS protected, *N*-benzoyl-rA, *N*-isobutyryl-rG, *N*-acetyl-rC and rU phosphoramidites (ChemGenes). 5'-Phosphate-ON was purchased from ChemGenes for the cTOS experiments as previously reported.[1] Chemical Phosphorylation Reagent (CPR) II CPG was purchased from Lumiprobe (MD, USA; cat. 46860). The 5′-amino-modifier phosphoramidite was synthesized as done in literature.[2] Post-synthetic amine conjugation of the free 3'-amino-modifier was performed after purification, according to the literature.[3]

Both DNA and RNA oligonucleotides (ONs) were assembled using standard reagents (ChemGenes; Wilmington, MA) and standard manufacturer protocols for the ABI 394 DNA synthesizer, with the exception that the 1st base was done using extended time (10 min) when extending off the secondary alcohol of diamino propanol for DNA. DMTr-removal reagent consisted of 3% trichloroacetic acid in dichloromethane, the activator consisted of 0.25 M 5-ethylthio tetrazole in acetonitrile, the oxidizer consisted of a 0.02 M solution of iodine in pyridine/water/tetrahydrofuran (8/16/76, v/v/v), and the capping reagents consisted of (Cap A) a solution of acetic anhydride/pyridine/tetrahydrofuran (10/10/80, v/v/v) and (Cap B) a 10% (v/v) solution of *N*-methylimidazole in tetrahydrofuran. All ONs were deprotected from the solid support using 1:2:2 (v/v) EtOH: $NH_4OH_{(aq)}$: $MeNH_{2(aq)}$ (500 µL to 1mL total volume) for 2 h at 65 °C and concentrated in a Savant SC 110A SpeedVac Plus under vacuum for just enough time to remove volatile (DNA only). It is important to note that amino modifiers are susceptible to adduct formation during the dry-down process; therefore, drying should be minimized and performed as mildly as possible. Gentle evaporation of the volatiles under a cooled airflow provided the best results. The RNA was concentrated completely to a dry pellet after adding 60 µL of 1 M Tris (pH 7.6) to mitigate adduct formation during the drying process.

Dimethylsulfoxide was purchased from Sigma-Aldrich. RNA oligomers were desilylated in DMSO/triethylamine trihydrofluoride (2:3, v/v, 100 µL / 150 µL) for 2 hours at 65 °C and then precipitated in cooled 1-butanol (ThermoScientific, Fisher Chemical™) overnight in the freezer. They were centrifuged to a pellet and the supernatant discarded. The pellet was further washed with 200 µL of 1-butanol. Tris base (molecular biology grade) was purchased from Fisher Bioreagents (ThermoScientific; Waltham, MA). Sep-Pak To C18 classic cartridge was purchased from Waters (Millford, MA). Anhydrous sodium acetate was purchased from Fisher Scientific.

Deprotected ONs were purified *via* Strong Anion-Exchange (SAX) Chromatography using a DNApac™ PA200 column with a Vanquish™ analytical purification high-performance liquid chromatography (HPLC) system with a standard gradient of 0-75% elution solvent (1 M NaCl in 10% MeCN, pH 7.61, 50 mM Tris buffer) and desalted using a Sep-Pak To C18 classic cartridge. The Sep-Pak C18 cartridge was conditioned with 10 mL of MeCN, 50% MeCN/Milli Q and 100 mM pH 7 NaOAc. The purified oligo was diluted to at least 2% MeCN (1:4 dilution with 100 mM NaOAc pH 7) and flowed through at least twice to load onto the column. The bound oligo was washed with water (~25 mL) and eluted from the column with 4 mL of 50% MeCN/Milli Q and concentrated.

The oligonucleotides UV measurements were taken at 260 nm using an Agilent BioTek Epoch Microplate Spectrophotometer. At least 3 readings were done of a given sample and averaged to a single value. The value was corrected by a blank measurement (average of at least 3 readings as well). Concentrations were calculated using the Beer-Lambert equation (molar extinction coefficients were estimated using the OligoAnalyzer™ Tool (Integrated DNA Technologies). Crude purity was determined by integrating UV absorbance signals from 3 to 20 minutes on the LC chromatogram, after the non-retained salts had eluted from the column.

On-solid support Functionalization Reactions

R-CPG Activation

A 1 µmol amount of controlled pore glass (CPG) was loaded into a 1 µmol standard twist ABI column. A saturated solution of DSC (30 mg) was prepared in a 50:50 (v/v) mixture of pyridine and acetonitrile (MeCN) (1 mL total volume) in a 2 mL Eppendorf tube. The mixture was vortexed vigorously for 15 seconds and passed through the column 4–5 times using disposable 1 mL syringes attached to each end of the column. After 2 hours, the reaction solution was removed, and the CPG was thoroughly washed with dry MeCN (10 mL) (see Discussion on coupling efficiency for further details).

Mono-Amine Incorporation

Conjugation reactions were carried out using 500 µL of a 50 mM amine solution prepared in either 50% (v/v) DMF/pyridine or MeCN/pyridine (25 equivalents). After a 10-minute reaction time, the solution was removed, and the solid support was sequentially washed with dry DMF, acetone, and MeCN (10 mL each). The column was then capped on the ABI synthesizer (if applicable) and extended as required. Cleavage and deprotection of the solid support were performed using AMA at 65 °C for 2 hours.

Bis-Amino Modifier Incorporation (i.e. Putrescine example)

The incorporation of diamino modifiers followed the general procedure described above, with the following modifications. The diamine was introduced using 500 µL of a 50 mM solution of the free diamine in 50:50 (v/v) DMSO/pyridine (25 equivalents) and reacted for 10 minutes. After a

total of 10 minutes, the reaction solution was removed, and the CPG was washed sequentially with DMSO, DMF, acetone, and MeCN (10 mL each).

Subsequently, the crude carbonate compound **1** was added as a 50 mM solution in DMF/pyridine (1 mL, 50 equivalents) and passed through the column 4–5 times. The mixture was allowed to react for 1 hour. Following the reaction, the solution was removed, and the CPG was washed with dry DMF, chloroform, acetone, hexanes, and MeCN (10 mL each). Finally, the solid support was capped on the ABI synthesizer and extended as required.

Compound **2** Conjugation

Conjugation was carried using a 50 mM stock of purified compound **2** in 50% (v/v) DMF/pyridine as described above. After conjugation, the solid support was then detritylated on the DNA synthesizer (x2) and then washed, sequentially, with H_2O, 50% v/v MeCN/H_2O, and MeCN (10 mL each).

On-column Amide Synthesis via NHS Esters (Aq. Conditions)

Conjugation was carried using 600 µL of a 100 mM stock of NHS ester in 100 mM HEPES pH 8 containing 50% DMF v/v (60 equivalents). The reagent was vortexed vigorously for 15 seconds and passed through the column 4–5 times using disposable 1 mL syringes attached to each end of the column. After 2 hours, the solid support was then washed, sequentially, with H_2O, 50% v/v MeCN/H_2O, and MeCN (10 mL each). The solid support was then washed on the synthesizer with MeCN for 30 s (3×), with a 3 min wait between washes. The extended wait time was necessary to remove residual water from the solid support.

On-column Amide Synthesis via NHS Esters (Org. Conditions)

Conjugation was carried using 600 µL of a 100 mM stock of NHS ester in 50% DMF: Py v/v (60 equivalents). The reagent was vortexed vigorously for 15 seconds and passed through the column 4–5 times using disposable 1 mL syringes attached to each end of the column. After 2 hours, the solid support was then washed, sequentially, with DMF, acetone, and MeCN (10 mL each). It was then washed on the synthesizer for 30s using MeCN (3x) with a 3-minute wait time in between washings.

On-column EDC-meditated Amide Synthesis[4]

The reaction was done as previously reported in literature, with the following modifications: 400 mM EDC•HCl was used instead of 800 mM.[4] First, 600 µL of a 100 mM stock solution of the desired carboxylate in 100 mM HEPES pH 8 containing 50% DMF v/v was prepared fresh in a 2 mL Eppendorf tube. To it, 4.78 µL of 1-Methylimidazole and 46 mg of EDC•HCl was added. The reaction solution was vortexed vigorously for 15 seconds and passed through the column 4–5 times using disposable 1 mL syringes attached to each end of the column. After 8 hours, the solid support was then washed, sequentially, with H_2O, 50% v/v MeCN/H_2O, and MeCN (10 mL each). Another aliquot was then added and left to react for 8 h. After 8 h, it was washed, sequentially,

with H_2O, 50% v/v MeCN/H_2O, and MeCN (10 mL each). Finally, the solid support was washed on the synthesizer with MeCN for 30 s (3×), with a 3 min wait between washes. The extended wait time was necessary to remove residual water from the solid support.

On-column Carbamate Synthesis (Aq. Conditions)

Conjugation was carried using 600 µL of a 100 mM stock of activated OSu carbonate (i.e., CBz-OSu, Alloc-OSu) in 100 mM HEPES pH 8 containing 50% DMF v/v (60 equivalents). The reagent was vortexed vigorously for 15 seconds and passed through the column 4–5 times using disposable 1 mL syringes attached to each end of the column. After 2 hours, the solid support was then washed, sequentially, with H_2O, 50% v/v MeCN/H_2O, and MeCN (10 mL each). It was then washed on the synthesizer for 30s using MeCN (3x) with a 3-minute wait time in between washings. We found that the wait time was necessary to remove excess water still on the CPG.

On-column Carbamate Synthesis (Org. Conditions)

Conjugation was carried using 600 µL of a 100 mM stock of commercial OSu (i.e. Alloc-OSu) in 50% DMF: Py v/v (60 equivalents). The reagent was vortexed vigorously for 15 seconds and passed through the column 4–5 times using disposable 1 mL syringes attached to each end of the column. After 2 hours, the solid support was then washed, sequentially, with H_2O, 50% v/v MeCN/H_2O, and MeCN (10 mL each). It was then washed on the synthesizer for 30s using MeCN (3x) with a 3-minute wait time in between washings. We found that the wait time was necessary to remove excess water still on the CPG.

cTOS Conditions (Amide NHS Ester Formation)

The solid support was pre-functionalized as previously described. The first strand was extended, followed by incorporation of the 3′-phosphate ON amidite (or X-amidite), and then extension of the second duplex strand containing an amino modifier. After synthesis, the solid support was detritylated twice to remove the MMTr protecting group, followed by sequential washing with H_2O, 50% (v/v) MeCN/H_2O, and MeCN (10 mL each). Conjugation of the NHS ester was performed under anhydrous organic conditions as described above for 2 h. After 2 hours, the solid support was then washed, sequentially, with DMF, acetone, and MeCN (10 mL each). Finally, the material was deprotected as described above using AMA at 65 °C for 2 hours.

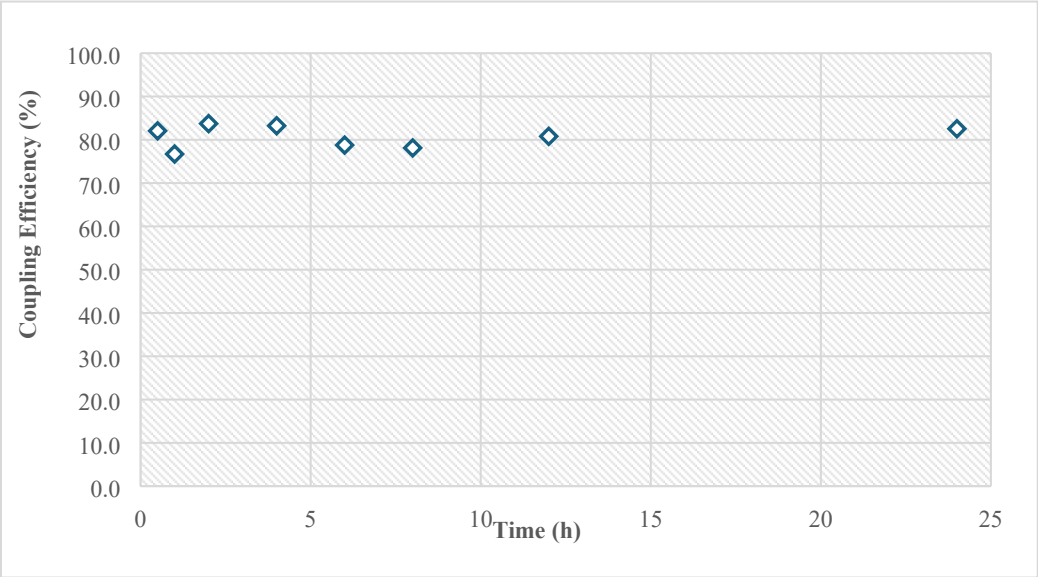
cTOS Conditions (Terminal Carbamate Formation)

The solid support was pre-functionalized as previously described. The first strand was extended, followed by incorporation of the 3′-phosphate ON amidite (or X-amidite), and then extension of the second duplex strand. After synthesis, the solid support was treated with DSC for 2 hours, washed with MeCN and subsequently coupled with the desired amine (50 mM, 600 µL), dissolved in 100 mM HEPES buffer (pH 8.0) containing 50% (v/v) DMF. The coupling reaction was allowed to proceed for 5 minutes, after which the solid support was sequentially washed with H_2O, 50% (v/v) MeCN/H_2O, and MeCN (10 mL each). Finally, the material was deprotected as described above using AMA at 65 °C for 2 hours.

cTOS Conditions (Post-Synthesis)

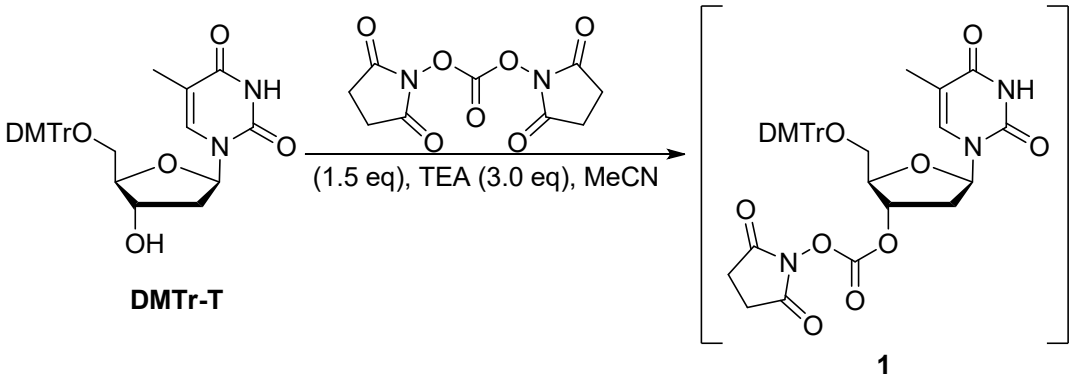
The solid support was pre-functionalized with **3** as previously described. The first strand was extended, followed by incorporation of the 3′-phosphate ON amidite (or X-amidite), and subsequent extension of the second duplex strand. After synthesis, the 5′-hydroxyl was first conjugated to the desired amine via DSC-mediated carbamate formation under aqueous conditions (to prevent sulfonyl cleavage), as described above. The solid support was then treated with BCN-OH (2 mg, 27 mM, DMSO, 500 µL) for 2 hours.[3] Following the reaction, the CPG was sequentially rinsed with DMSO, DMF, acetone, and MeCN (10 mL each).

Discussion on ***R***-CPG Coupling Efficiency



We have reported that the sulfonyl-containing linker has an optimal DSC solid-support activation of 2 – 6 h.[5] We observed yield lowering past the 6 h mark presumably due to immolation of the sulfonyl linker, which was expected as even mild deprotection conditions (50 mM K_2CO_3 in anhydrous methanol can immolate the linker after 8 h). Interestingly, the DSC activation of the CPR II CPG reached approximately 80 % efficiency after 30 min and remained consistent over the span of 24 h. Beginning for manufacturer estimated loading of x µmol / g, we estimated a loading of y µmol / g over two steps (DSC activation, followed by coupling with DMT-dT_{NH2}) via trityl monitoring. Satisfied with this loading, we conducted a head-to-head comparison of two sequences, one control sequence (T_{10}p) and the experiment (T_9-dT_{NH2}), both containing 10 thymidyl units. We were pleased to observe slightly lower isolated yields (89%, 8 h time point) for the T_9-dT_{NH2} relative to the control experiment (T_{10}p), validating that the introduction of amino-modifiers using our strategy did not suffer from significant yield loss. Consequently, we chose to perform the functionalization reactions following a 2-hour on-column DSC reaction.

Chemical Synthesis

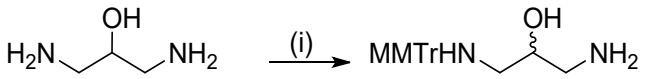


Supporting Scheme S1. Synthesis of carbonate **1**.

*(2R,3S,5R)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl (2,5-dioxopyrrolidin-1-yl) carbonate (**1**)*

Compound **1** was synthesized according to literature.[5]

1-amino-3-(((4-methoxyphenyl)diphenylmethyl)amino)propan-2-ol (**2**)



1,3-Diamino-2-propanol **2**

In a flame dried 100 mL RBF, with its atmosphere briefly exchanged with argon, 1,3-Diamino-2-propanol (193 mg, 2.142 mmol) was dissolved in dry MeCN (10.7mL, 0.2M). DBU (959 µL, 6.425 mmol, 3.0 equiv.) was added to the solution and allowed to stir for 10 minutes. After complete solubilization, MMTr-Cl (331 mg, 1.071 mmol, 0.5 equiv.) was added to it and the solution turned yellow. After 20 minutes, the solution became clear and colourless once again. At this point, MMTr-Cl (331 mg, 1.071 mmol, 0.5 equiv.) was added once more and allowed to stir for 30 minutes. After the solution turned colourless once more, it was concentrated under vacuo to a yellow-orange gum. It was immediately purified by flash column chromatography using a stepwise gradient of 5-10% MeOH in DCM (containing 1% v/v TEA) resulting in a desired product as a white solid (403mg, 52%).

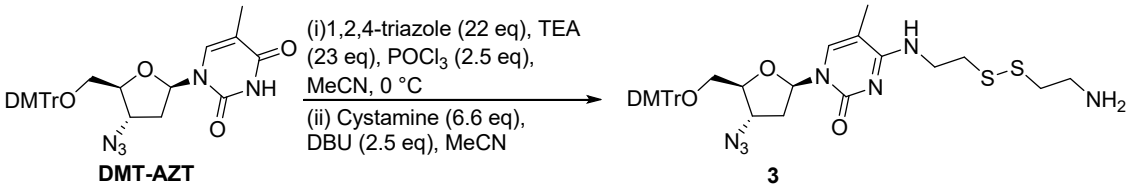
R_f: 0.26 (10% MeOH in DCM containing 1% v/v TEA).

^{1}H NMR (300 MHz, DMSO-d_6) δ 7.46-7.38 (m, 4H, Ar), 7.33-7.24 (m, 6H, Ar), 7.21-7.14 (m, 2H, Ar), 6.90-6.84 (m, 2H, Ar), 5.25 (bs, 4H, OH, NH, NH2), 3.81-3.69 (m, 4H, OCH & ArOCH3), 2.87 (dd,1H, NCH, *J* = 3 & 13 Hz), 2.63 (dd, 1H, NCH, *J* = 8 & 13 Hz), 2.15-2.02 (m, 2H, 2 x NCH). Residual triethylamine is observed as 2.46 & 0.95 ppm.

^{13}C NMR (75 MHz, DMSO-d6) δ 157.86, 146.83, 138.37, 130.10, 128.78, 128.16, 126.47, 113.51, 70.15, 69.75, 55.45, 48.08, 46.16, 44.91, 40.83, 40.56, 40.28, 40.00, 39.72, 39.45, 39.17, 12.00.Triethyl amine was present at 12.00 and 46.16.

LRMS (ESI-MS) *m*/*z* calculated for $C_{23}H_{27}N_2O_2^+$ 363.21: found $[M+H]^+$ 363.21.

HRMS (ESI-MS) *m*/*z* calculated for $[C_{23}H_{26}N_2O_2+Na]^+$ 385.1887: found $[M+Na]^+$ 385.1912.

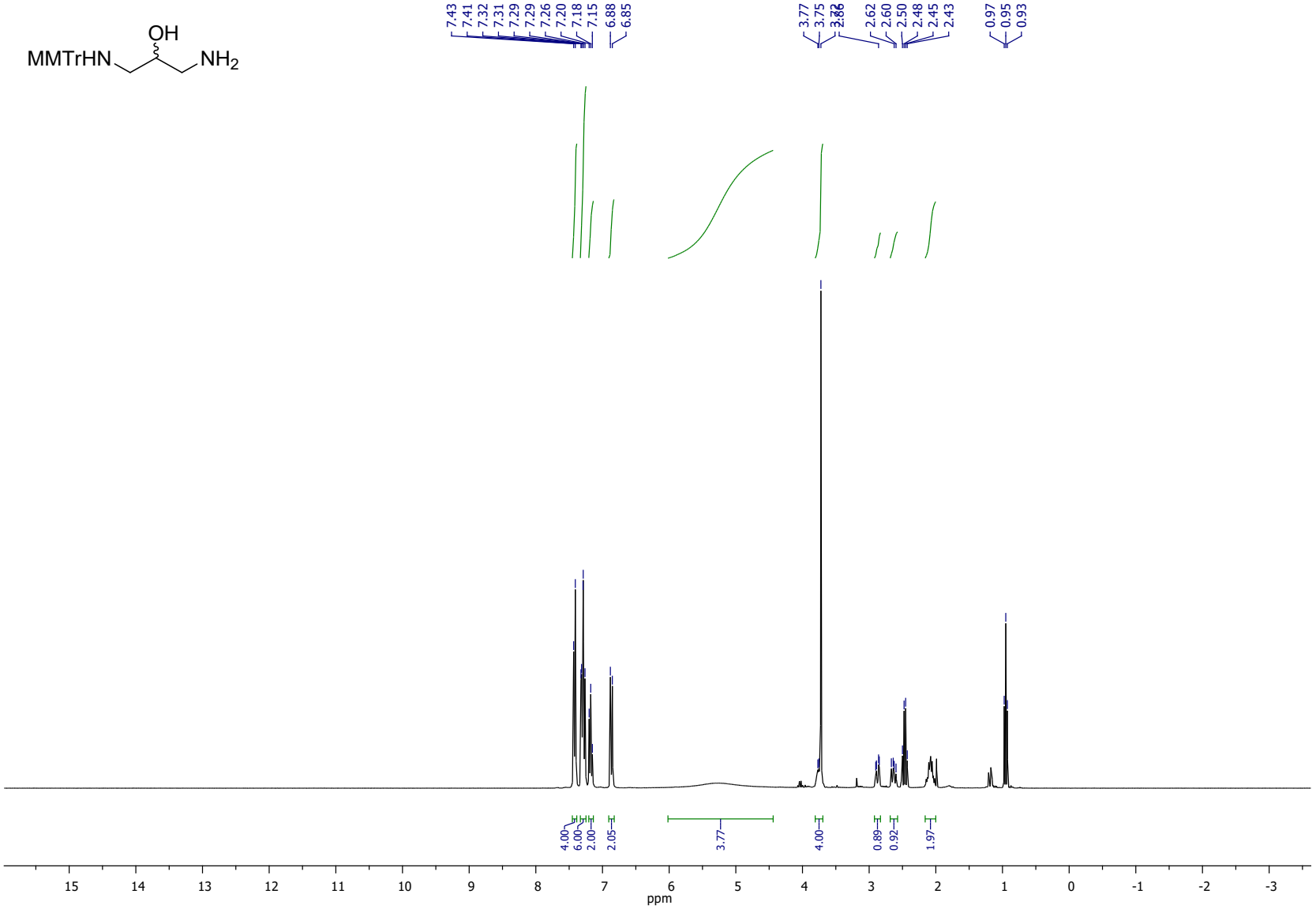


Supporting Scheme S2. Synthesis of amino-containing nucleoside **3**.

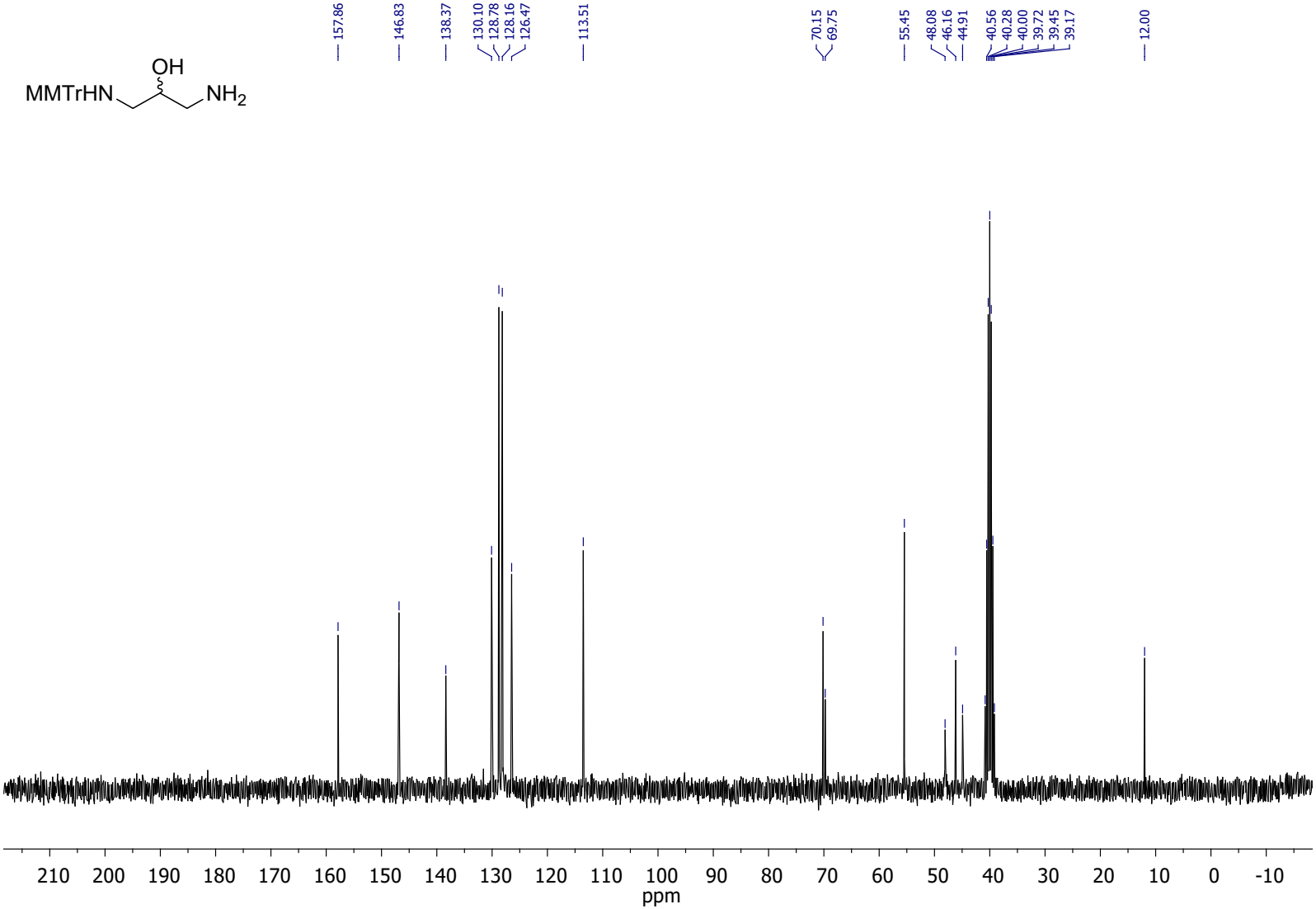
*4-((2-((2-aminoethyl)disulfanyl)ethyl)amino)-1-((2R,4S,5S)-4-azido-5-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)tetrahydrofuran-2-yl)-5-methylpyrimidin-2(1H)-one (**3**)*

Compound **3** was synthesized according to literature.[3]

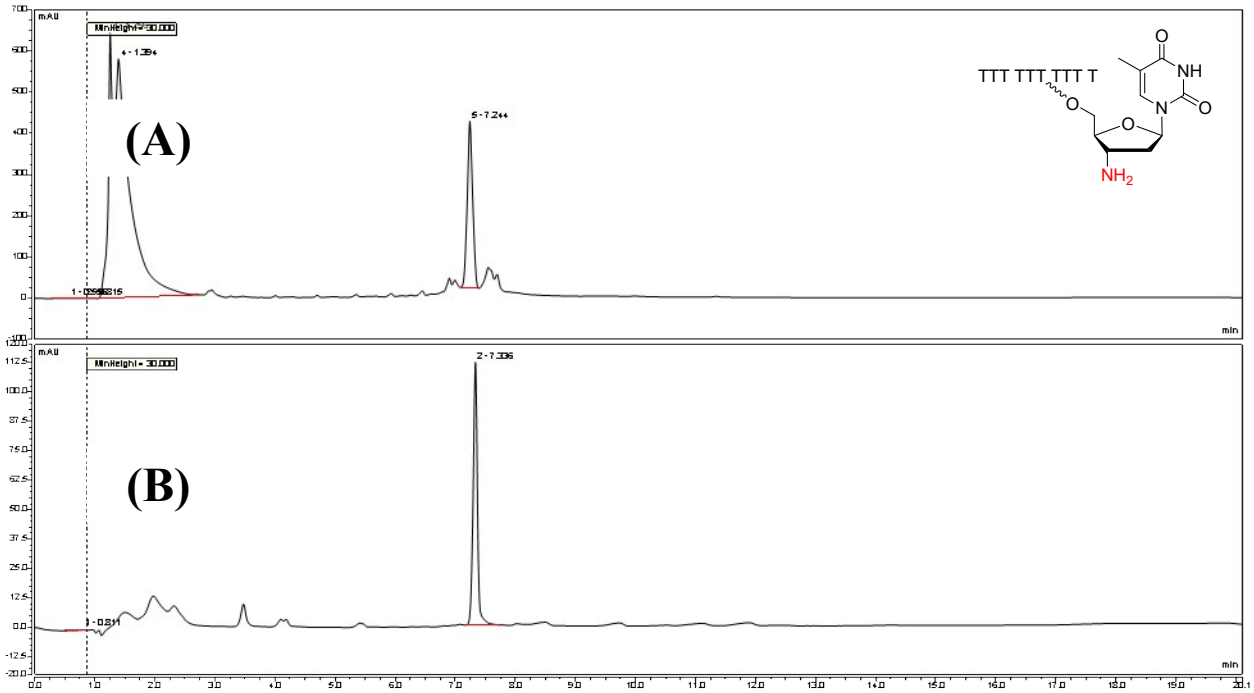
Supporting Figure S1- 300 MHz ^{1}H NMR spectrum of compound (**2**) (in DMSO-d_6).



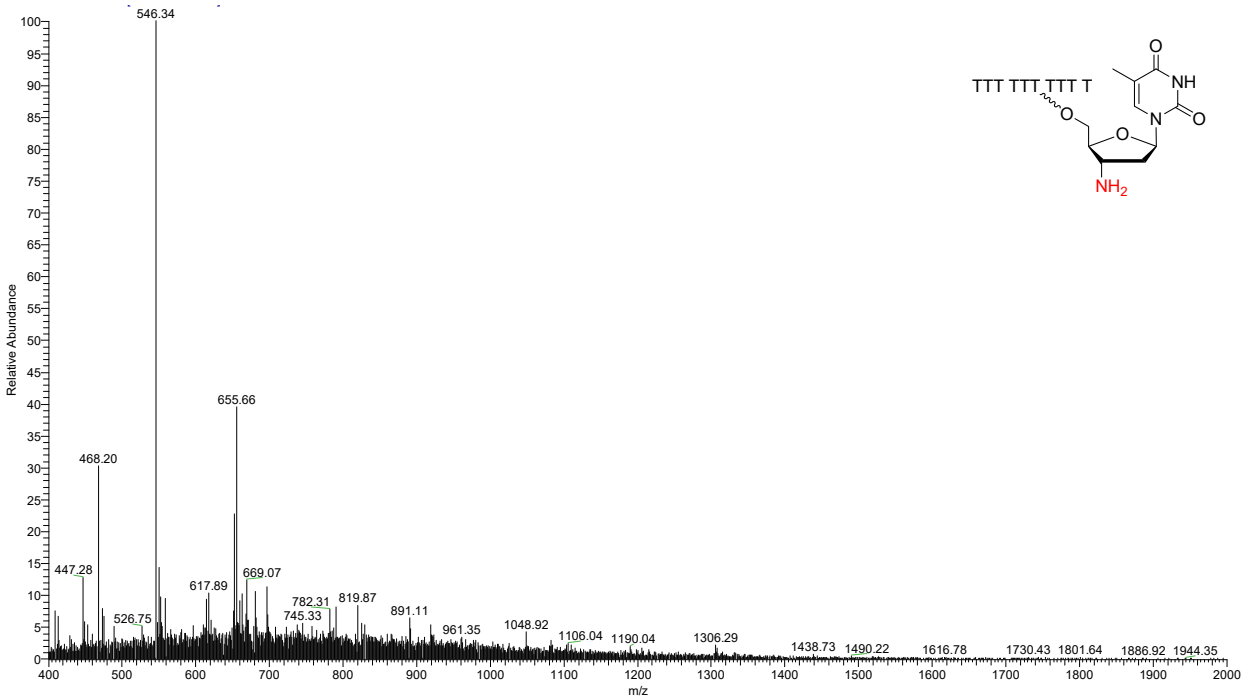
Supporting Figure S2-75 MHz ^{13}C NMR spectrum of compound (**2**) (in DMSO-d_6).



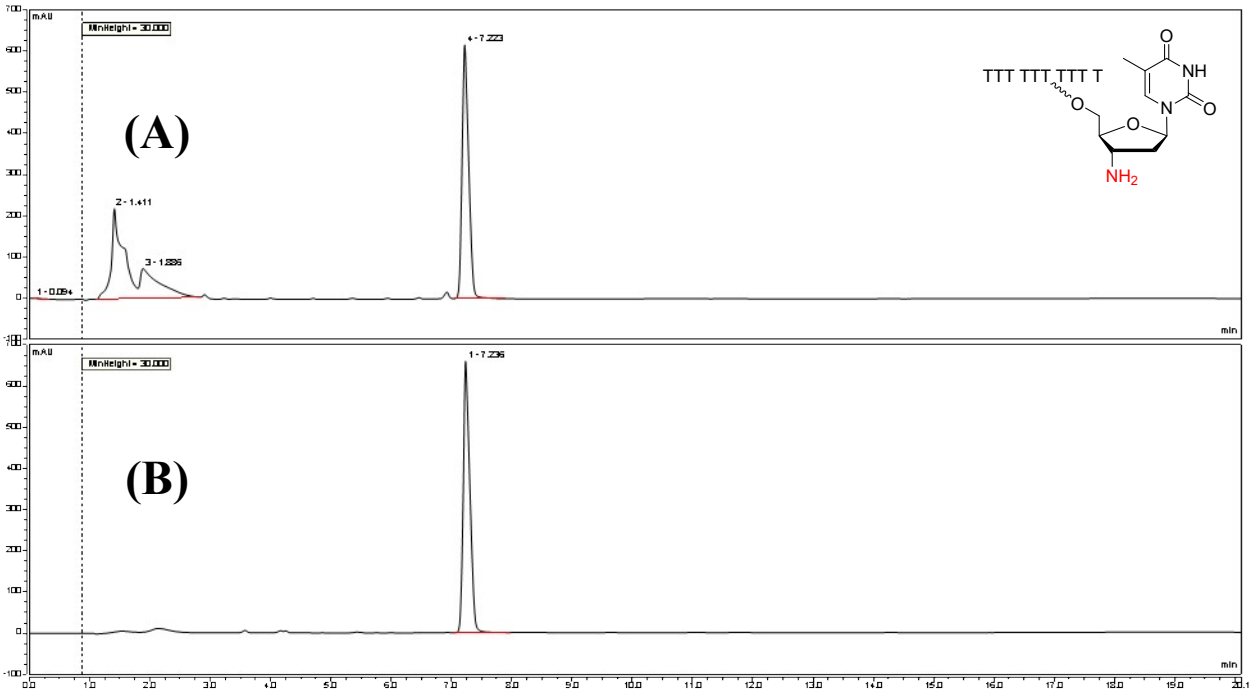
Supporting Figure S3- SAX-HPLC profile of TTT TTT TTT T T_{NH2} as a crude-analytical using ***S***-CPG (A), and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm.



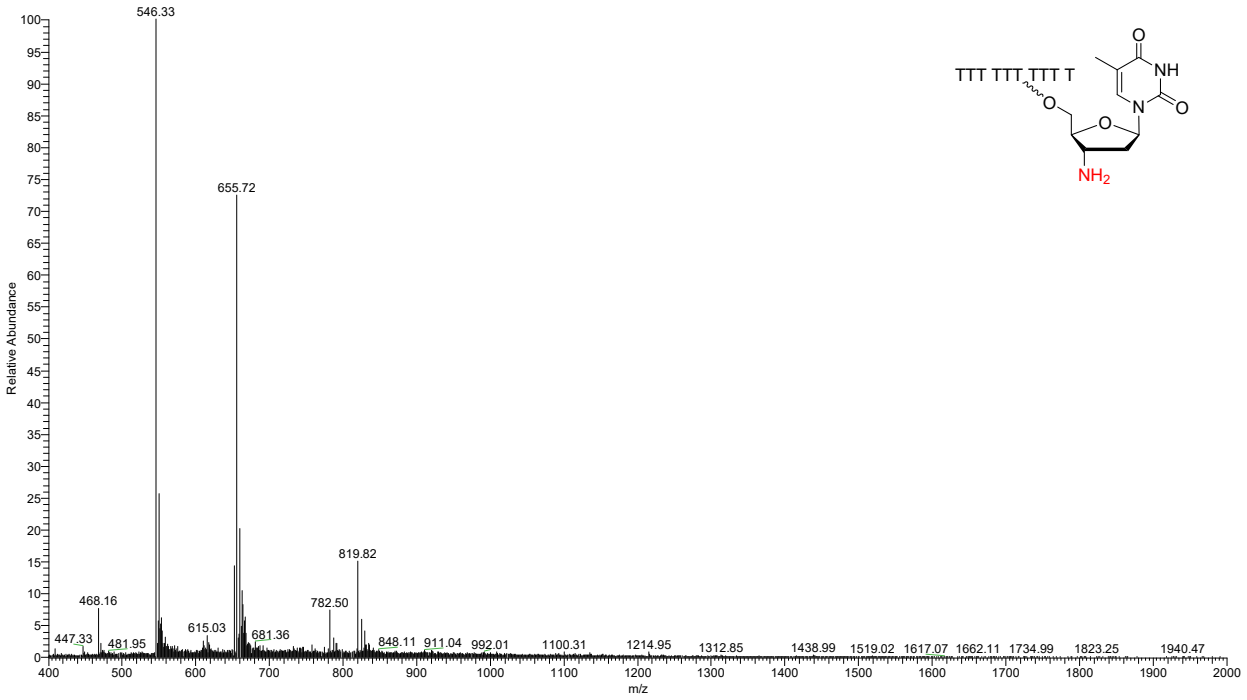
Supporting Figure S4- LR-ESI-MS of T_{10} T_{NH2} (via ***S***-CPG). Expected Masses: $[M-7H]^{-7}$ 467.93, $[M-6H]^{-6}$ 546.09, $[M-5H]^{-5}$ 655.51; found 468.20, 546.34, 655.66.



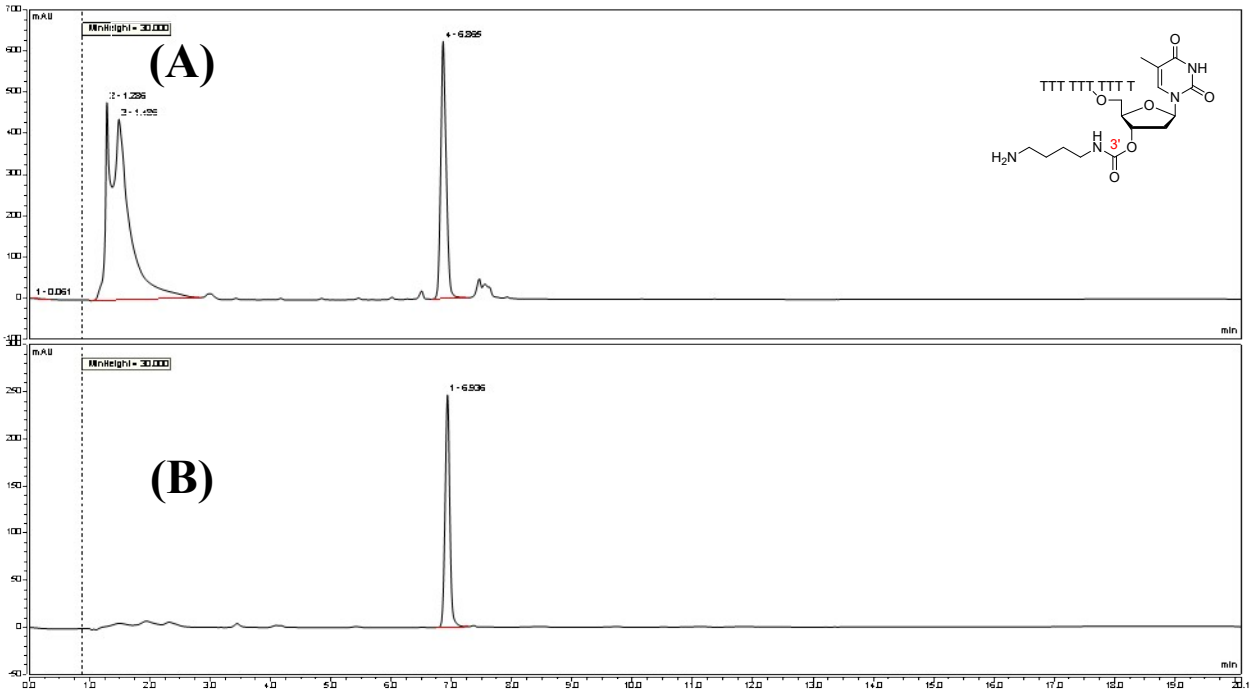
Supporting Figure S5- SAX-HPLC profile of TTT TTT TTT T T_{NH2} as a crude-analytical using ***R***-CPG (A), and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm.



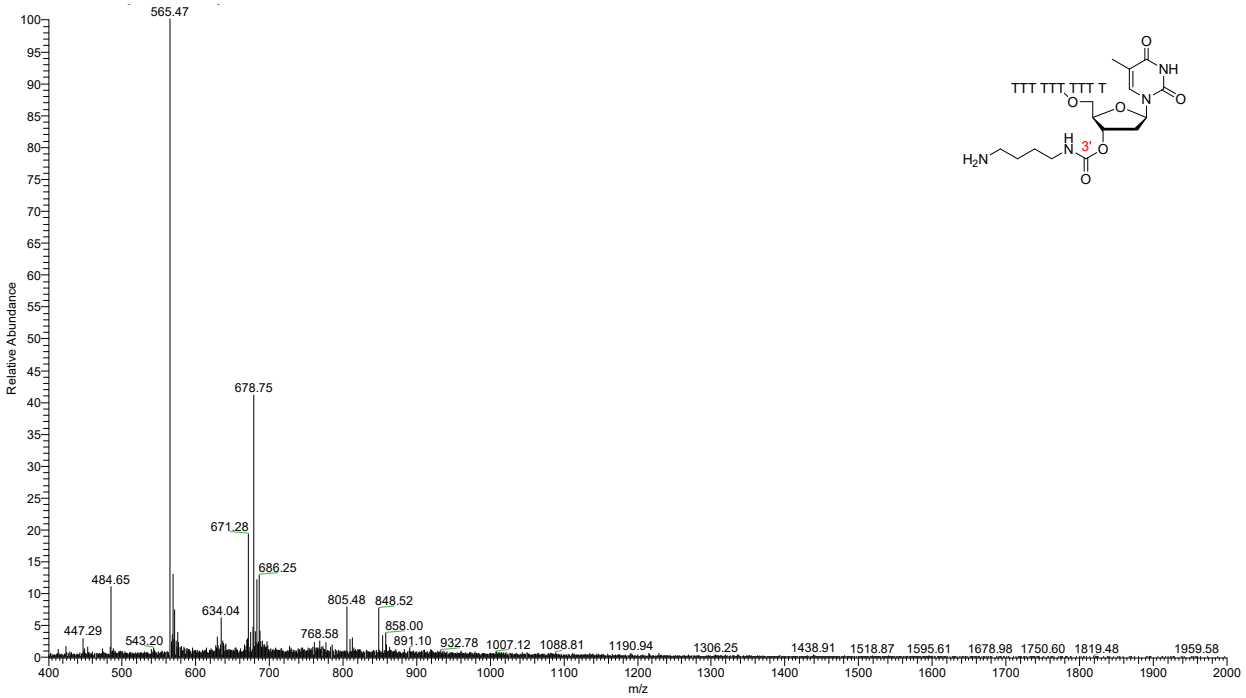
Supporting Figure S6- LR-ESI-MS of T_{10} T_{NH2} (via ***R***-CPG). Expected Masses: $[M-7H]^{-7}$ 467.93, $[M-6H]^{-6}$ 546.09, $[M-5H]^{-5}$ 655.51; found 468.16, 546.33, 655.72.



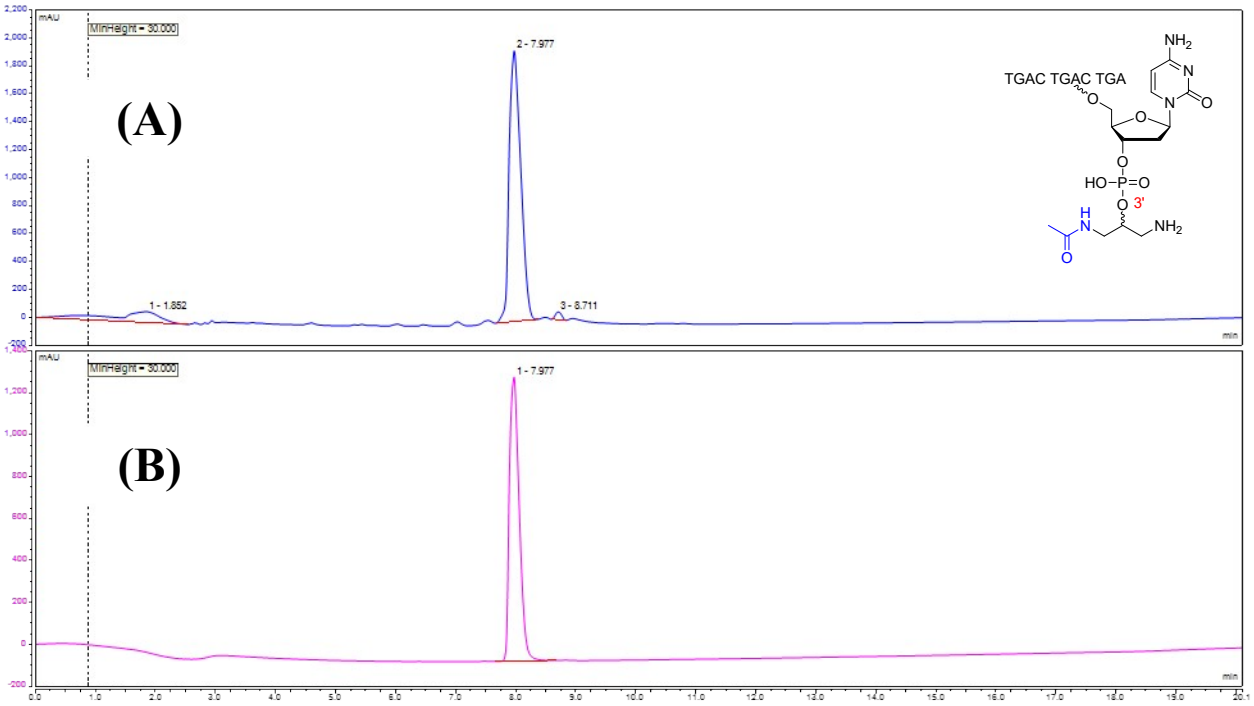
Supporting Figure S7- SAX-HPLC profile of TTT TTT TTT T $T_{putrescine}$ as a crude-analytical using ***R***-CPG (A), and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. Note that SAX-HPLC trace depicted in (**A**) is identical as the one shown in Fig. **2D**, and is reproduced here to show the difference between crude and purified traces of the sample.



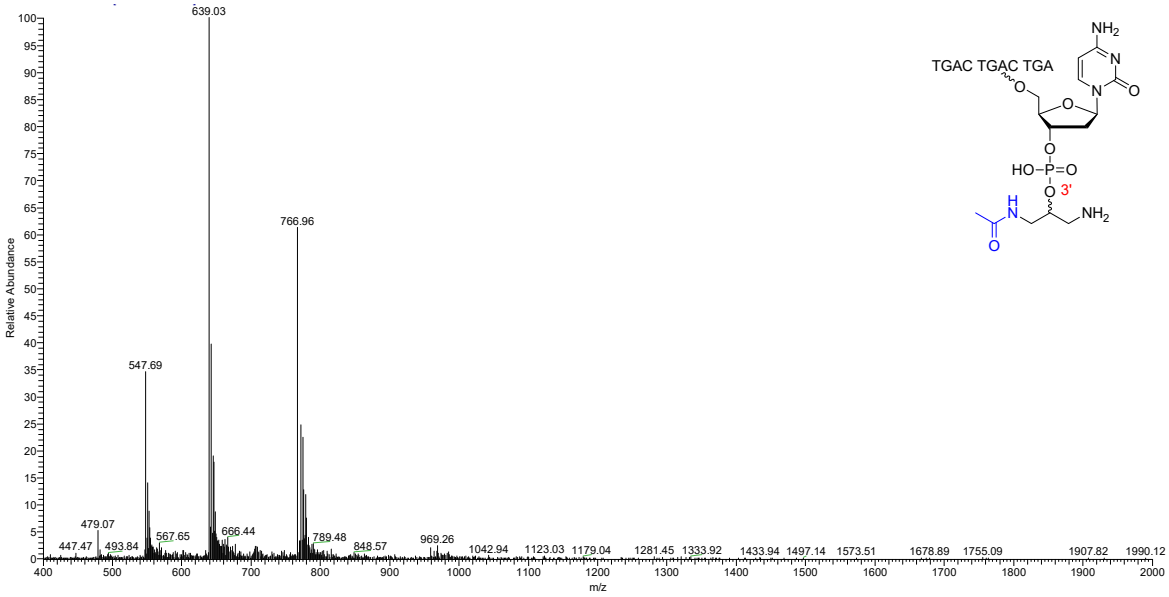
Supporting Figure S8- LR-ESI-MS of T_{10} $T_{putrescine}$. Expected Masses: $[M-7H]^{-7}$ 484.37, $[M-6H]^{-6}$ 565.27, $[M-5H]^{-5}$ 678.52; found 484.65, 565.47, 678.75.



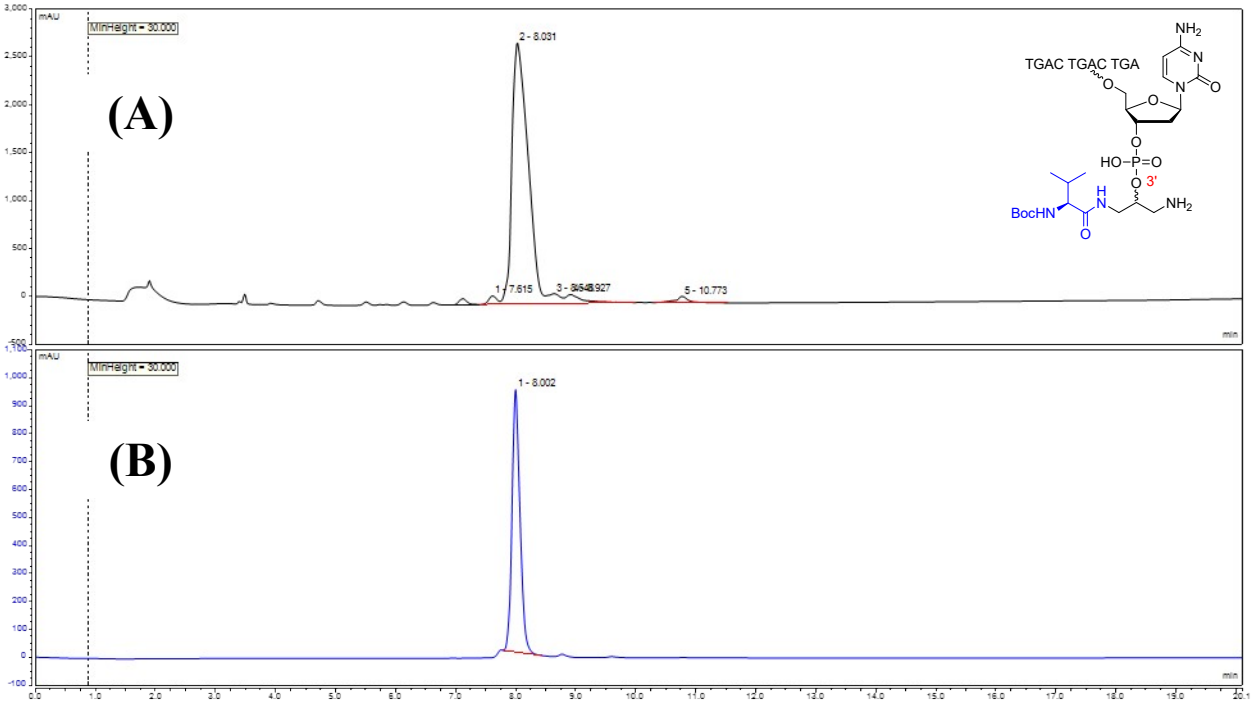
Supporting Figure S9- SAX-HPLC profile of TGAC TGAC TGAC$_{(diamino\ propanol\ +\ N\text{-}Ac\text{-}OSu)}$ as a crude-analytical (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. The NHS ester was conjugated in aqueous conditions.



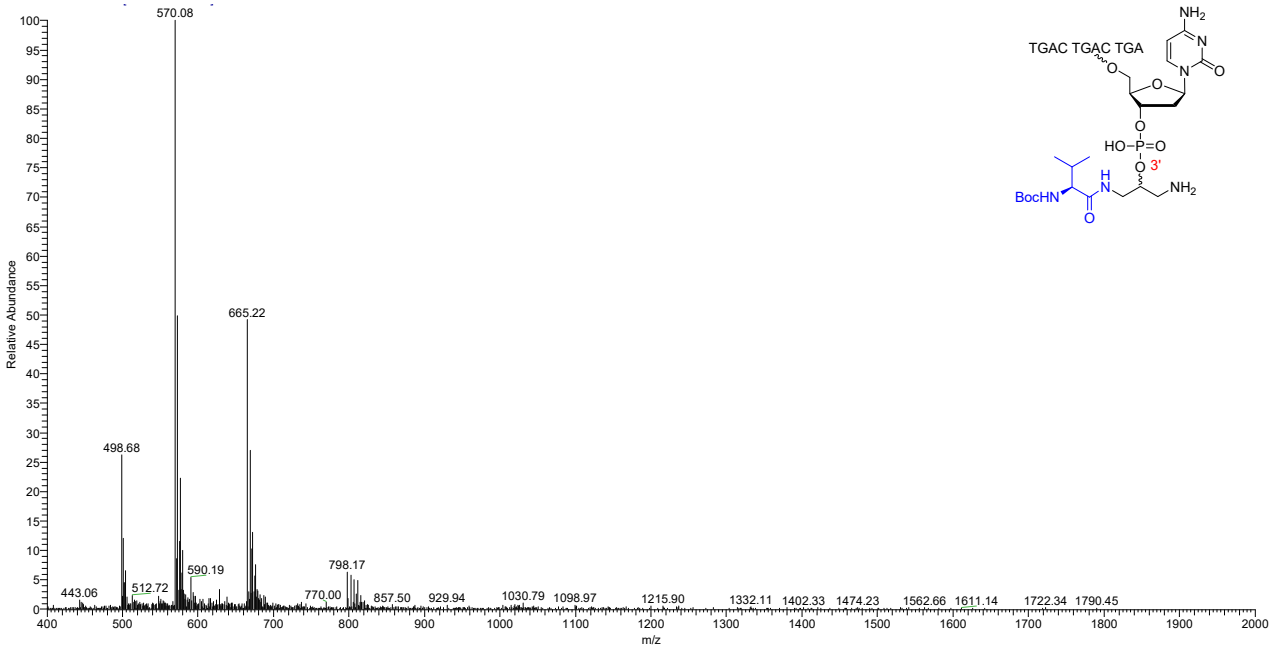
Supporting Figure S10- LR-ESI-MS of TGAC TGAC TGAC$_{(\text{diamino propanol + N-Ac-OSu})}$. Expected Masses: $[M-7H]^{-7}$ 547.67, $[M-6H]^{-6}$ 639.11, $[M-5H]^{-5}$ 767.14; found 547.69, 639.03, 766.96.



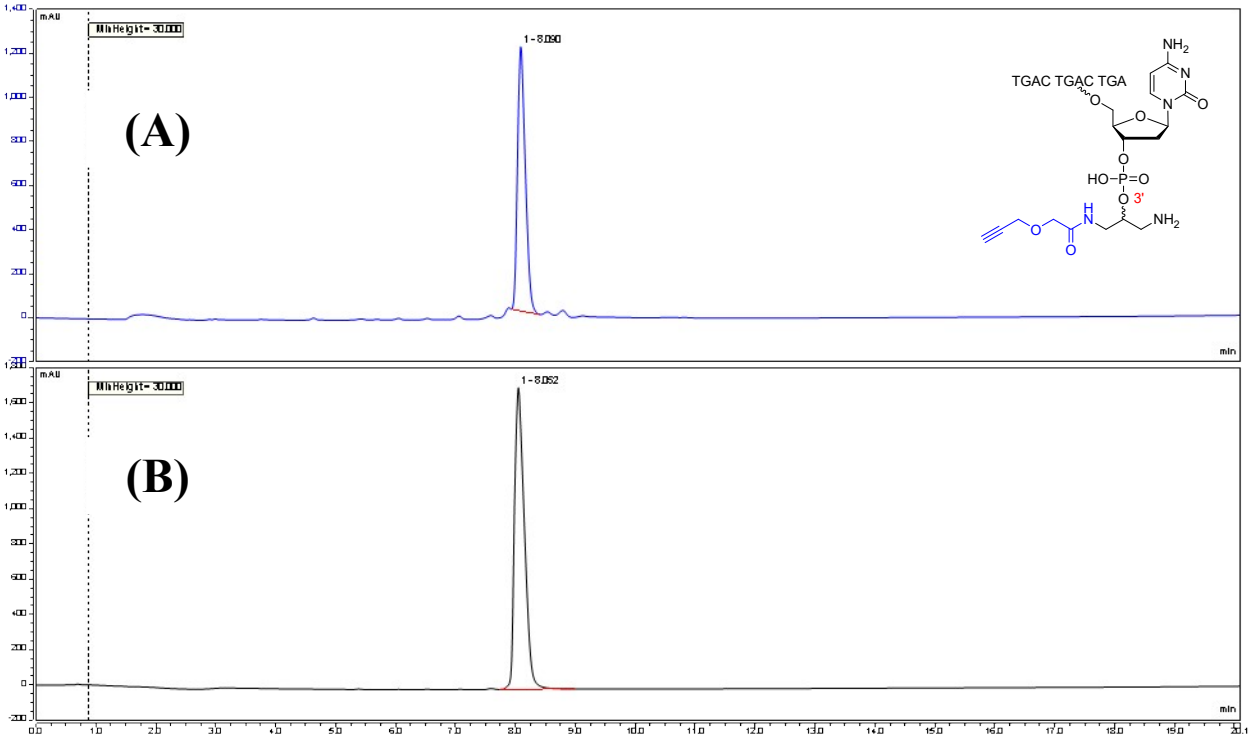
Supporting Figure S11- SAX-HPLC profile of TGAC TGAC TGAC$_{(diamino\ propanol\ +\ Boc\ Val\text{-}OSu)}$ as a crude-analytical (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. The NHS ester was conjugated in aqueous conditions. Note that SAX-HPLC trace depicted in (**A**) is identical as the one shown in Fig. **4B**, and is reproduced here to show the difference between crude and purified traces of the sample.



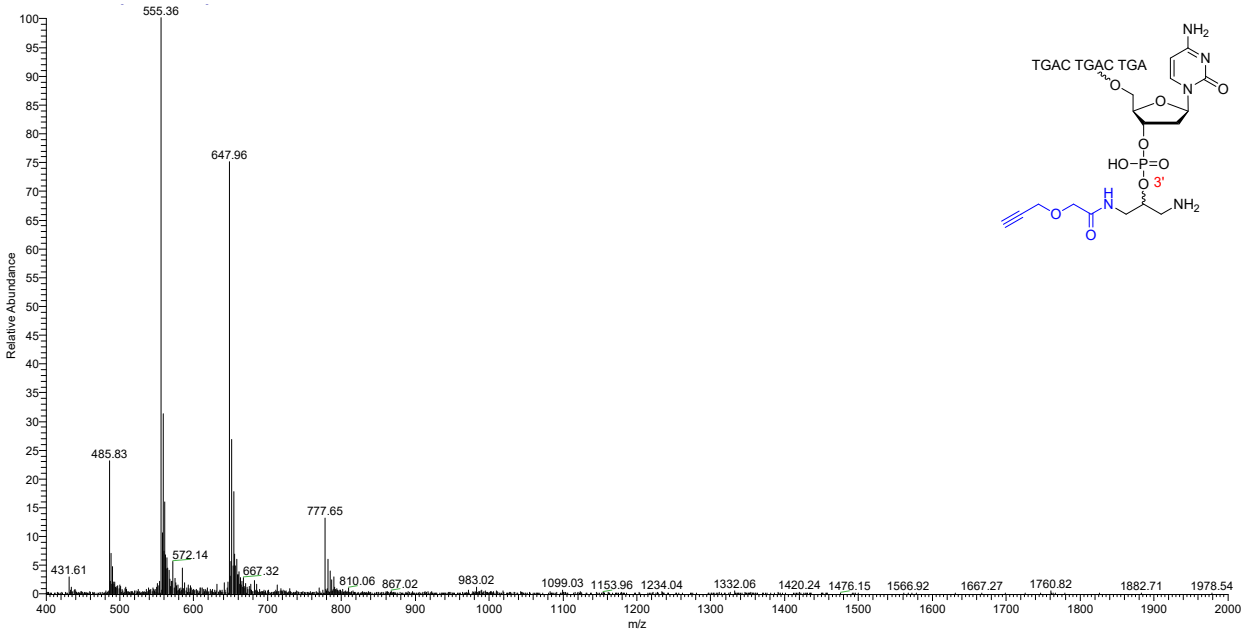
Supporting Figure S12- LR-ESI-MS of TGAC TGAC TGAC$_{(diamino\ propanol\ +\ Boc\ Val\text{-}OSu)}$. Expected Masses: $[M-8H]^{-8}$ 498.47, $[M-7H]^{-7}$ 569.82, $[M-6H]^{-6}$ 664.96; found 498.68, 570.08, 665.22.



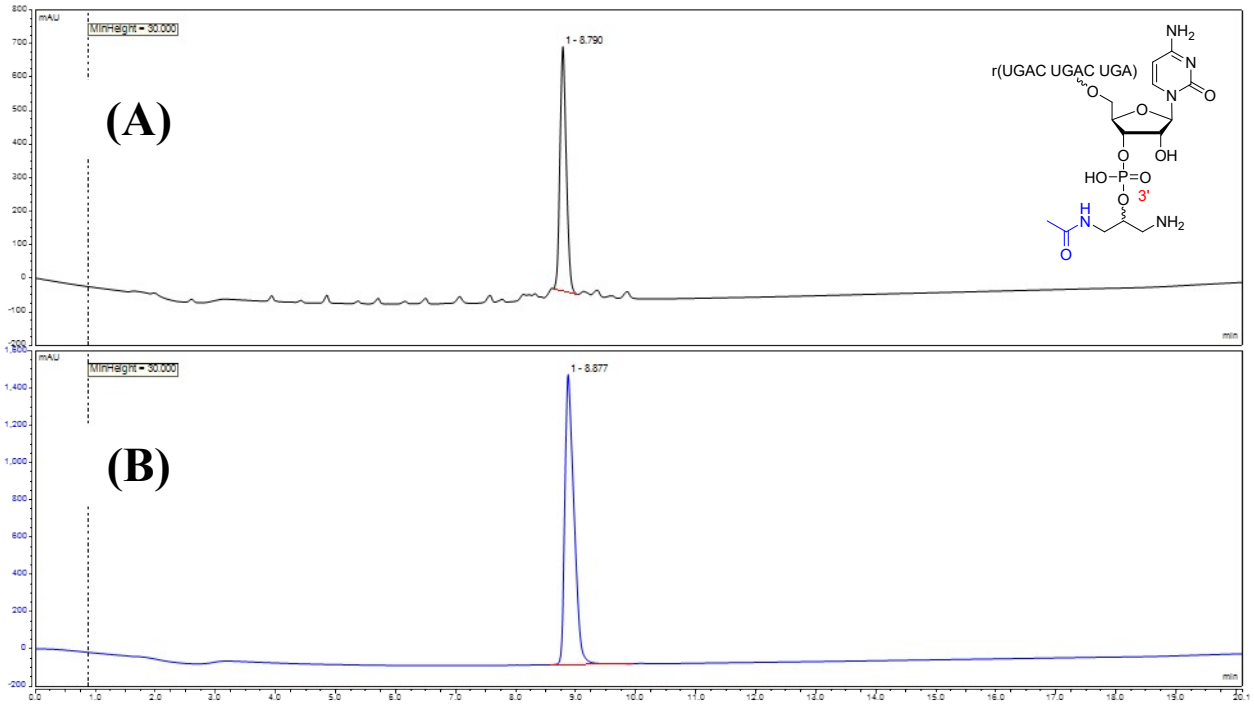
Supporting Figure S13- SAX-HPLC profile of TGAC TGAC TGAC$_{(diamino\ propanol\ +\ propargyl\text{-}OSu)}$ as a crude-analytical (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. The NHS ester was conjugated in aqueous conditions. Note that SAX-HPLC trace depicted in (**A**) is identical as the one shown in Fig. **4B**, and is reproduced here to show the difference between crude and purified traces of the sample.



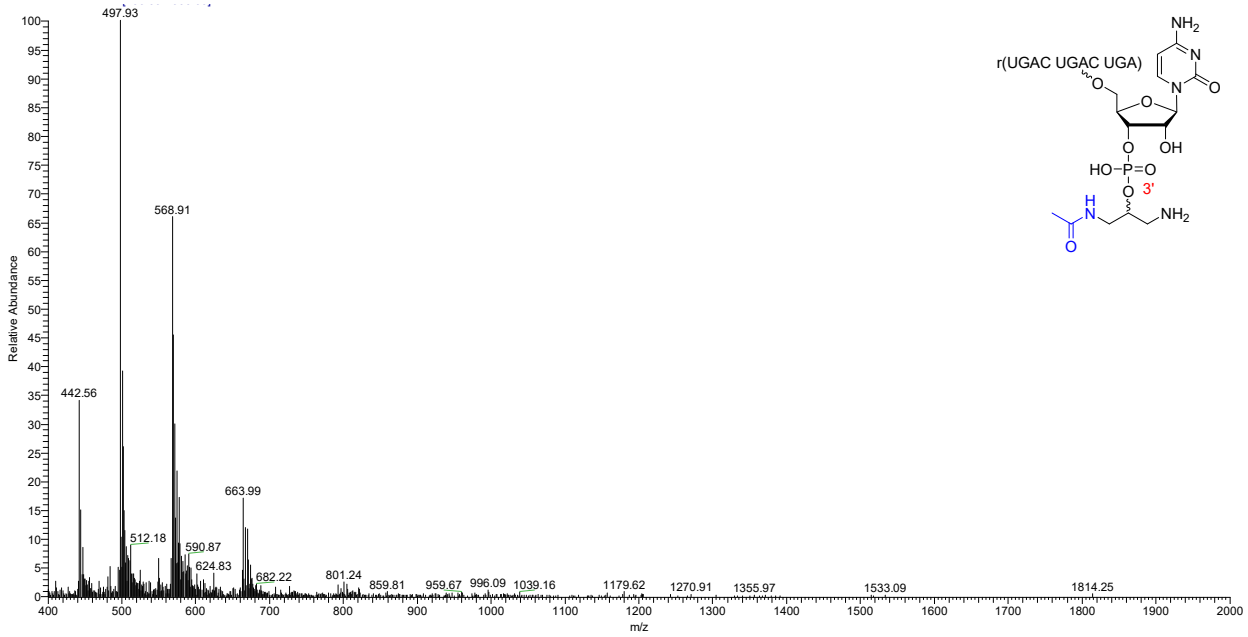
Supporting Figure S14- LR-ESI-MS of TGAC TGAC TGAC$_{(diamino\ propanol\ +\ propargyl\text{-}OSu)}$. Expected Masses: $[M-7H]^{-7}$ 555.09, $[M-6H]^{-6}$ 647.78, $[M-5H]^{-5}$ 777.53; found 555.36, 647.96, 777.65.



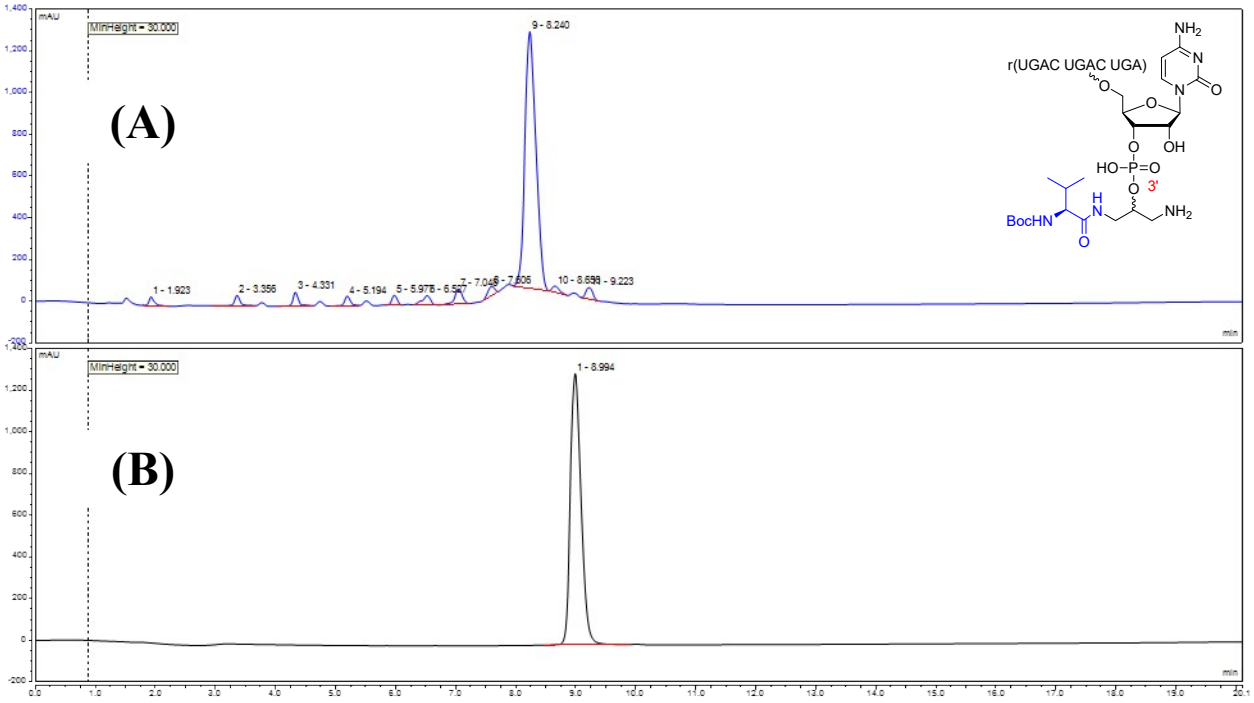
Supporting Figure S15- SAX-HPLC profile of r(UGAC UGAC UGAC) $_{\text{(diamino propanol + N-Ac-OSu)}}$ as a crude-analytical (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. The NHS ester was conjugated in aqueous conditions.



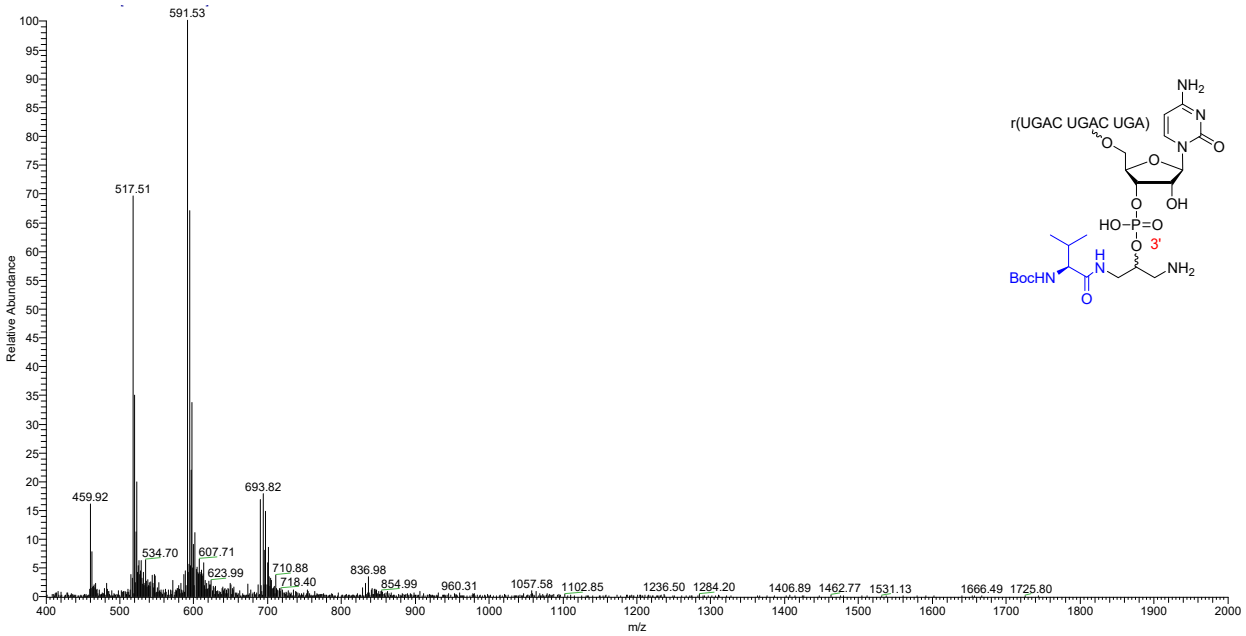
Supporting Figure S16- LR-ESI-MS of r(UGAC UGAC UGAC) (diamino propanol + N-Ac-OSu). Expected Masses: $[M-9H]^{-9}$ 442.17, $[M-8H]^{-8}$ 497.93, $[M-7H]^{-7}$ 568.79; found 442.56, 497.93, 568.91.



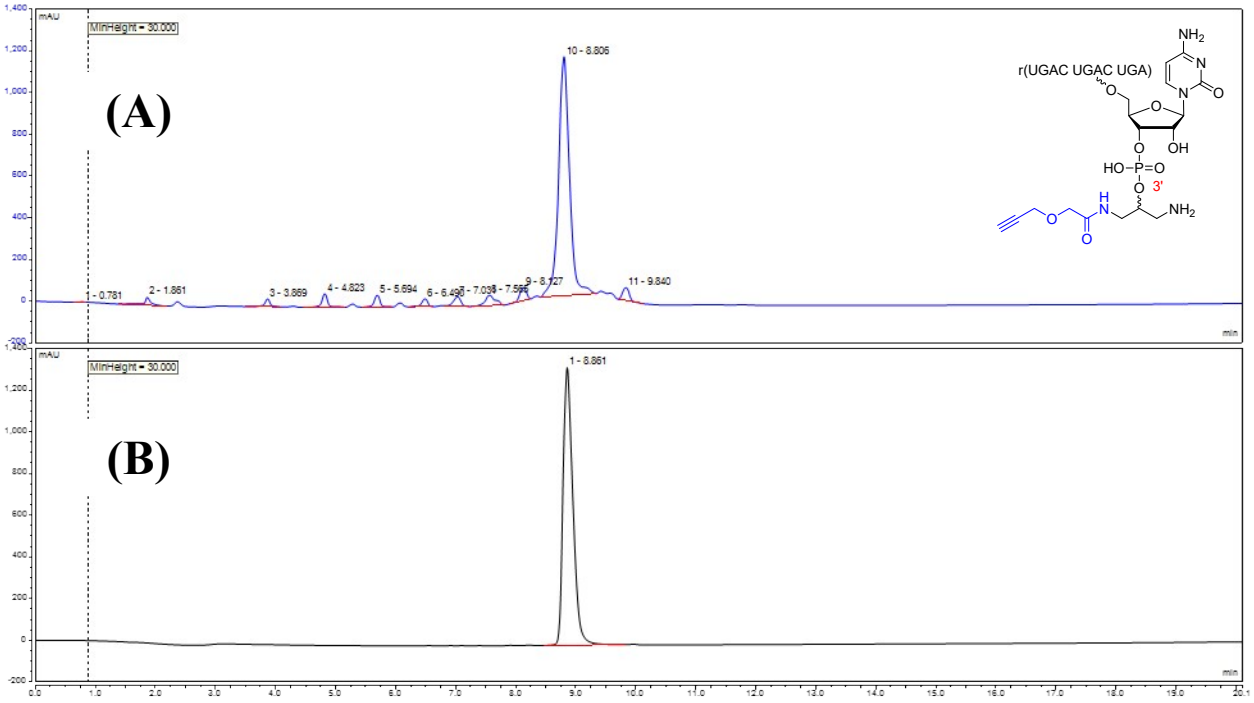
Supporting Figure S17- SAX-HPLC profile of r(UGAC UGAC UGAC) (diamino propanol + Boc Val-OSu) as a crude-analytical (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. The NHS ester was conjugated in aqueous conditions. Note that SAX-HPLC trace depicted in (**A**) is identical as the one shown in Fig. **4B** and is reproduced here to show the difference between crude and purified traces of the sample.



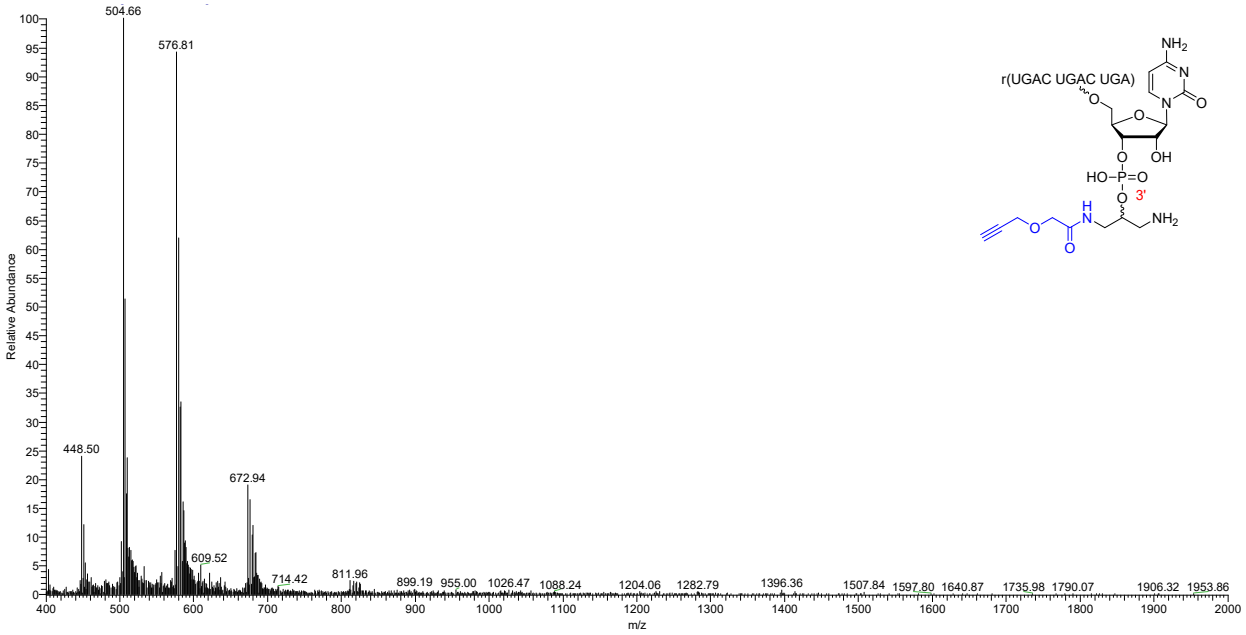
Supporting Figure S18- LR-ESI-MS of r(UGAC UGAC UGAC) (diamino propanol + Boc Val-OSu). Expected Masses: $[M-9H]^{-9}$ 459.63, $[M-8H]^{-8}$ 517.21, $[M-7H]^{-7}$ 591.24; found 459.92, 517.51, 591.53.



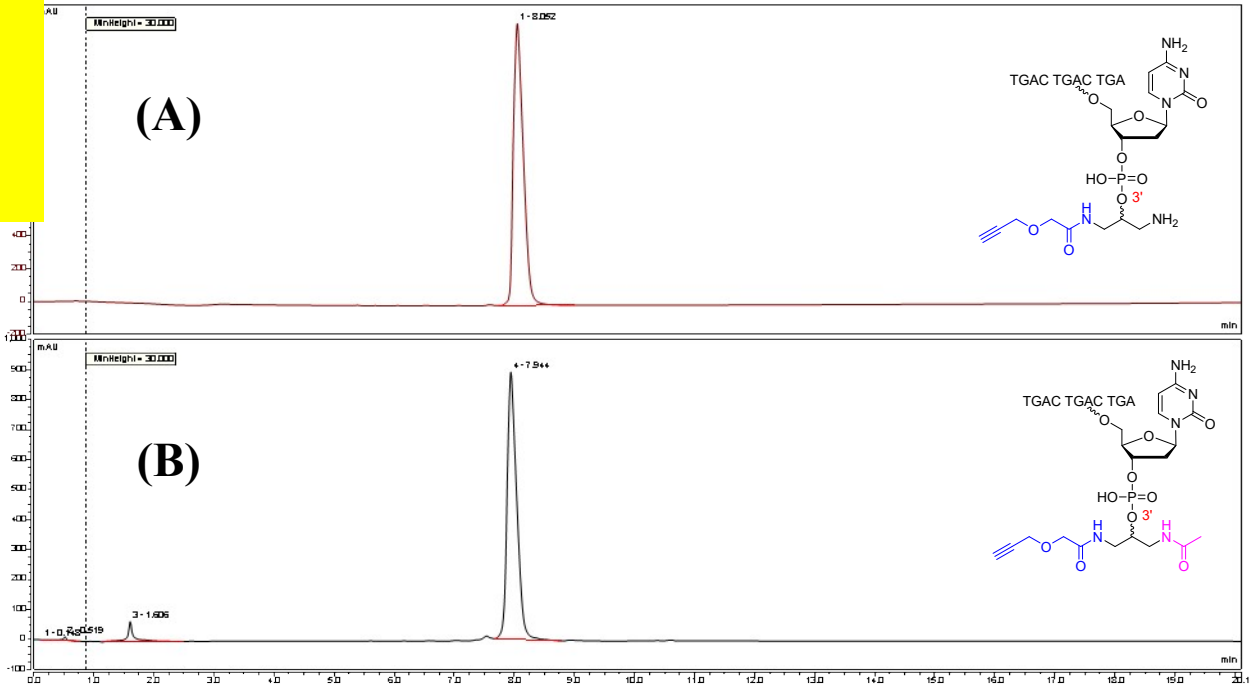
Supporting Figure S19- SAX-HPLC profile of r(UGAC UGAC UGAC) (diamino propanol + propargyl-OSu) as a crude-analytical (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. The NHS ester was conjugated in aqueous conditions. Note that SAX-HPLC trace depicted in (**A**) is identical as the one shown in Fig. **4B** and is reproduced here to show the difference between crude and purified traces of the sample.



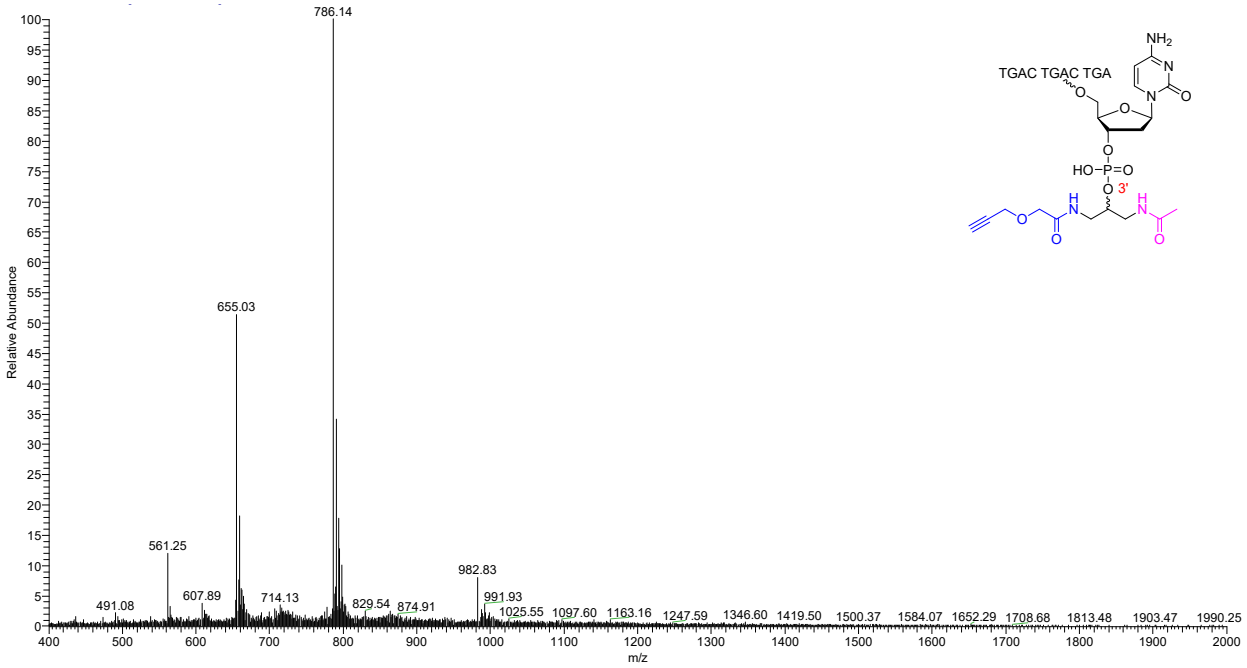
Supporting Figure S20- LR-ESI-MS of r(UGAC UGAC UGAC) (diamino propanol + propargyl-OSu). Expected Masses: $[M-9H]^{-9}$ 448.17, $[M-8H]^{-8}$ 504.32, $[M-7H]^{-7}$ 576.51; found 448.50, 504.66, 576.81.



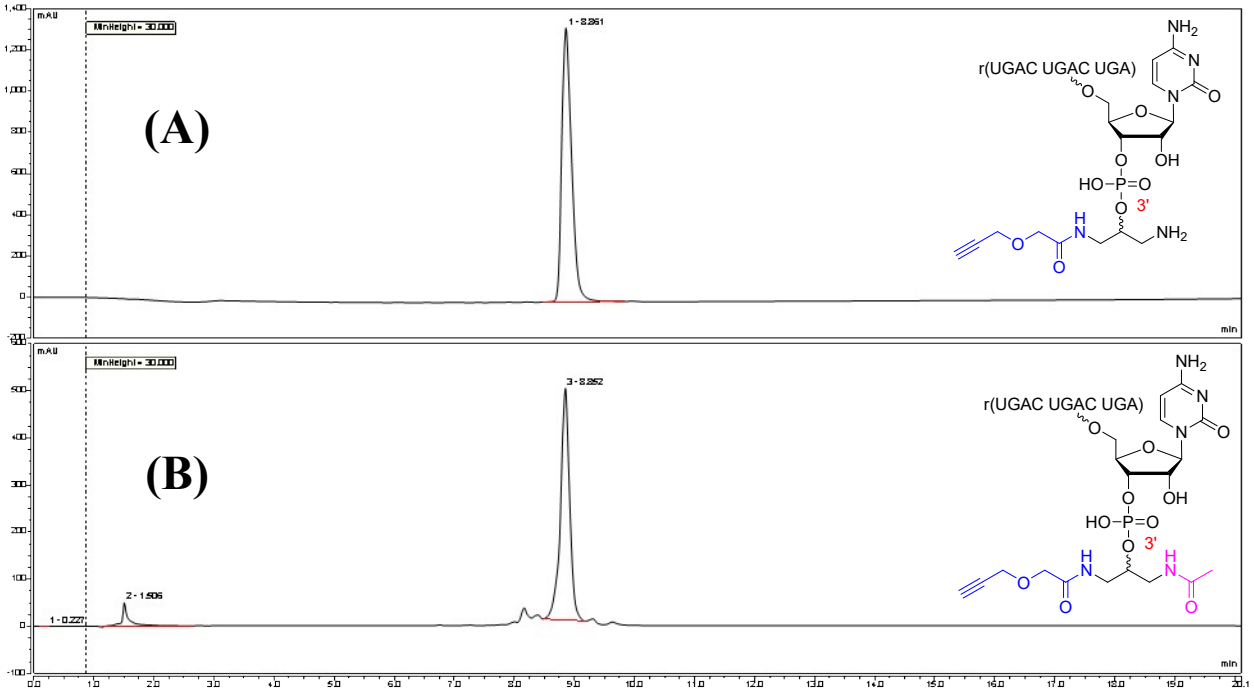
pporting Figure S21- SAX-HPLC profile of TGAC TGAC TGAC$_{(diamino\ propanol\ +\ propargyl\text{-}OSu+\ Ac\text{-}OSu)}$ as a ified starting material (A) and crude-reaction analytical (B). The column was eluted using a ear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% tonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow e of 1 mL/min. Monitoring was accomplished at 260 nm. The oligonucleotide was desalted er coupling with N-Ac OSu and subsequently used without additional purification.[3]



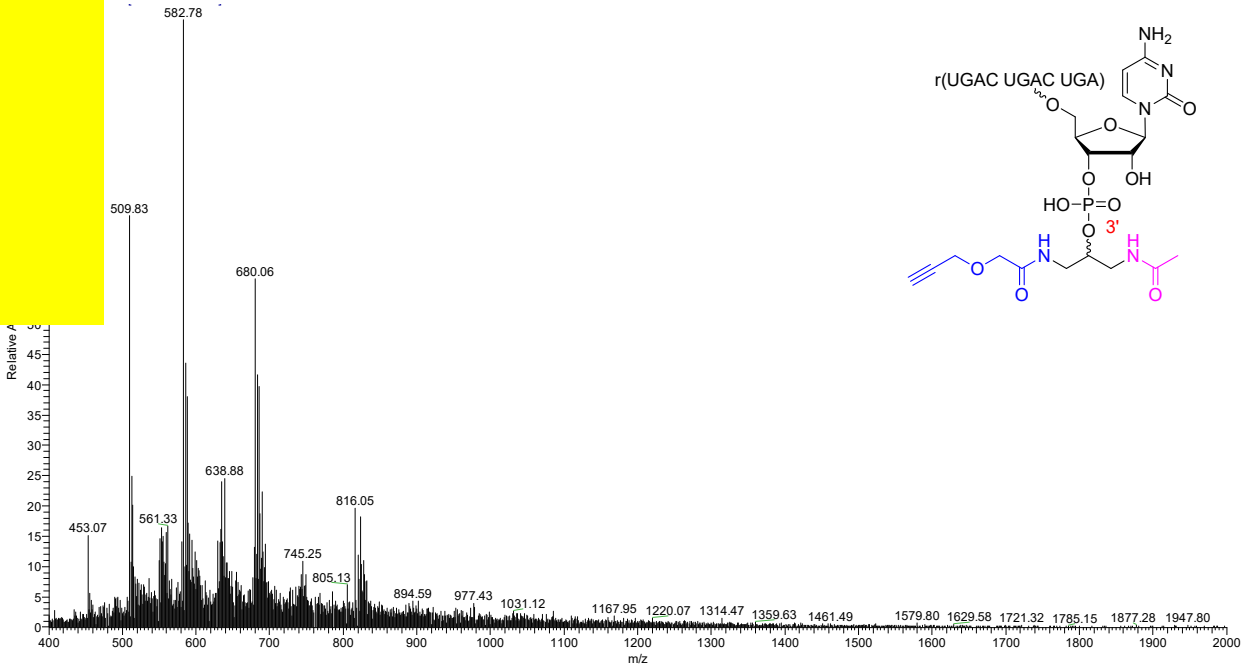
Supporting Figure S22- LR-ESI-MS of TGAC TGAC TGAC$_{(diamino\ propanol\ +\ propargyl\text{-}OSu\ +\ Ac\text{-}OSu)}$. Expected Masses: $[M-7H]^{-7}$ 561.10, $[M-6H]^{-6}$ 654.78, $[M-5H]^{-5}$ 785.94; found 561.25, 655.03, 786.14.



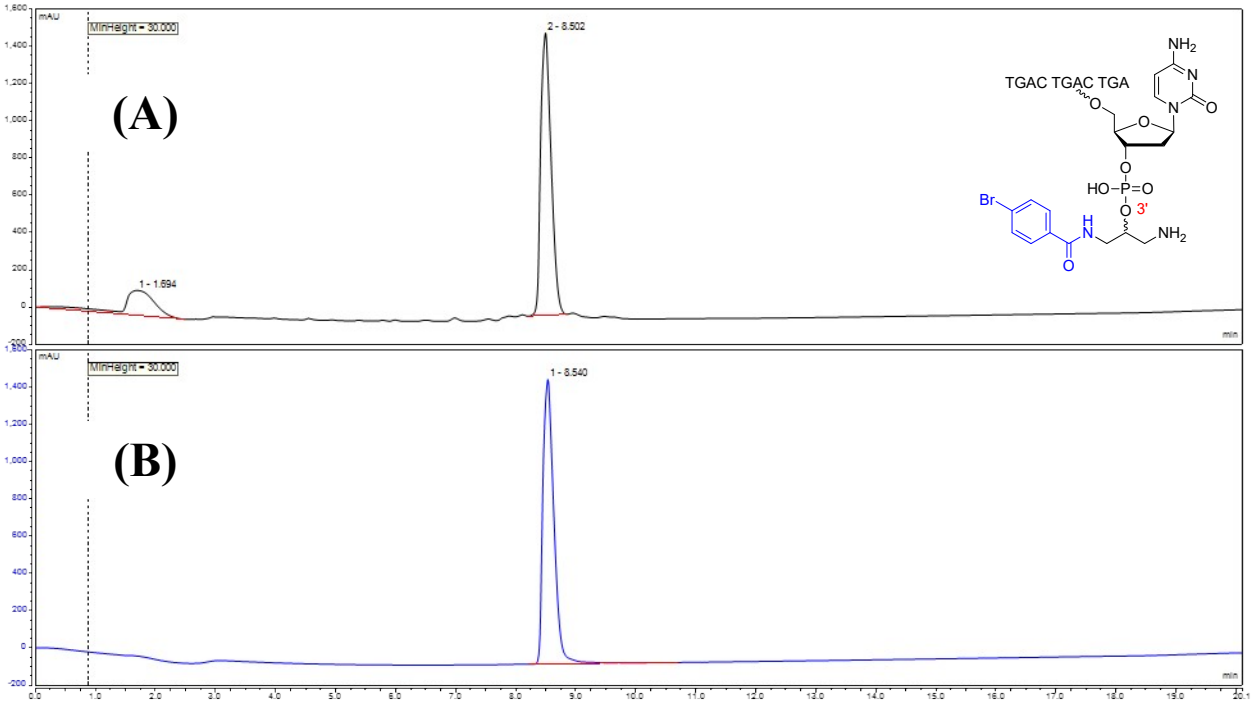
Supporting Figure S23- SAX-HPLC profile of r(UGAC UGAC UGAC) (diamino propanol + propargyl-OSu+ Ac-OSu) as purified starting material (A) and crude-reaction analytical (B).The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. The oligonucleotide was desalted after coupling with N-Ac OSu and subsequently used without additional purification.[3]



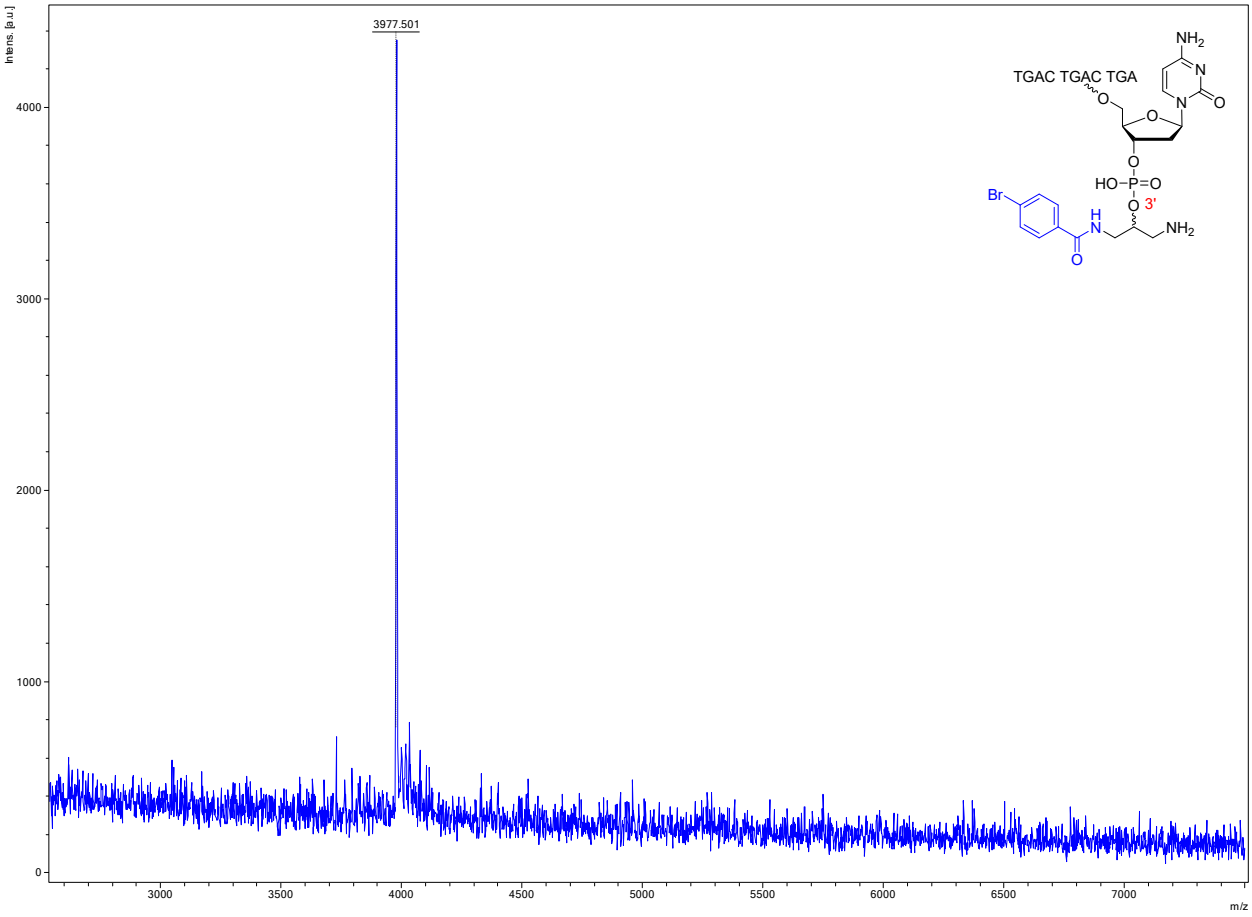
ing Figure S24- LR-ESI-MS of r(UGAC UGAC UGAC) (diamino propanol + propargyl-OSu+ Ac-OSu).
d Masses: $[M-8H]^{-8}$ 509.57, $[M-7H]^{-7}$ 582.51, $[M-6H]^{-6}$ 679.76; found 509.83, 582.78,



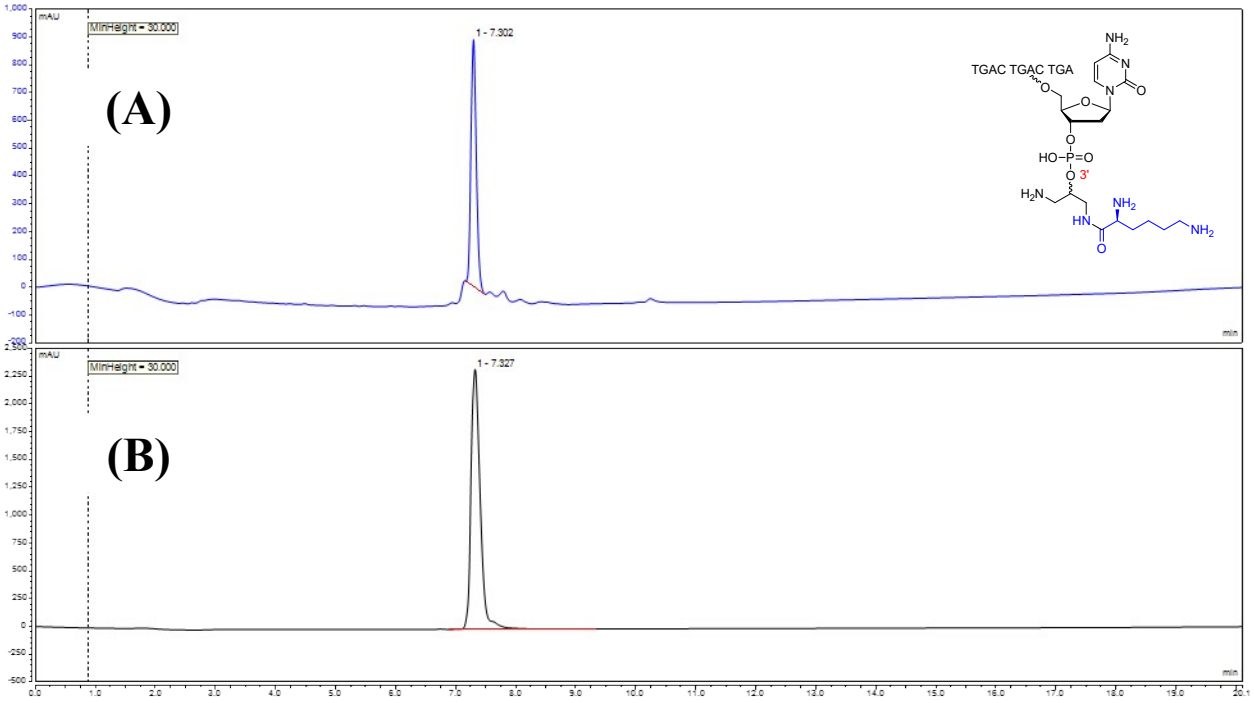
Supporting Figure S25- SAX-HPLC profile of TGAC TGAC TGAC$_{(diamino\ propanol\ +\ 4\text{-}Br\ Bz)}$ as a crude-analytical (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. Note that SAX-HPLC trace depicted in (**A**) is identical as the one shown in Fig. **5B**, and is reproduced here to show the difference between crude and purified traces of the sample.



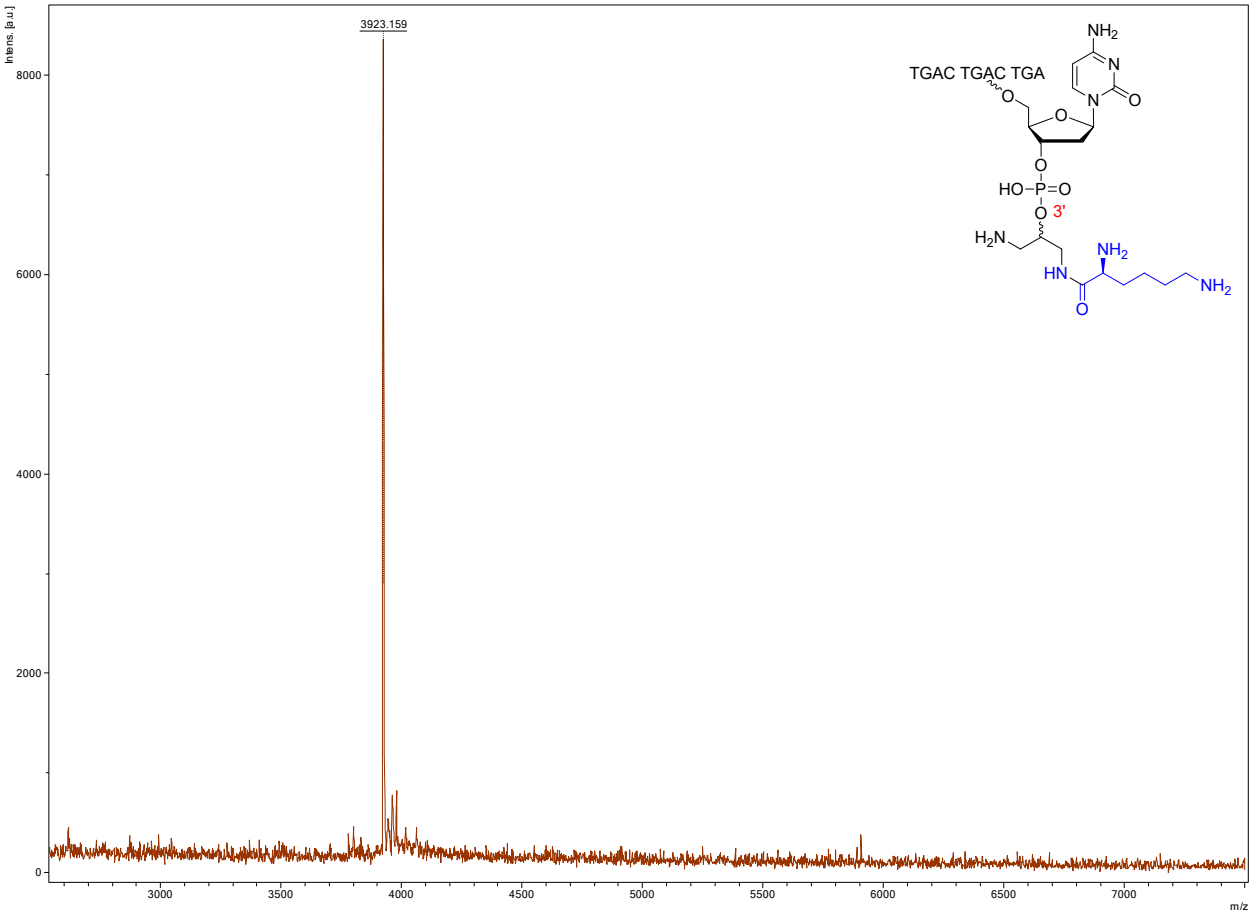
Supporting Figure S26- MALDI-TOF/TOF of TGAC TGAC TGAC$_{(\text{diamino propanol + 4-Br Bz})}$. Expected Mass: $[M-H]^-$ 3977.620; found 3977.501.



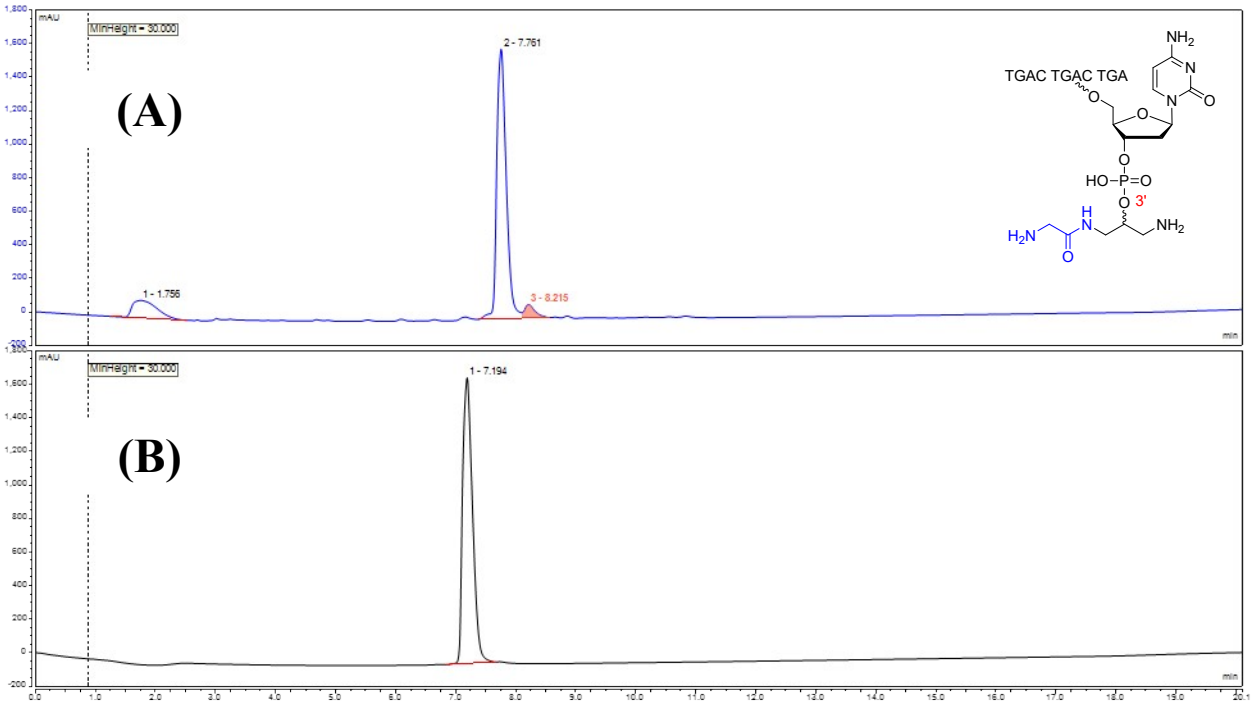
Supporting Figure S27- SAX-HPLC profile of TGAC TGAC TGAC$_{(diamino\ propanol\ +\ N\alpha,N\varepsilon\text{-}di\text{-}Fmoc\text{-}L\text{-}lysine)}$ as a crude-analytical (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. Note that SAX-HPLC trace depicted in (**A**) is identical as the one shown in Fig. **5B**, and is reproduced here to show the difference between crude and purified traces of the sample.



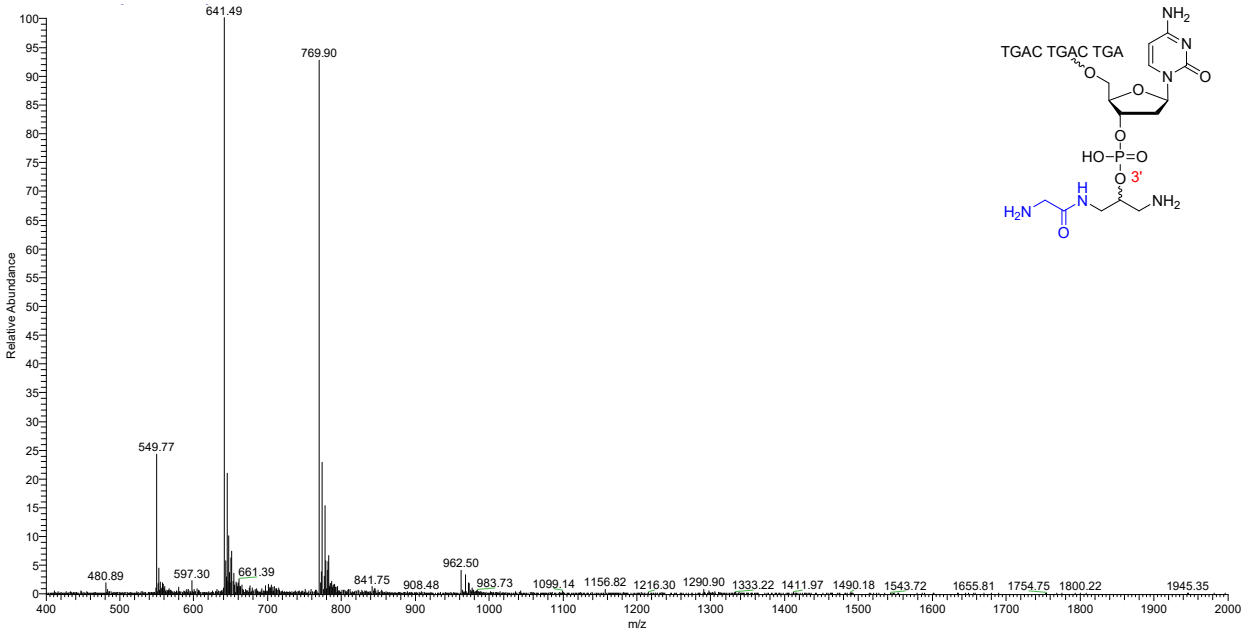
Supporting Figure S28- MALDI-TOF/TOF MS of TGAC TGAC TGAC$_{(\text{diamino propanol + N}\alpha,\text{N}\varepsilon\text{-di-Fmoc-L-lysine})}$. Expected Mass: $[M-H]^-$ 3923.778; found 3923.159.



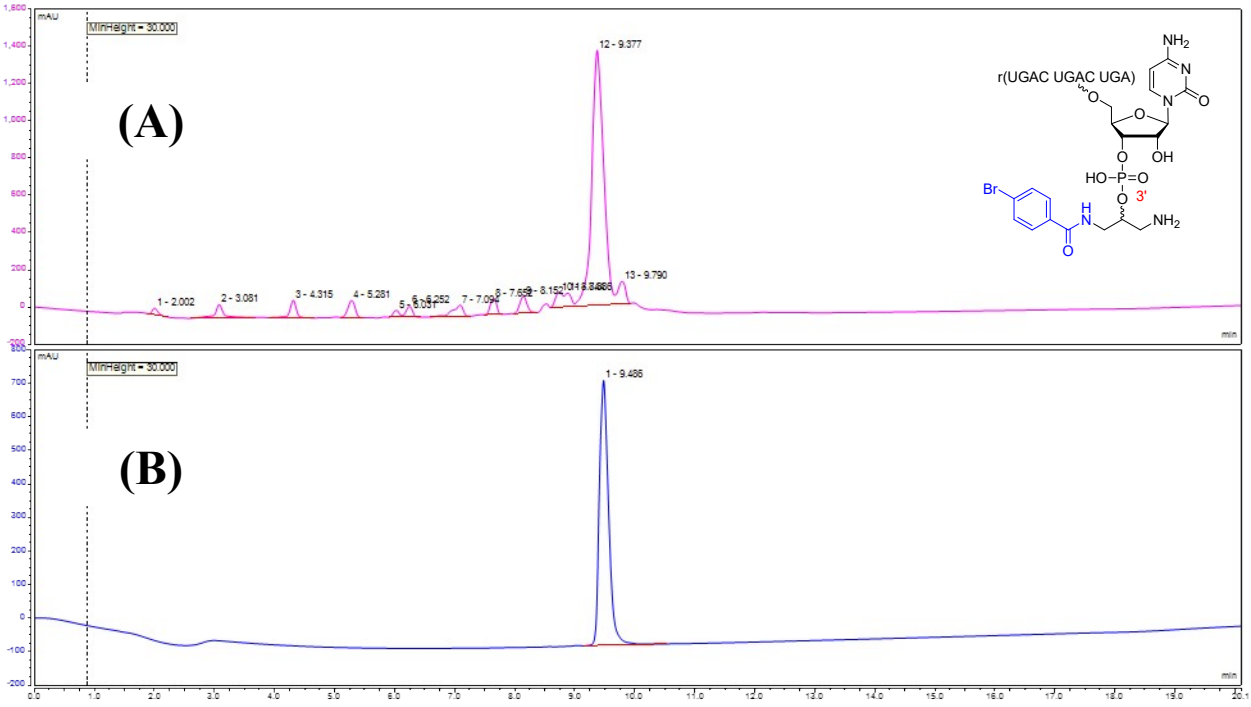
Supporting Figure S29- SAX-HPLC profile of TGAC TGAC TGAC$_{(diamino\ propanol\ +\ Gly\ Fmoc)}$ as a crude-analytical (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm.



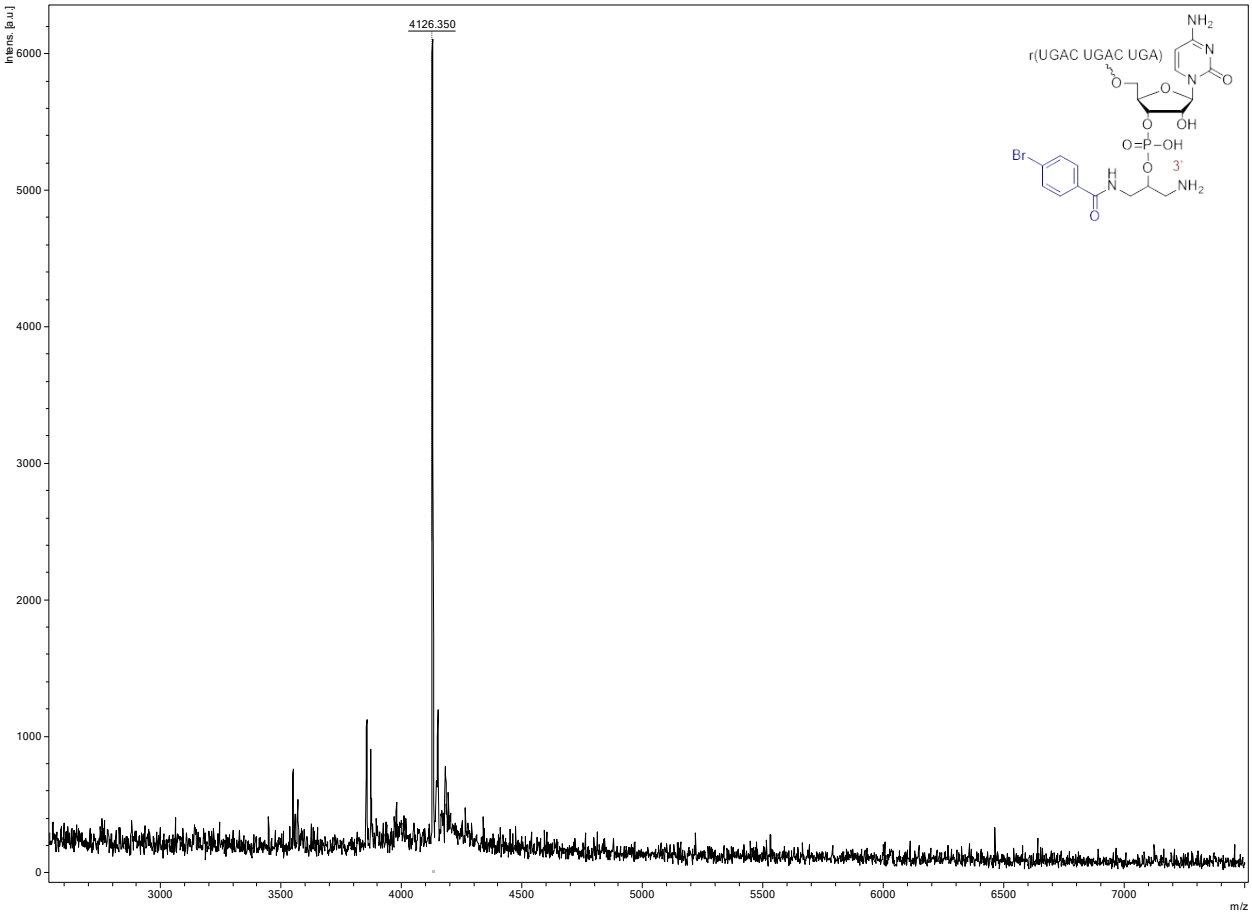
Supporting Figure S30- LR-ESI-MS of TGAC TGAC TGAC$_{(diamino\ propanol\ +\ Gly\ Fmoc)}$. Expected Masses: $[M-7H]^{-}$ 549.52, $[M-6H]^{-6}$ 641.28, $[M-5H]^{-5}$ 769.74; found 549.77, 641.49, 769.90.



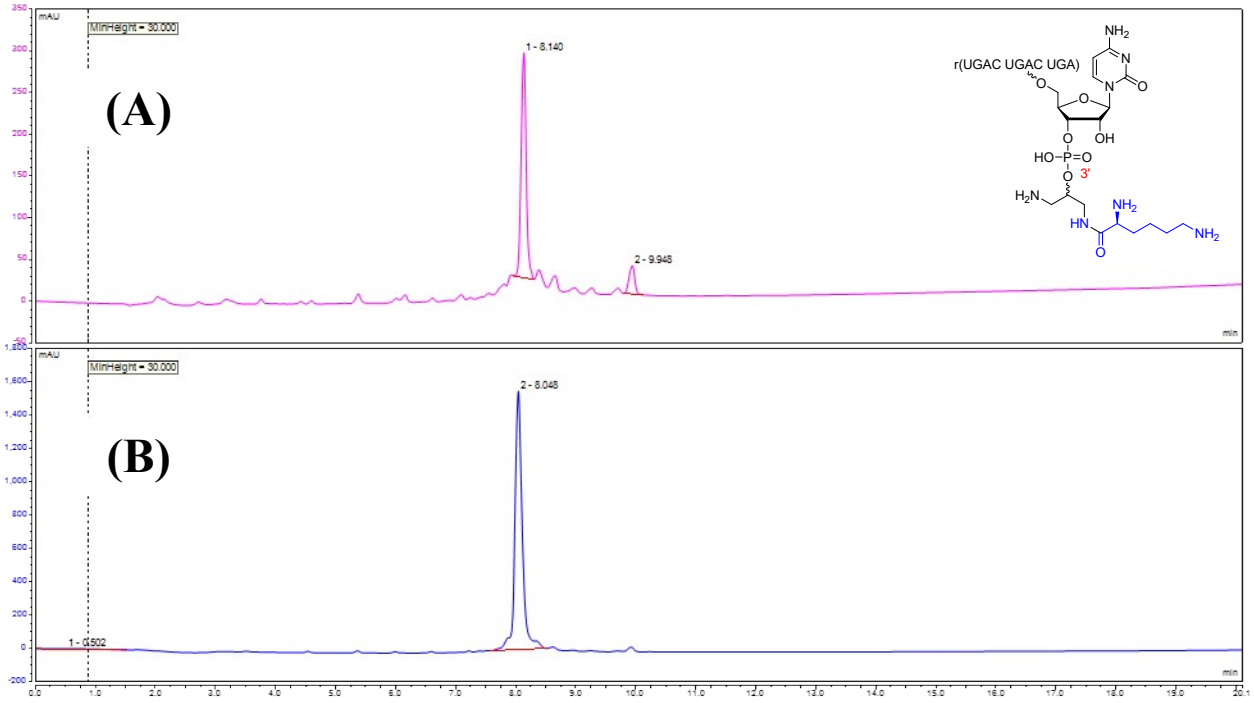
Supporting Figure S31- SAX-HPLC profile of r(UGAC UGAC UGAC)$_{(\text{diamino propanol + 4-Br Bz})}$ as a crude-analytical (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. Note that SAX-HPLC trace depicted in (**A**) is identical as the one shown in Fig. **5B**, and is reproduced here to show the difference between crude and purified traces of the sample.



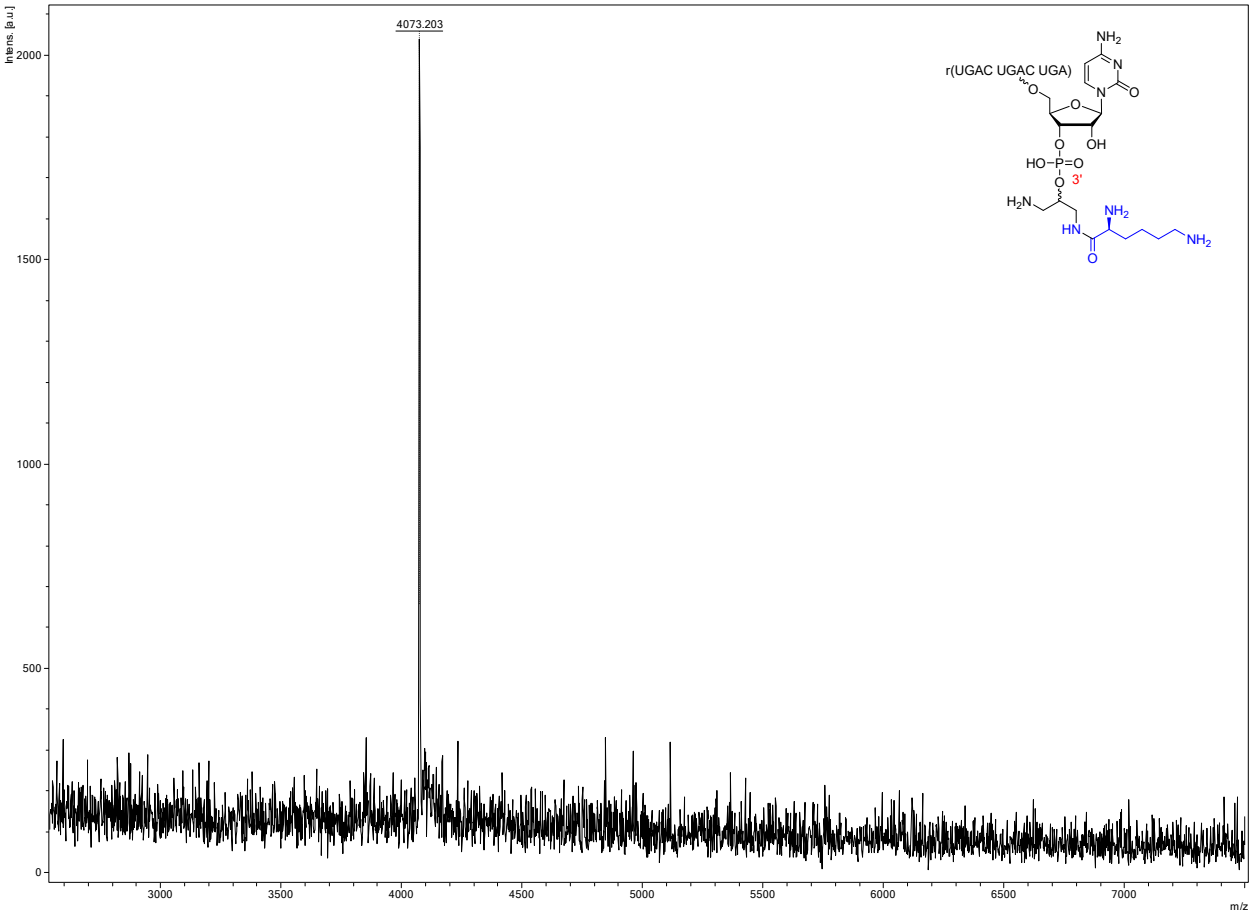
Supporting Figure S32- MALDI-TOF/TOF MS of r(UGAC UGAC UGAC) (diamino propanol + 4-Br Bz). Expected Mass: $[M-H]^-$ 4126.508; found 4126.350.



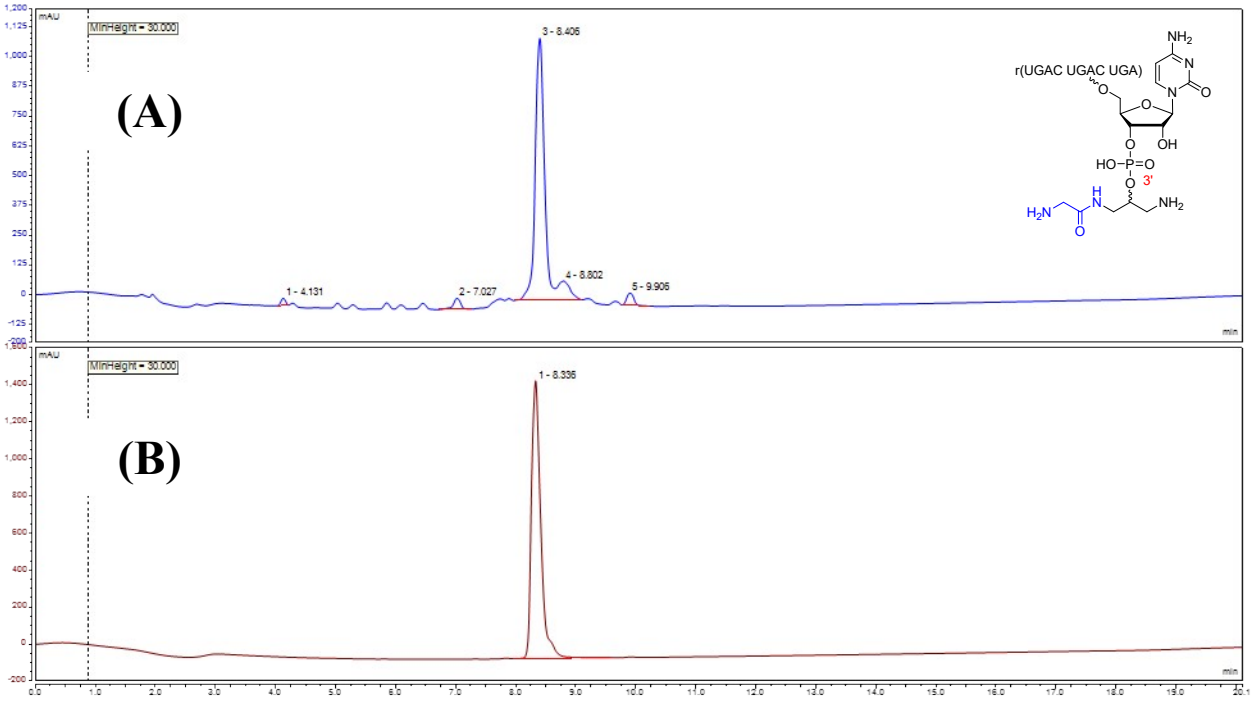
Supporting Figure S33- SAX-HPLC profile of r(UGAC UGAC UGAC) (diamino propanol + Nα,Nε-di-Fmoc-L-lysine) as a crude-analytical (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. Note that SAX-HPLC trace depicted in (**A**) is identical as the one shown in Fig. **5B**, and is reproduced here to show the difference between crude and purified traces of the sample.



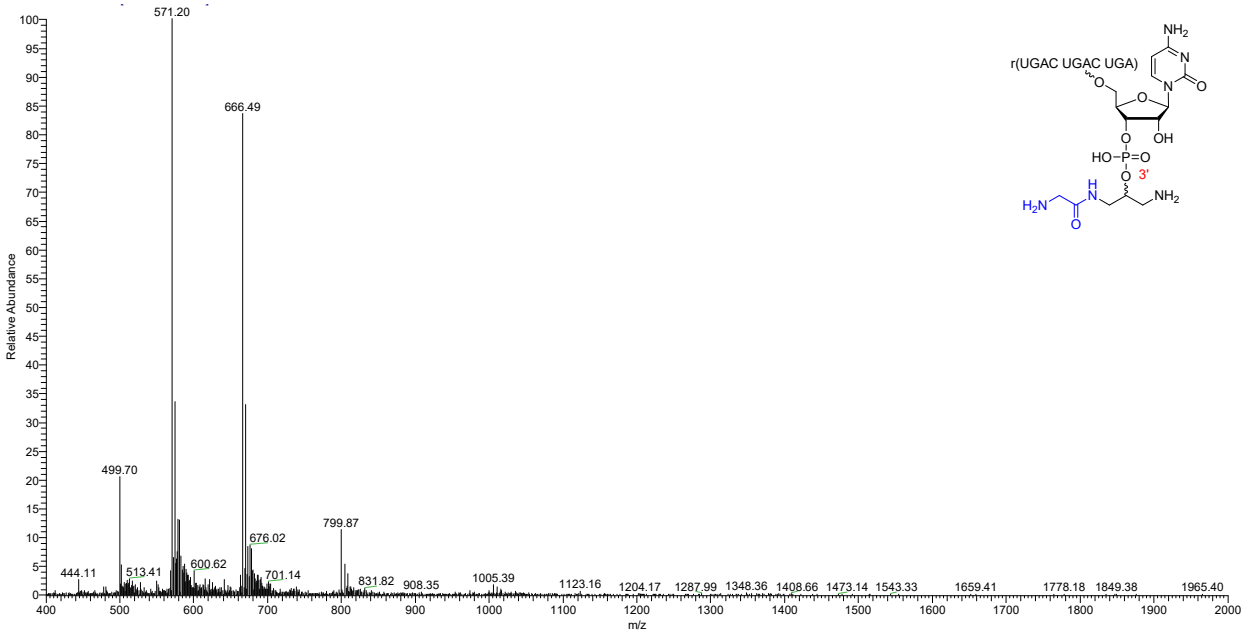
Supporting Figure S34- MALDI-TOF/TOF MS of r(UGAC UGAC UGAC) (diamino propanol + Nα,Nε-di-Fmoc-L-lysine). Expected Mass: $[M-H]^-$ 4073.670; found 4073.203.



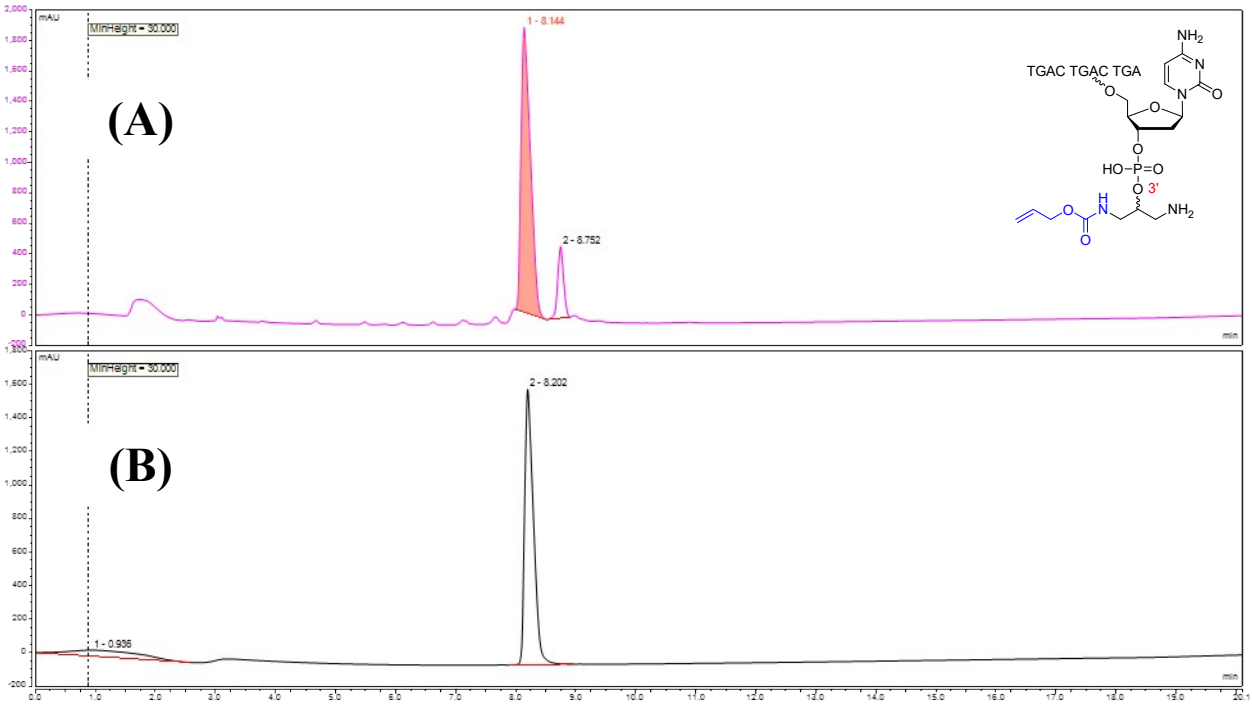
Supporting Figure S35- SAX-HPLC profile of r(UGAC UGAC UGAC) $_{(diamino\ propanol\ +\ Gly\ Fmoc)}$ as a crude-analytical (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm.



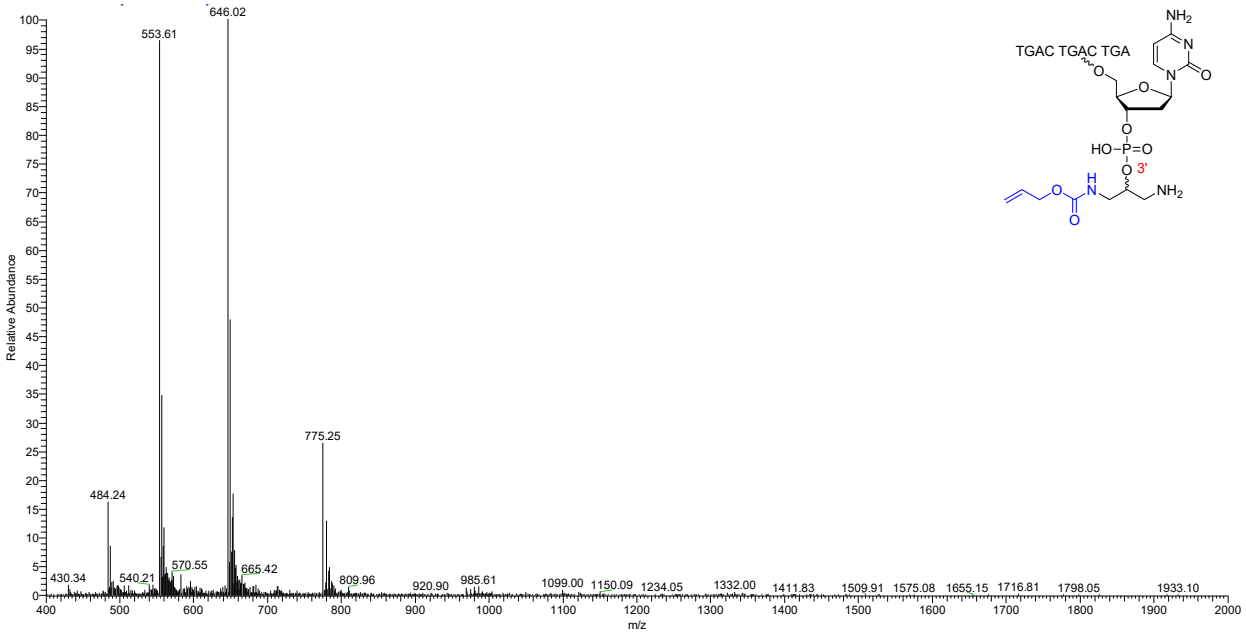
Supporting Figure S36- LR-ESI-MS of r(UGAC UGAC UGAC) (diamino propanol + Gly Fmoc). Expected Masses: $[M-8H]^{-8}$ 499.44, $[M-7H]^{-7}$ 571.94, $[M-6H]^{-6}$ 666.26; found 499.70, 571.20, 666.49.



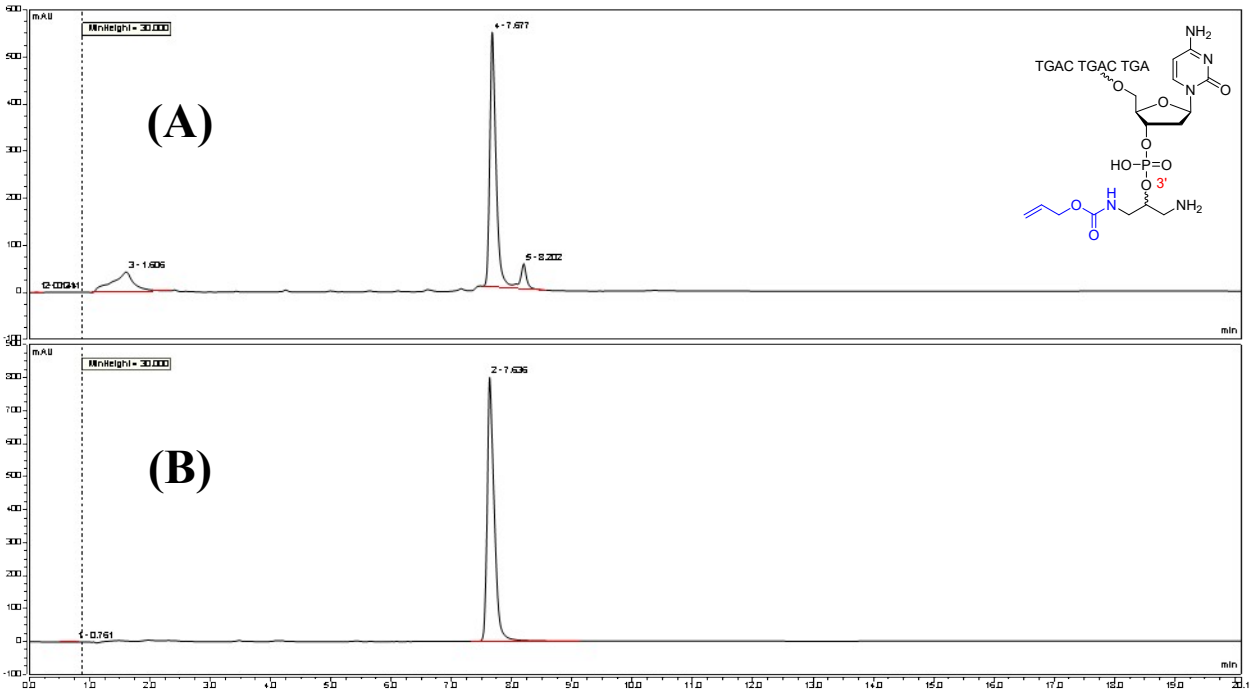
Supporting Figure S37- SAX-HPLC profile of TGAC TGAC TGAC$_{(diamino\ propanol\ +\ Alloc\text{-}OSu)}$ as a crude-analytical (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. Conjugation was performed in aqueous conditions.



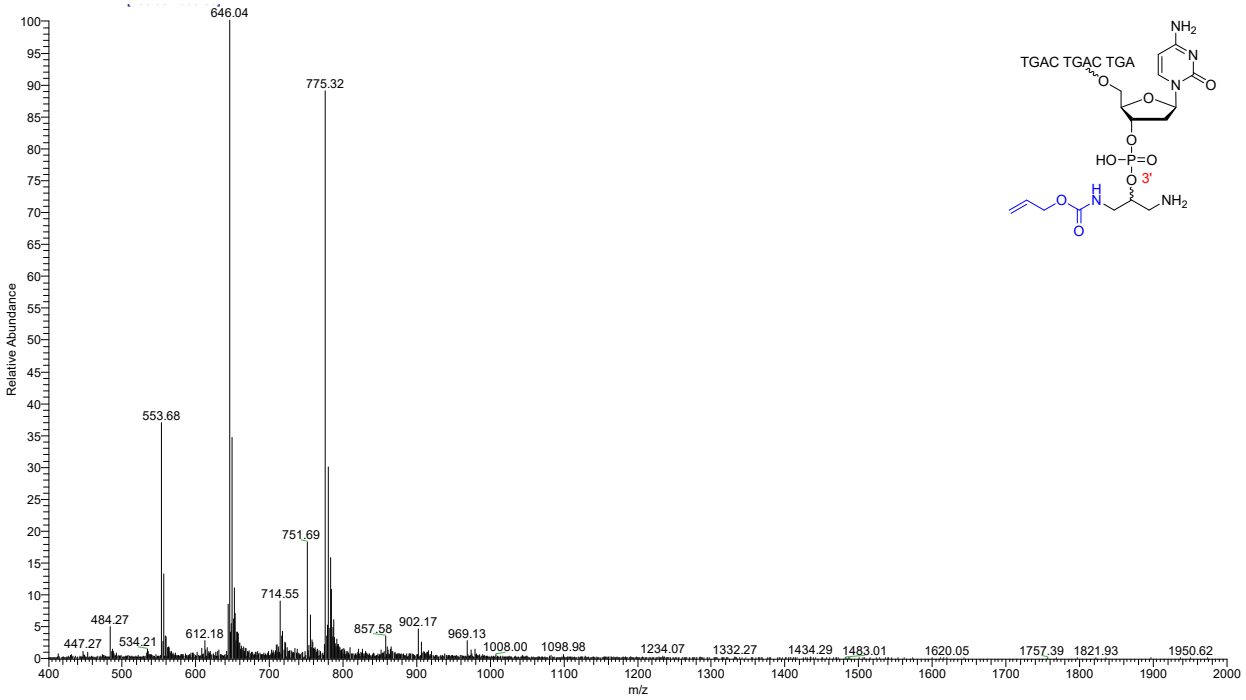
Supporting Figure S38- LR-ESI-MS of TGAC TGAC TGAC$_{(diamino\ propanol\ +\ Alloc\text{-}OSu)}$. Expected Masses: $[M\text{-}7H]^{-7}$ 553.38, $[M\text{-}6H]^{-6}$ 645.78, $[M\text{-}5H]^{-5}$ 775.14; found 553.61, 646.02, 775.25.



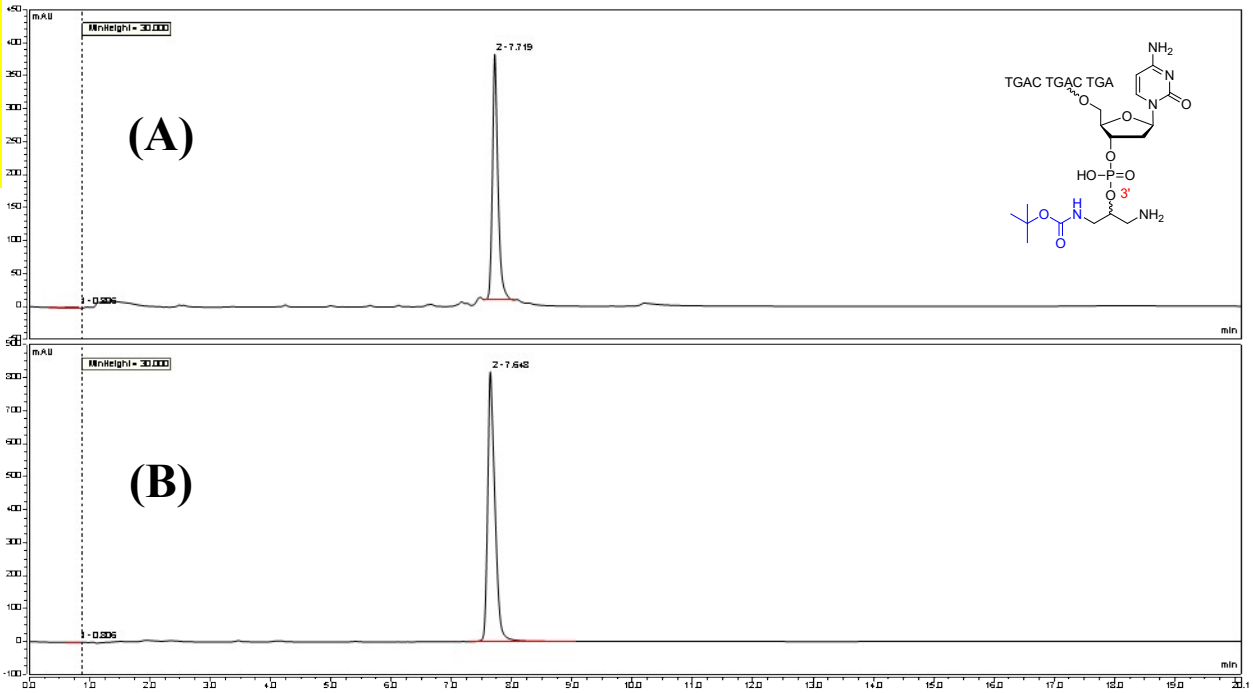
Supporting Figure S39- SAX-HPLC profile of TGAC TGAC TGAC$_{(diamino\ propanol\ +\ Alloc\text{-}OSu)}$ as a crude-analytical (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. Conjugation was performed in organic conditions. Note that SAX-HPLC trace depicted in (**A**) is identical as the one shown in Fig. **6B**, and is reproduced here to show the difference between crude and purified traces of the sample.



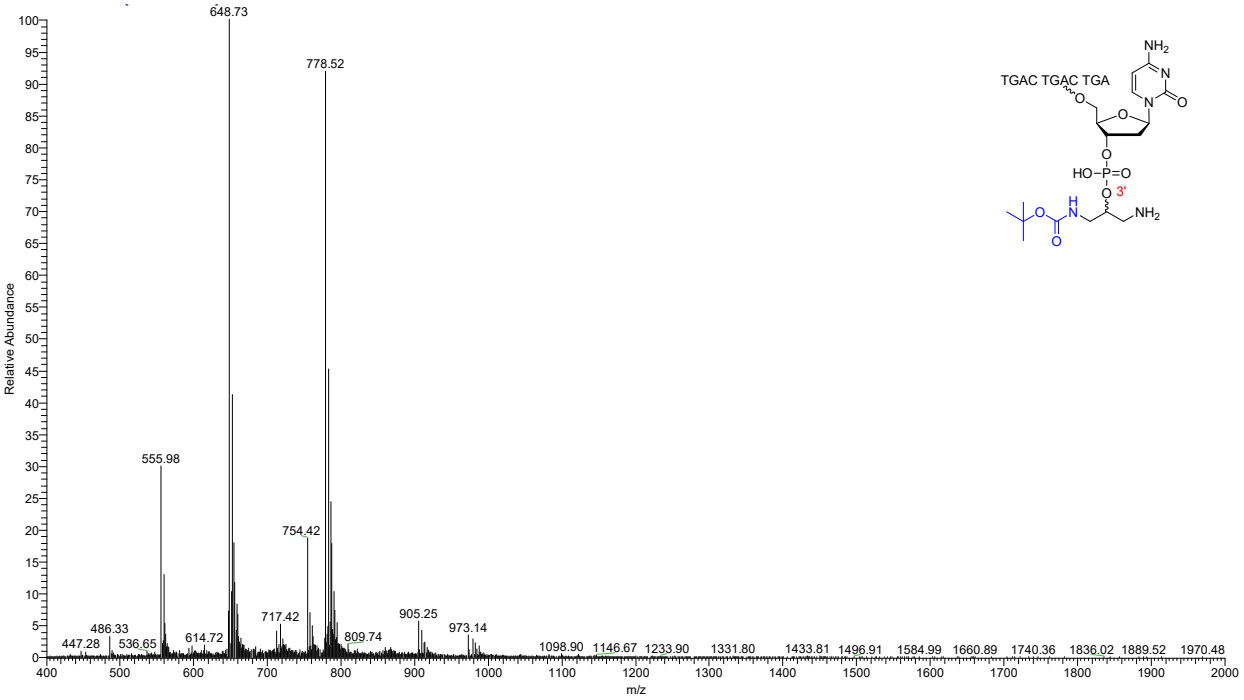
Supporting Figure S40- LR-ESI-MS of TGAC TGAC TGAC$_{(diamino\ propanol\ +\ Alloc\text{-}OSu)}$. Expected Masses: $[M-7H]^{-7}$ 553.38, $[M-6H]^{-6}$ 645.78, $[M-5H]^{-5}$ 775.14; found 553.68, 646.04, 775.32.



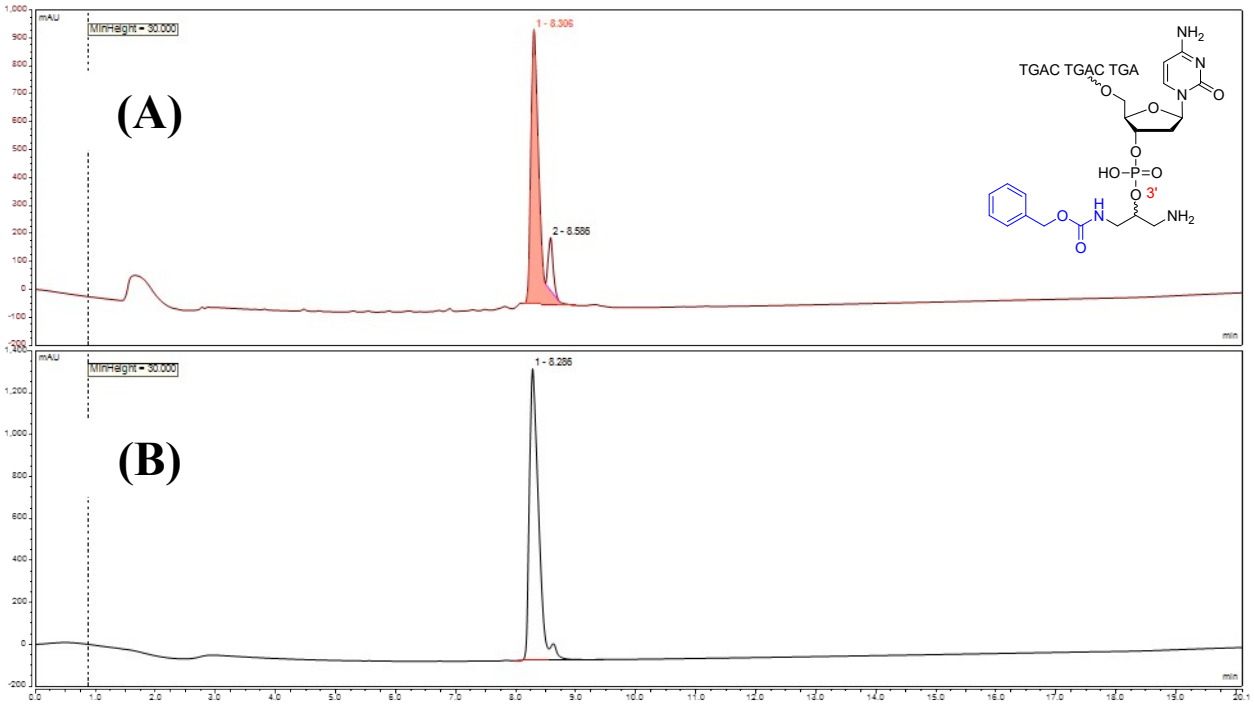
Supporting Figure S41- SAX-HPLC profile of TGAC TGAC TGAC$_{(diamino\ propanol\ +\ Boc\text{-}OSu)}$ as a crude-analytical (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. Conjugation was performed in organic conditions. Note that SAX-HPLC trace depicted in (**A**) is identical as the one shown in Fig. **6B**, and is reproduced here to show the difference between crude and purified traces of the sample.



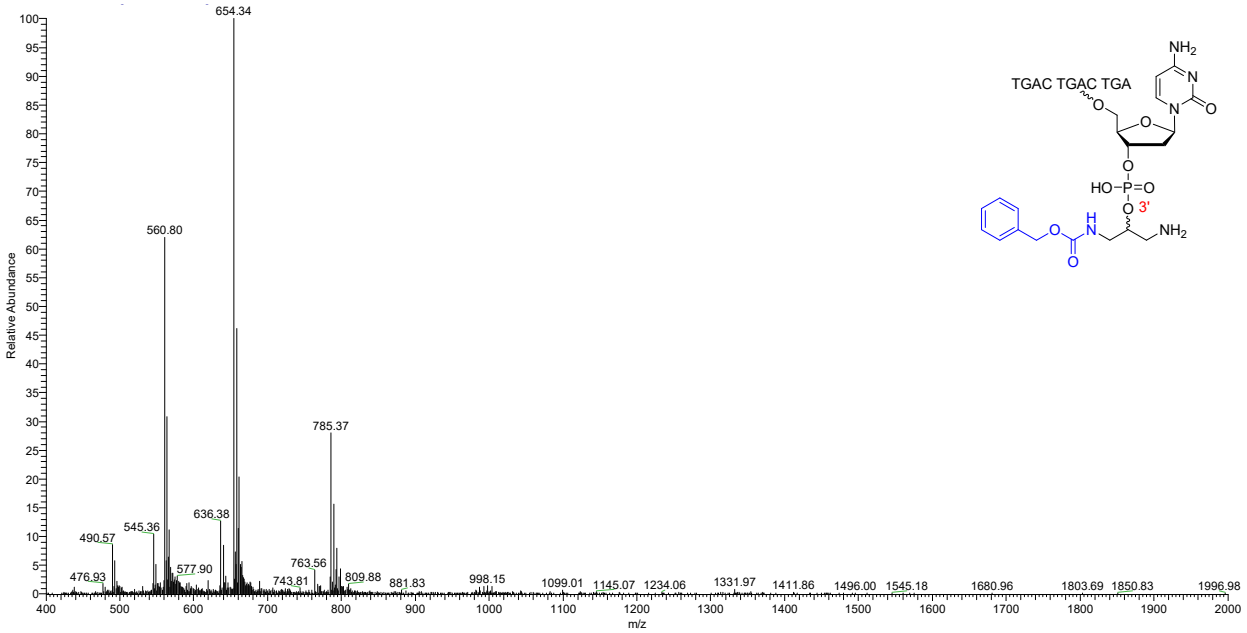
Supporting Figure S42- LR-ESI-MS of TGAC TGAC TGAC$_{(diamino\ propanol\ +\ Boc\text{-}OSu)}$. Expected Masses: $[M-7H]^{-7}$ 555.67, $[M-6H]^{-6}$ 648.45, $[M-5H]^{-5}$ 778.34; found 555.98, 648.73, 778.52.



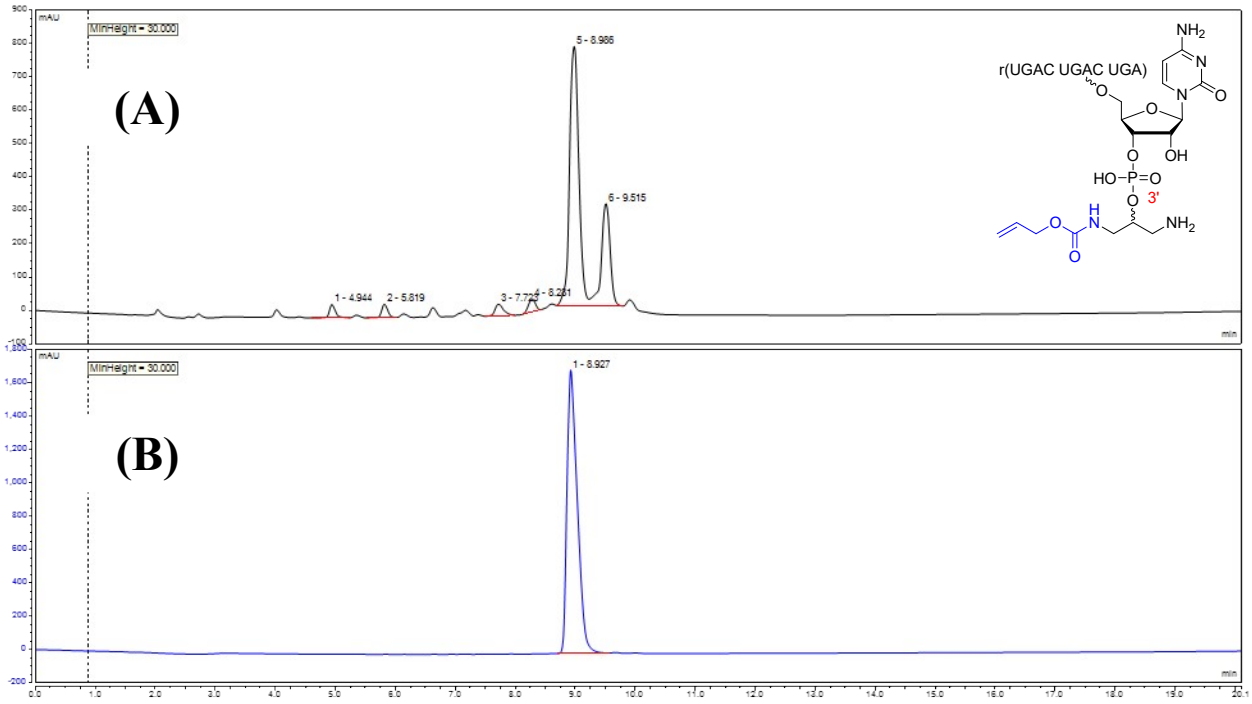
Supporting Figure S43- SAX-HPLC profile of TGAC TGAC TGAC$_{(diamino\ propanol\ +\ CBz\text{-}OSu)}$ as a crude-analytical (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. Conjugation was performed in aqueous conditions.



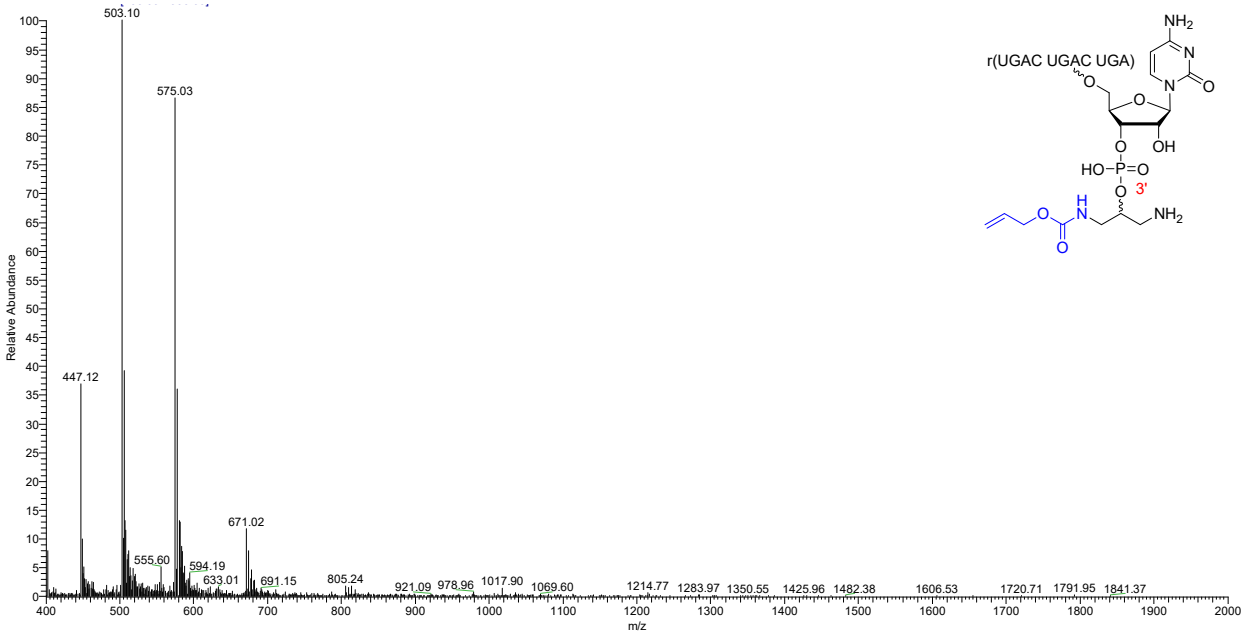
Supporting Figure S44- LR-ESI-MS of TGAC TGAC TGAC$_{(\text{diamino propanol + CBz-OSu})}$. Expected Masses: $[M-7H]^{-7}$ 560.53, $[M-6H]^{-6}$ 654.11, $[M-5H]^{-5}$ 785.14; found 560.80, 654.34, 785.37.



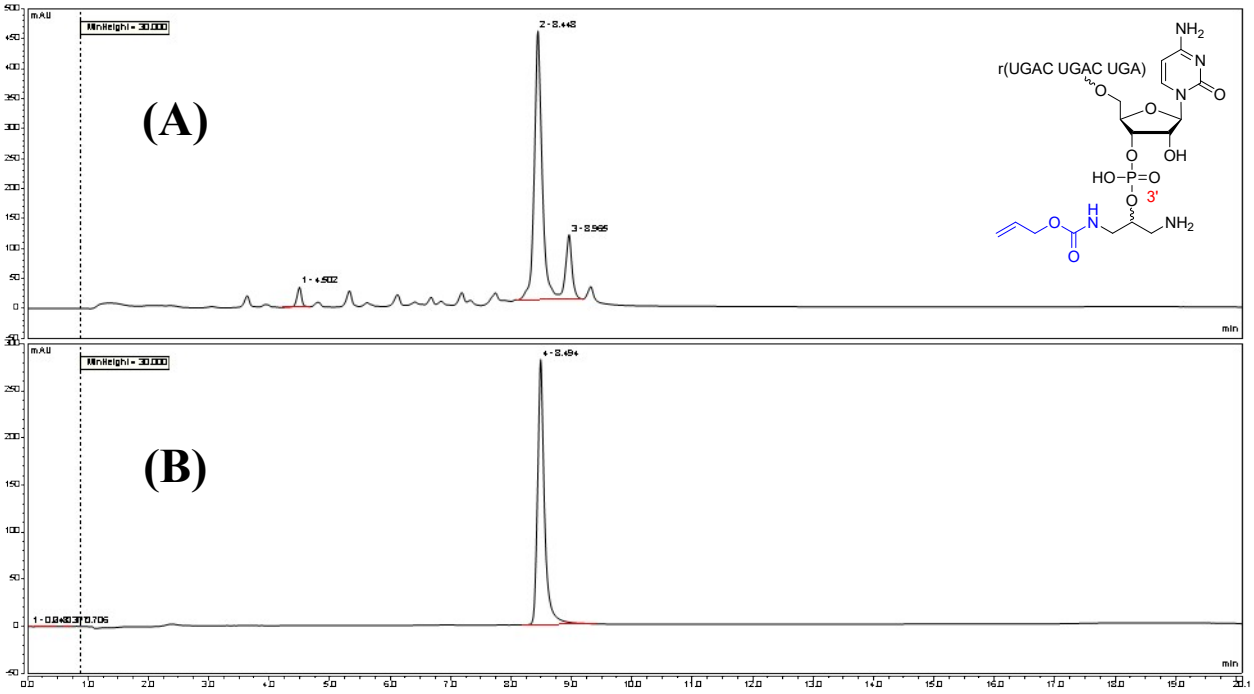
Supporting Figure S45- SAX-HPLC profile of r(UGAC UGAC UGAC) $_{(\text{diamino propanol + Alloc-OSu})}$ as a crude-analytical (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. Conjugation was performed in aqueous conditions.



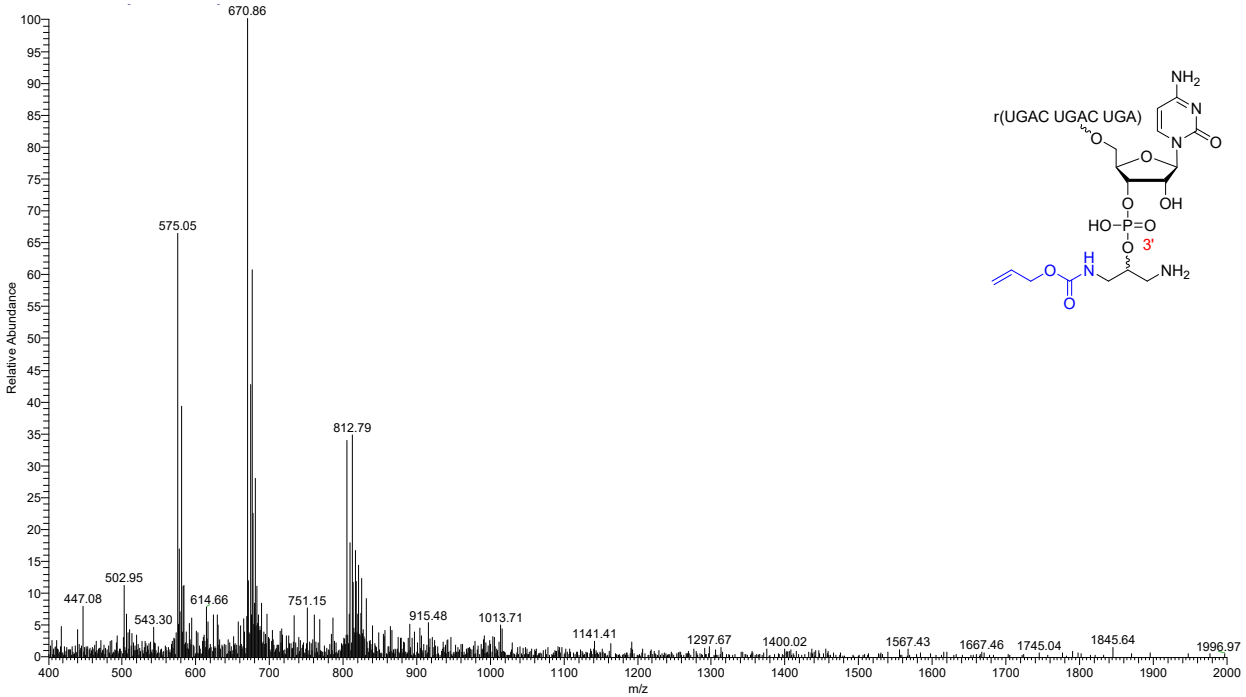
Supporting Figure S46- LR-ESI-MS of r(UGAC UGAC UGAC) $_{(\text{diamino propanol + Alloc-OSu})}$. Expected Masses: $[M-9H]^{-9}$ 446.84, $[M-8H]^{-8}$ 502.82, $[M-7H]^{-7}$ 574.79; found 447.12, 503.10, 575.03.



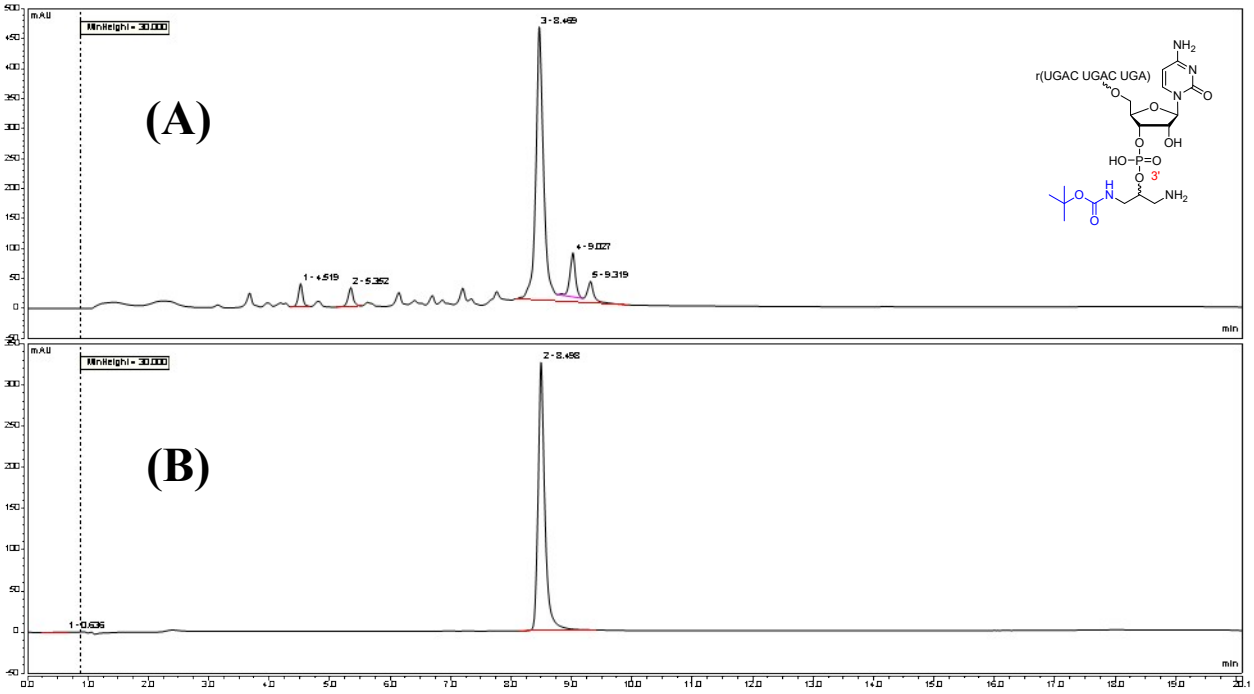
Supporting Figure S47- SAX-HPLC profile of r(UGAC UGAC UGAC) (diamino propanol + Alloc-OSu) as a crude-analytical (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. Conjugation was performed in organic conditions. Note that SAX-HPLC trace depicted in (**A**) is identical as the one shown in Fig. **6B**, and is reproduced here to show the difference between crude and purified traces of the sample.



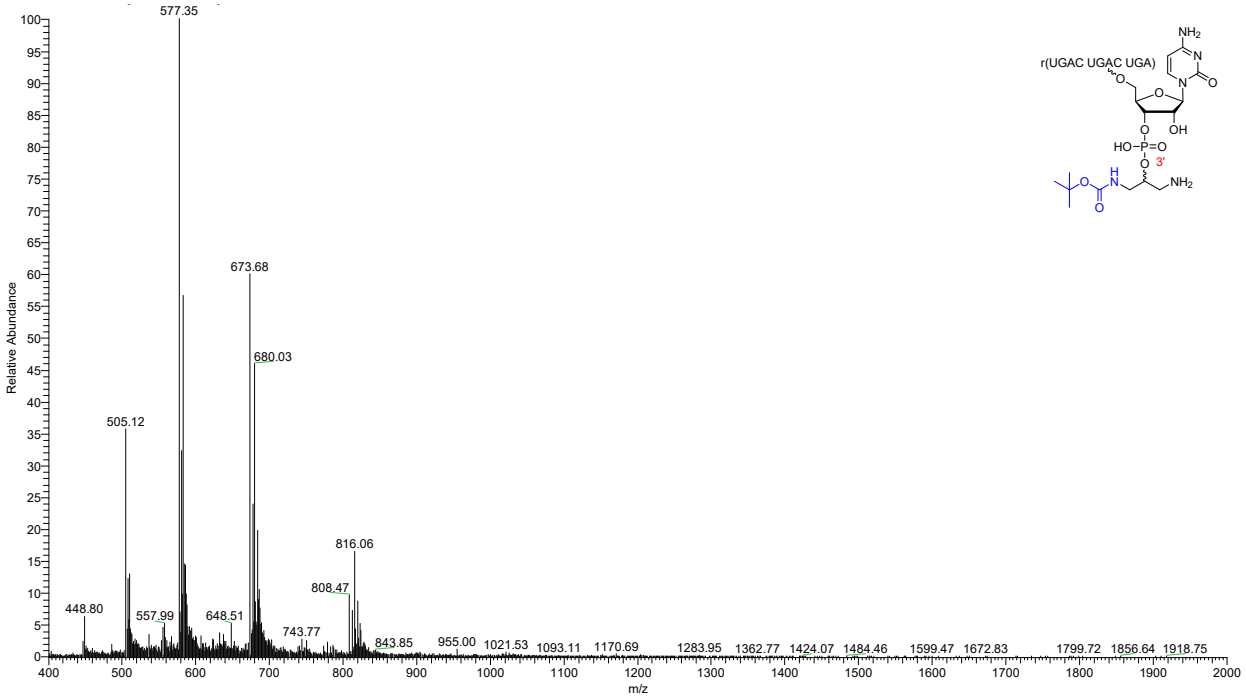
Supporting Figure S48- LR-ESI-MS of r(UGAC UGAC UGAC) (diamino propanol + Alloc-OSu). Expected Masses: $[M-8H]^{-8}$ 502.82, $[M-7H]^{-7}$ 574.79, $[M-6H]^{-6}$ 670.76; found 502.95, 575.03, 670.86.



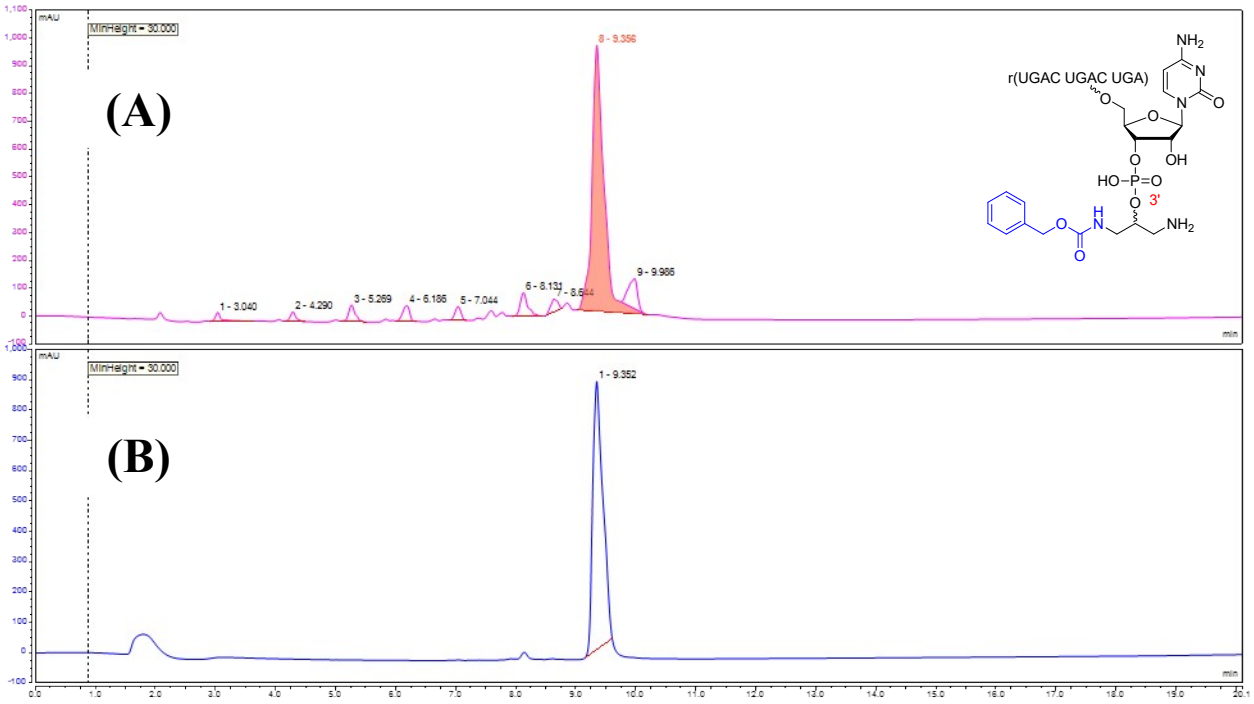
Supporting Figure S49- SAX-HPLC profile of r(UGAC UGAC UGAC) $_{(\text{diamino propanol + Boc-OSu})}$ as a crude-analytical (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. Conjugation was performed in organic conditions. Note that SAX-HPLC trace depicted in (**A**) is identical as the one shown in Fig. **6B**, and is reproduced here to show the difference between crude and purified traces of the sample.



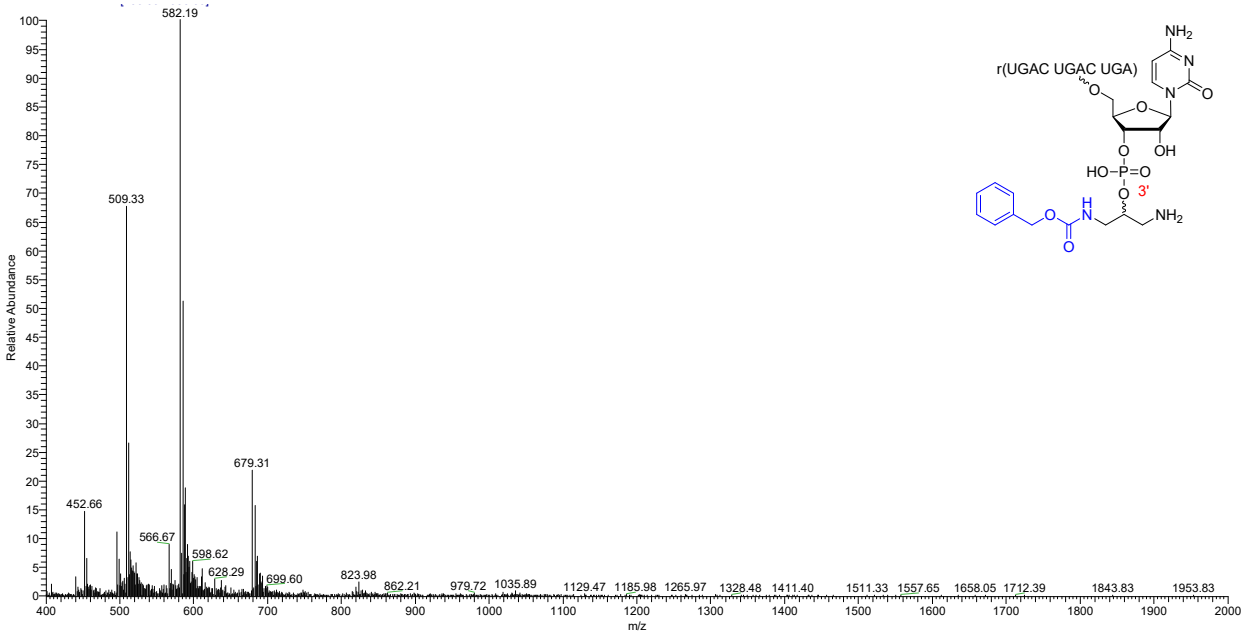
Supporting Figure S50- LR-ESI-MS of r(UGAC UGAC UGAC) (diamino propanol + Boc-OSu). Expected Masses: $[M-8H]^{-8}$ 504.82, $[M-7H]^{-7}$ 577.08, $[M-6H]^{-6}$ 673.43; found 505.12, 577.35, 673.68.



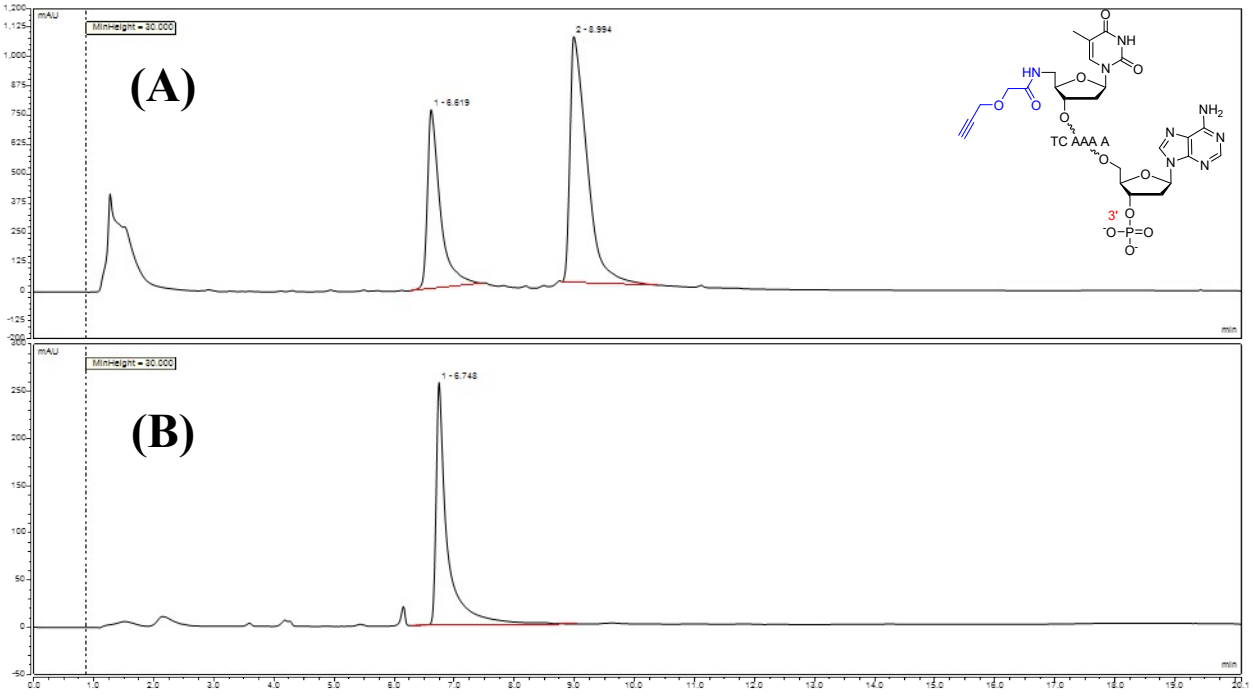
Supporting Figure S51- SAX-HPLC profile of r(UGAC UGAC UGAC) $_{(diamino\ propanol\ +\ CBz\text{-}OSu)}$ as a crude-analytical (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. Conjugation was performed in aqueous conditions.



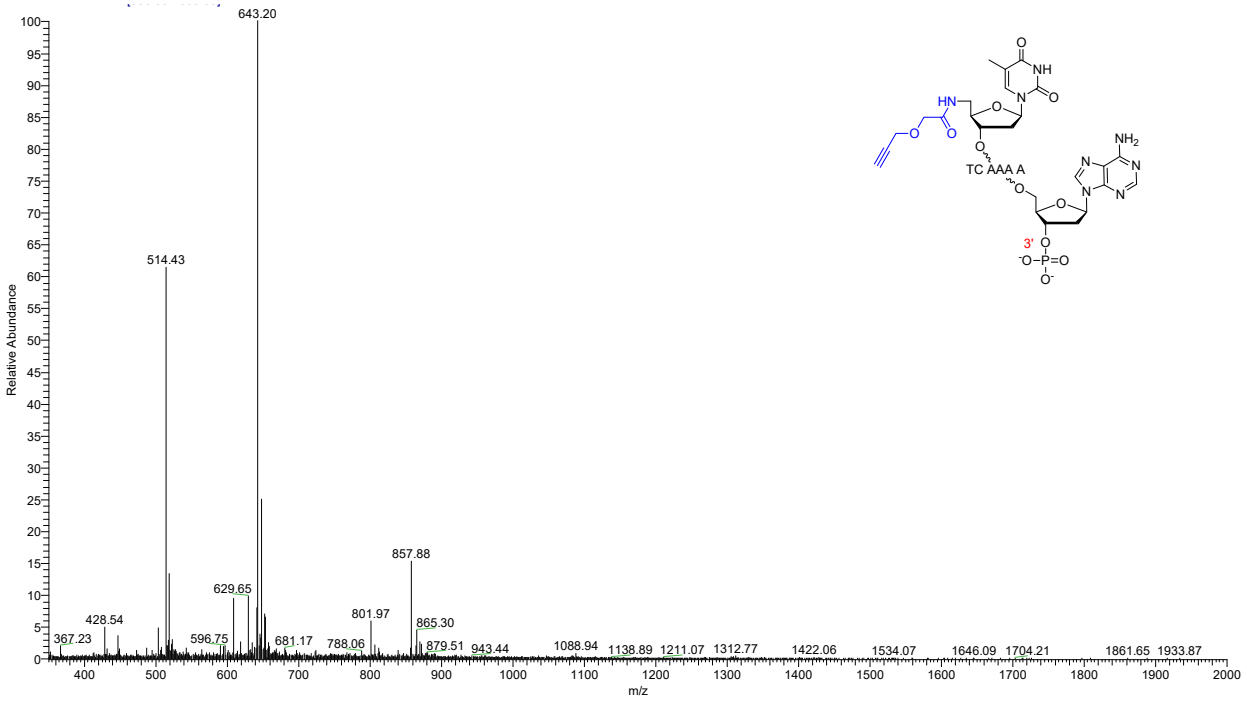
Supporting Figure S52- LR-ESI-MS of r(UGAC UGAC UGAC) (diamino propanol + CBz-OSu). Expected Masses: $[M-9H]^{-9}$ 452.39, $[M-8H]^{-8}$ 509.07, $[M-7H]^{-7}$ 581.94; found 452.66, 509.33, 582.19.



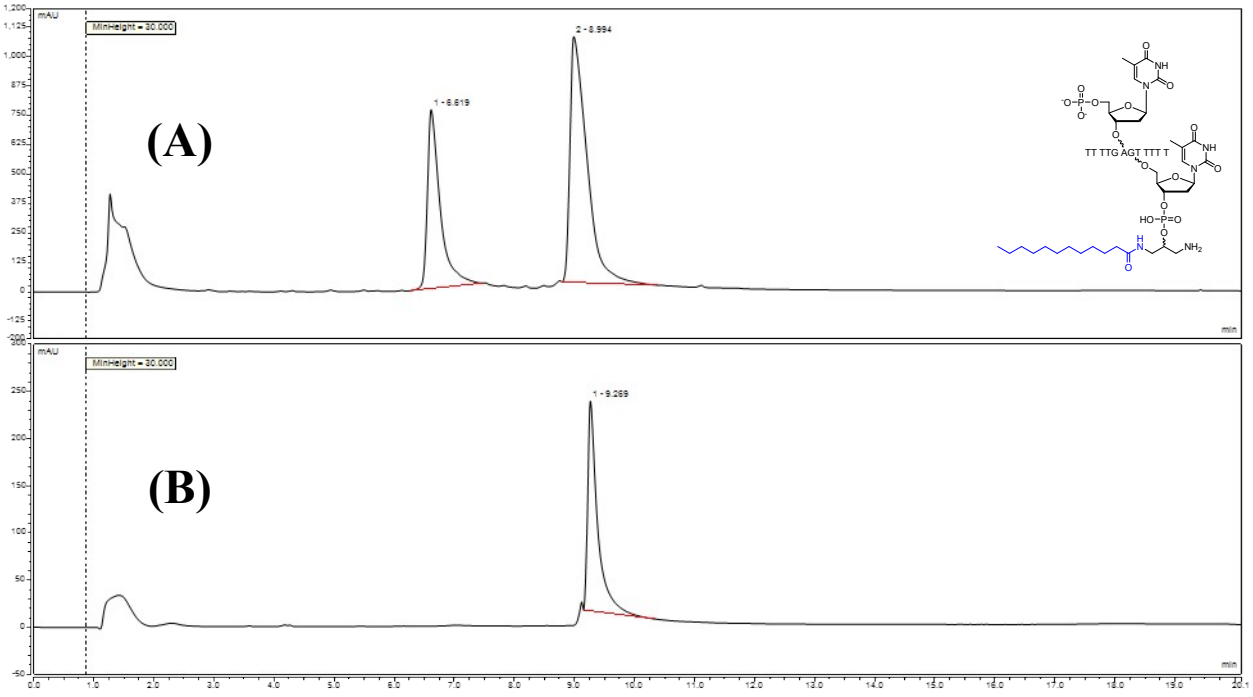
Supporting Figure S53- SAX-HPLC profile of $^{(Propargyl\ NHS\ Ester\ +\ NH2)}$TTC AAA AAp as a crude-analytical TOS experiment represented in Fig. 7 (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. Note that SAX-HPLC trace depicted in (**A**) is identical as the one shown in Fig. **7C**, and is reproduced here to show the difference between crude and purified traces of the sample.



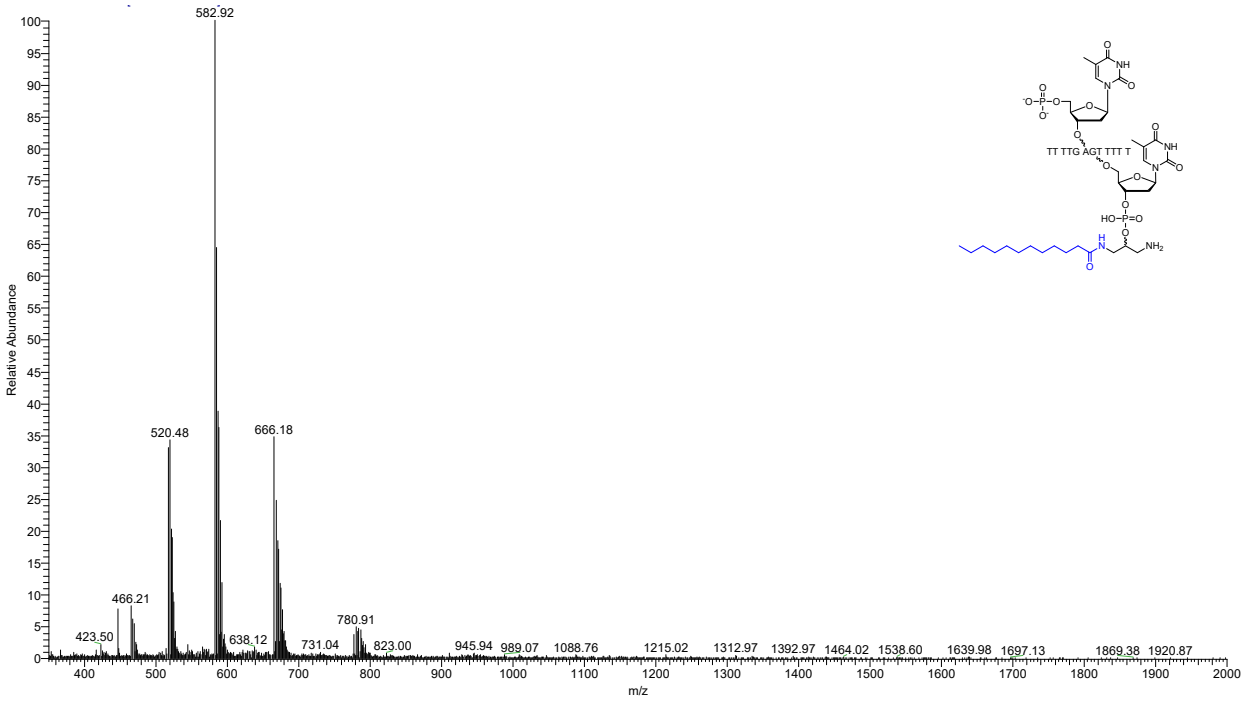
Supporting Figure S54- LR-ESI-MS of $^{(Propargyl\ NHS\ Ester\ +\ NH2)}$TTC AAA AAp. Expected Masses: $[M-5H]^{-5}$ 514.09, $[M-4H]^{-4}$ 642.86, $[M-3H]^{-3}$ 857.82; found 514.43, 643.20, 857.88.



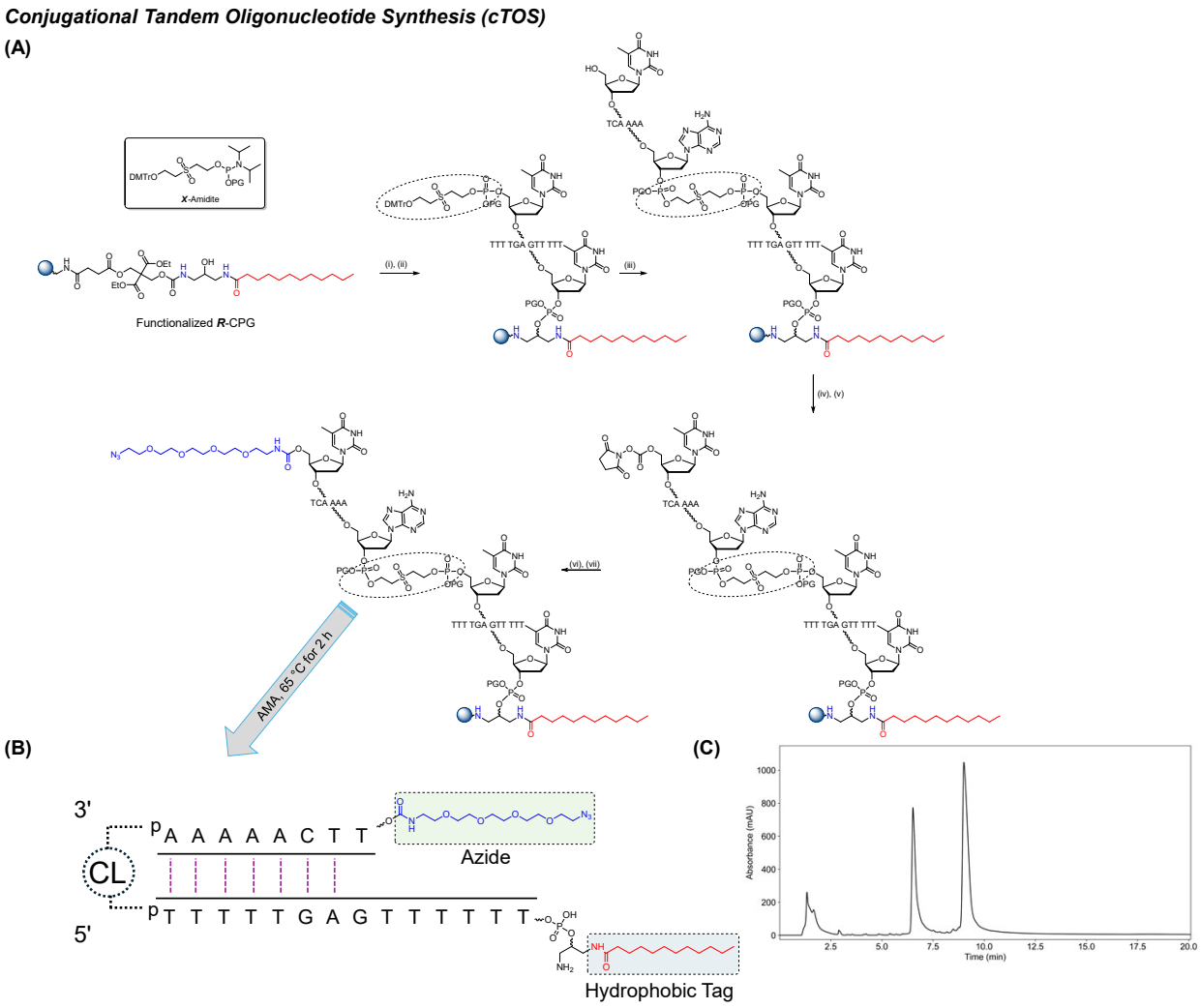
Supporting Figure S55- SAX-HPLC profile of pTTT TTG AGT TTT TT$_{(diamino\ propanol + Lauric\text{-}OSu)}$ as a crude-analytical of the TOS experiment represented in Fig. 7 (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. Note that SAX-HPLC trace depicted in (**A**) is identical as the one shown in Fig. **7C**, and is reproduced here to show the difference between crude and purified traces of the sample.



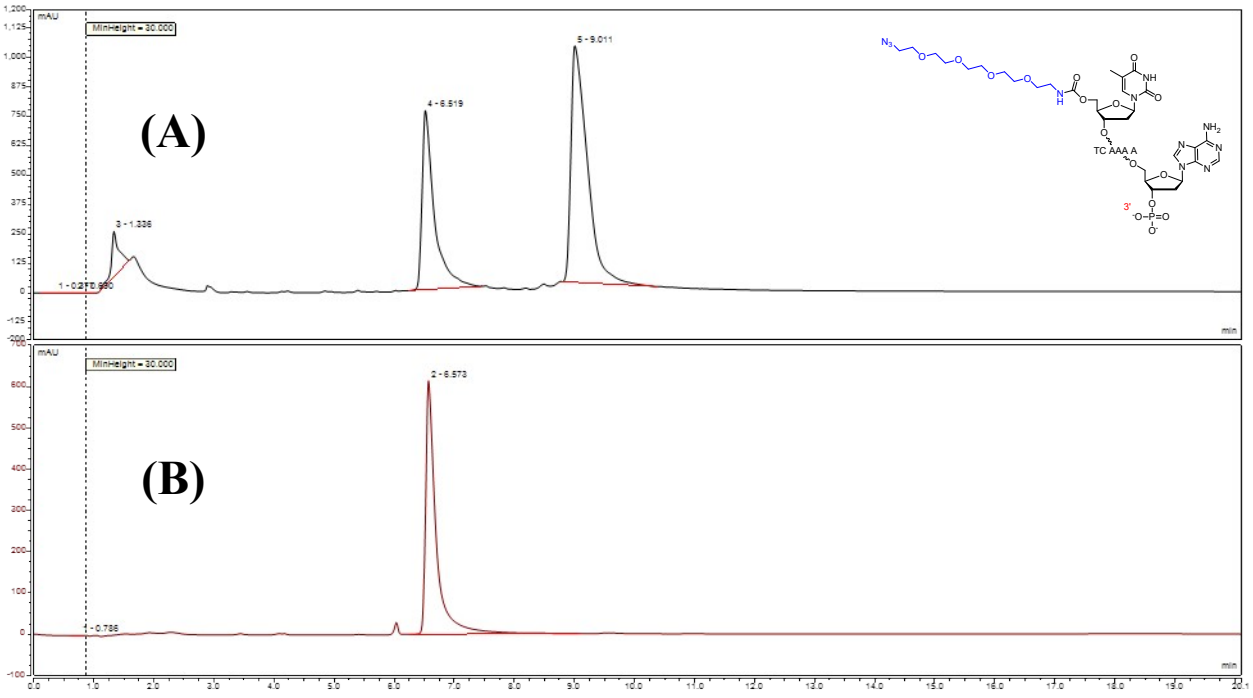
Supporting Figure S56- LR-ESI-MS of pTTT TTG AGT TTT TT$_{(diamino\ propanol\ +\ Lauric\text{-}OSu)}$. Expected Masses: $[M-8H]^{-8}$ 582.60, $[M-7H]^{-7}$ 666.12; found 582.92, 666.18.



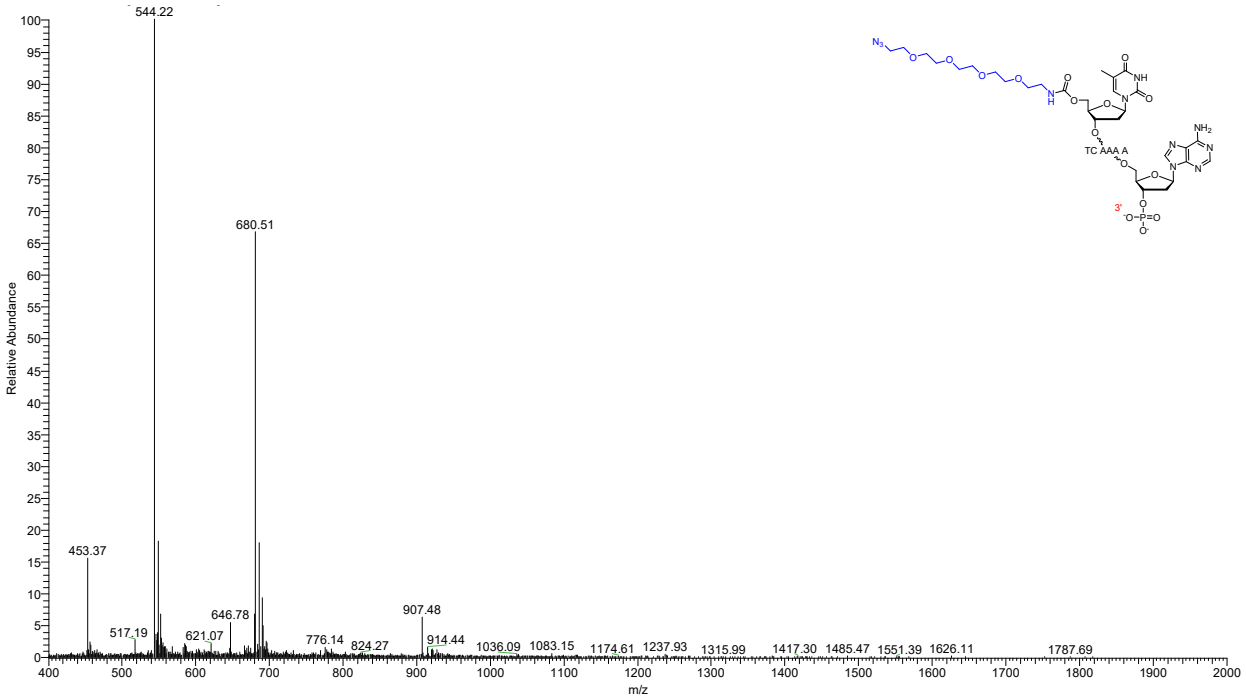
Supporting Figure S57. Tandem synthesis of a DNA duplex bearing ligand modifiers on both strands. ***R***-CPG was functionalized as previously described and conjugated at the 5′-end via DSC-mediated carbamate synthesis. **(A)** Overall scheme for the solid-phase conjugation of two duplex strands. (i) Extension of the first duplex strand (TTT TTG AGT TTT TT). (ii) Incorporation of a base-labile sulfonyl **X**-amidite. (iii) Extension of the second duplex strand (^{HO}TTC AAA AA). (iv) On-solid-support mixed carbonate synthesis using a saturated solution of *N,N*′-disuccinimidyl carbonate (DSC) in 1:1 (v/v) MeCN/pyridine (500 µL) for 2 h. (v) Washing of the solid support with MeCN (10 mL). (vi) Coupling of amine-PEG$_4$-azide (50 mM, 600 µL) in 100 mM HEPES buffer (pH 8.0) containing 50% (v/v) DMF for 5 min. (vii) Washing of the solid support with H_2O, 50% (v/v) MeCN/H_2O, and MeCN (10 mL each). **(B)** Schematic representation of conjugational tandem oligonucleotide synthesis (cTOS) of the duplex. Hybridization is expected to occur only after cleavage and deprotection under native conditions (purification may be required). **(C)** Crude SAX-HPLC trace of the cTOS mixture containing the conjugated DNA strands. The yields of the purified oligonucleotide, calculated relative to the manufacturer's specified loading and the amount of solid support used, are provided in **Table S2**. PG and CL denote the ethyl-cyano protecting group and cleavable linker, respectively**.**



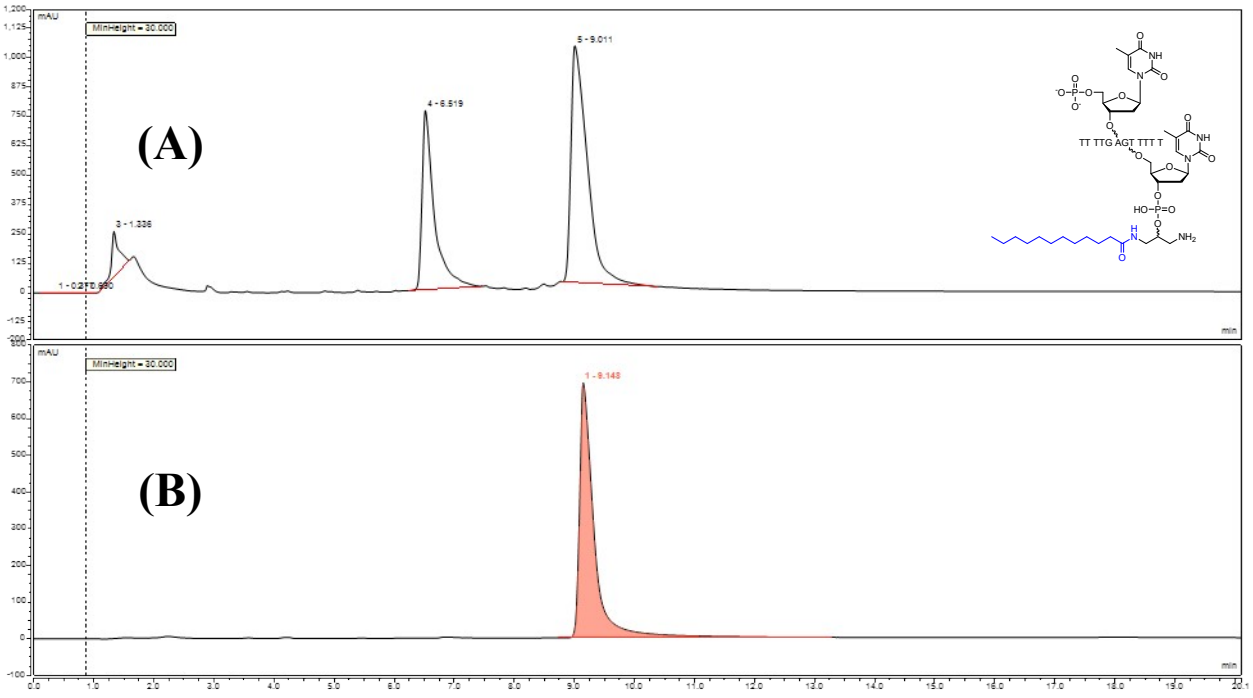
Supporting Figure S58- SAX-HPLC profile of $^{(Amine\text{-}Peg4\text{-}Azide + HO)}$TTC AAA AAp as a crude-analytical of the TOS experiment represented in Fig. S58 (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm.



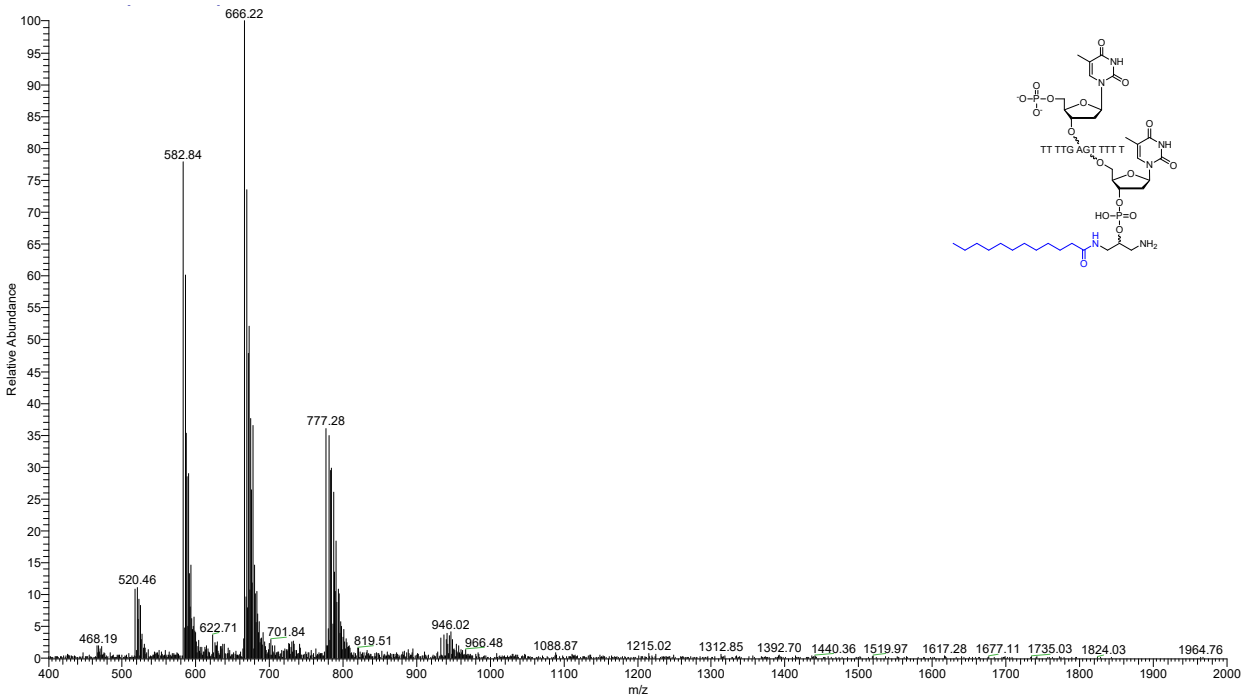
Supporting Figure S59- LR-ESI-MS of $^{(\text{Amine-Peg4-Azide + HO})}$TTC AAA AAp. Expected Masses: $[M-6H]^{-6}$ 453.09, $[M-5H]^{-5}$ 543.90, $[M-4H]^{-4}$ 680.13; found 453.37, 544.22, 680.51.



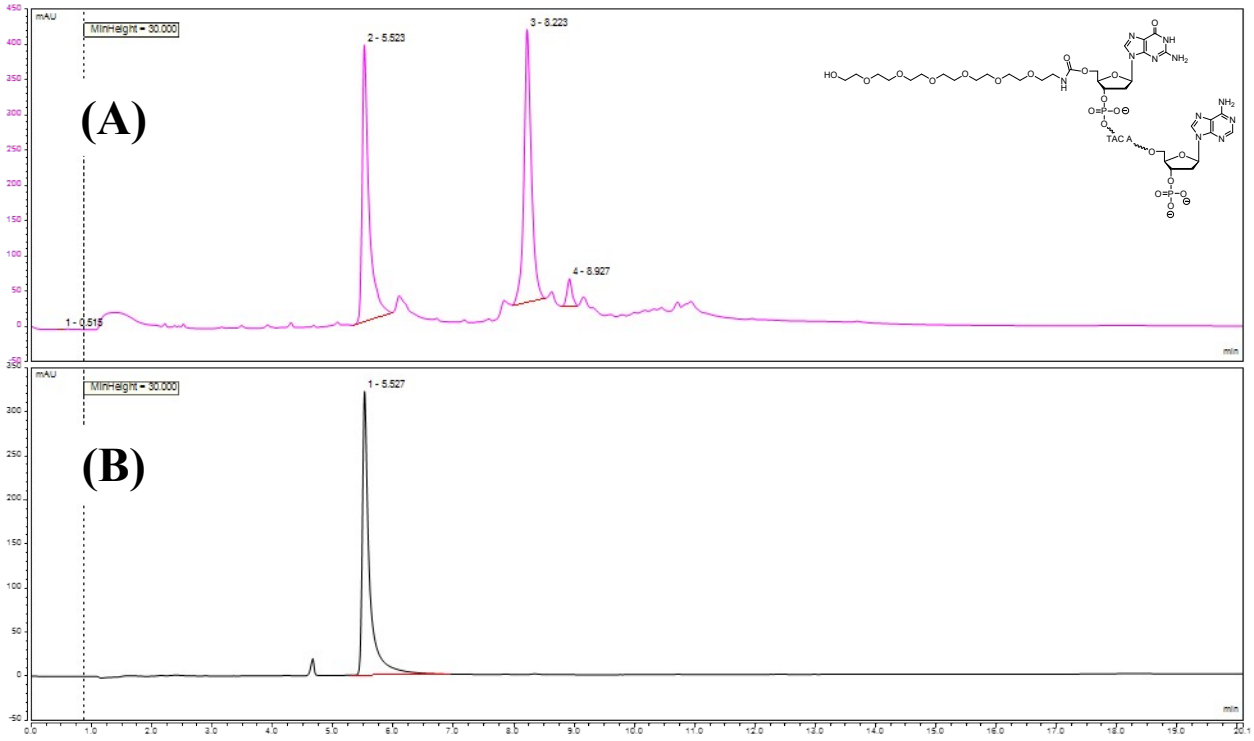
Supporting Figure S60- SAX-HPLC profile of pTTT TTG AGT TTT $TT_{(diamino\ propanol\ +\ Lauric\text{-}OSu)}$ as a crude-analytical of the TOS experiment represented in Fig. S58 (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm.



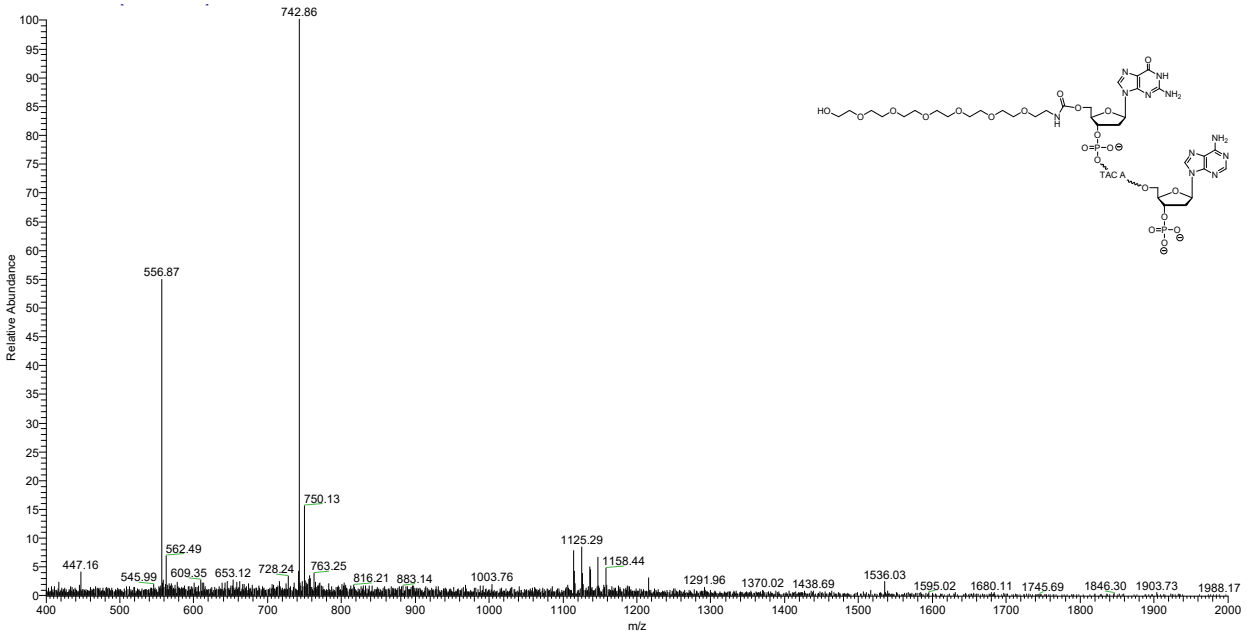
Supporting Figure S61- LR-ESI-MS of pTTT TTG AGT TTT TT$_{(diamino\ propanol\ +\ Lauric\text{-}OSu)}$. Expected Masses: $[M-8H]^{-8}$ 582.60, $[M-7H]^{-7}$ 666.12, $[M-6H]^{-6}$ 777.31; found 582.84, 666.22, 777.28.



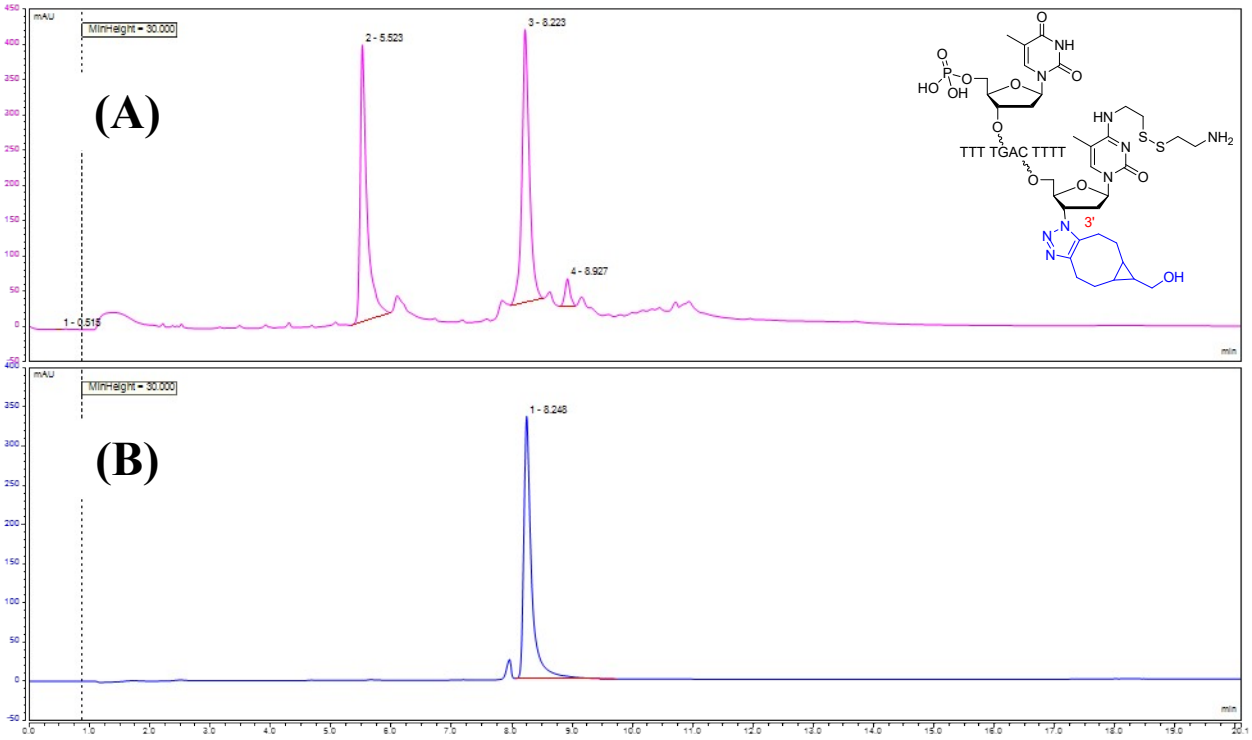
Supporting Figure S62- SAX-HPLC of GTCA $AAp_{(Amine\text{-}Peg7\text{-}Alcohol)}$ as a crude-analytical of the TOS experiment represented in Fig. 8 (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. Note that SAX-HPLC trace depicted in (**A**) is identical as the one shown in Fig. **8C**, and is reproduced here to show the difference between crude and purified traces of the sample.



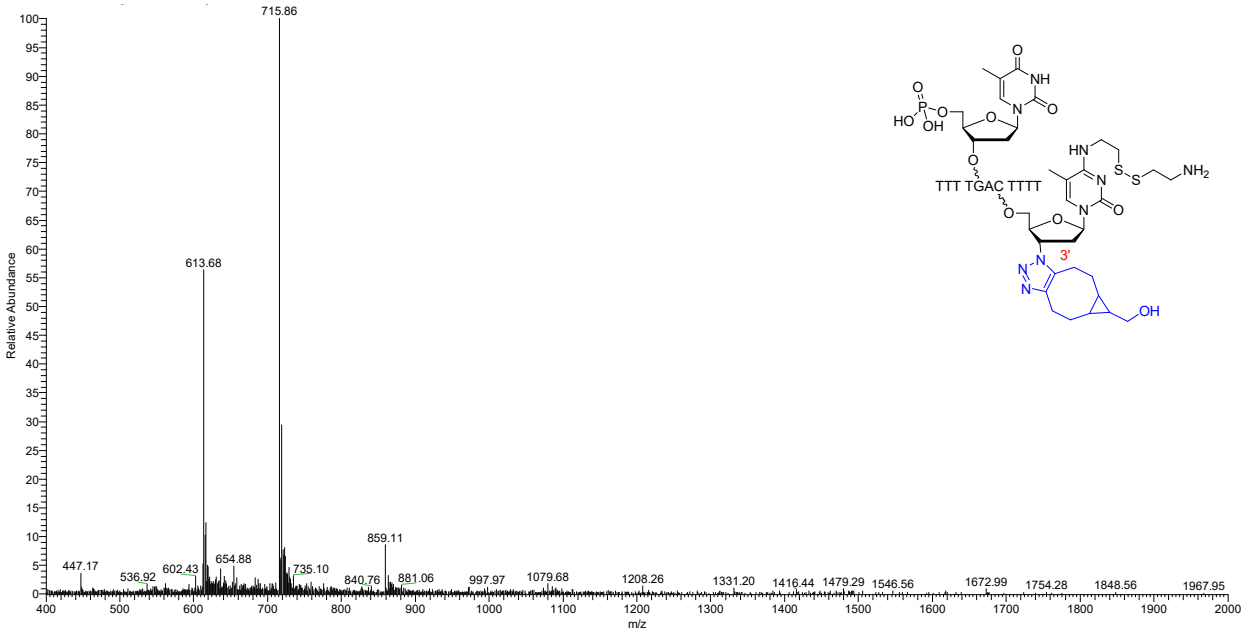
Supporting Figure S63- LR-ESI-MS of GTCA AAp $_{(Amine\text{-}Peg7\text{-}Alcohol)}$. Expected Masses: $[M\text{-}4H]^{-4}$ 556.62, $[M\text{-}3H]^{-3}$ 742.50; found 556.87, 742.86.



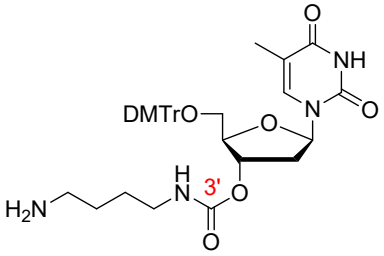
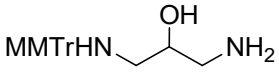
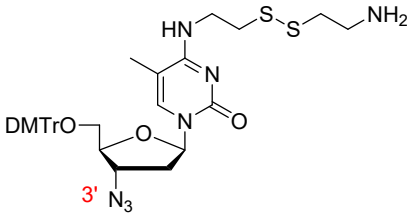
Supporting Figure S64- SAX-HPLC profile of pTTT TTG ACT TTT $C_{(Azide + BCN\text{-}OH)}$ as a crude-analytical of the TOS experiment represented in Fig. 8 (A) and purified (B). The column was eluted using a linear gradient of 0-75% buffer B over 20 min (buffer A: 50 mM Tris HCl, pH 7.61, 10% acetonitrile and buffer B: 50mMTris HCl, pH 7.61, 10% acetonitrile, 1 M NaCl) at 55 °C and flow rate of 1 mL/min. Monitoring was accomplished at 260 nm. Note that SAX-HPLC trace depicted in (**A**) is identical as the one shown in Fig. **8C**, and is reproduced here to show the difference between crude and purified traces of the sample.



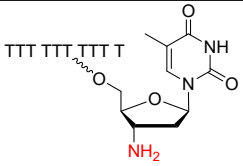
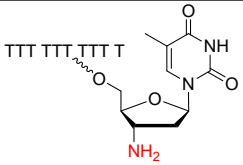
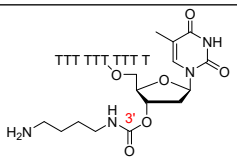
Supporting Figure S65- LR-ESI-MS of pTTT TTG ACT TTT C $_{(Azide + BCN\text{-}OH).}$ Expected Masses: $[M-7H]^{-7}$ 613.53, $[M-6H]^{-6}$ 715.79, $[M-5H]^{-5}$ 859.15; found 613.68, 715.86, 859.11.

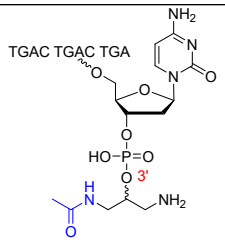
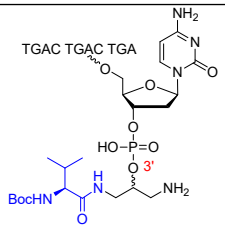
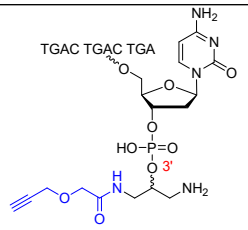
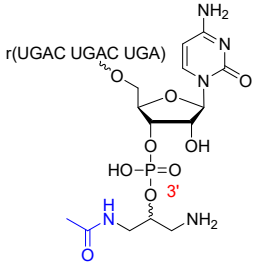
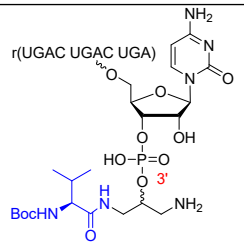
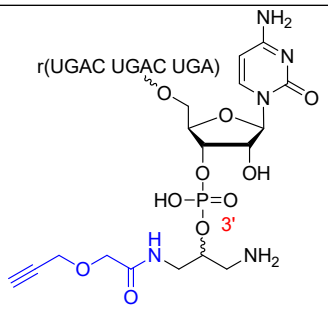


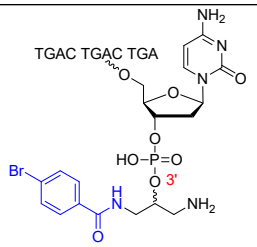
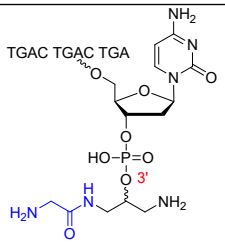
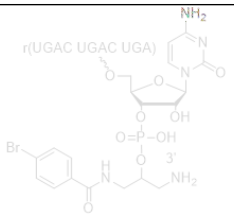
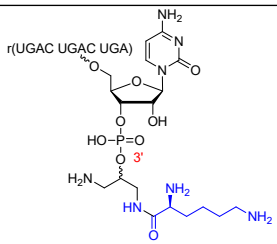
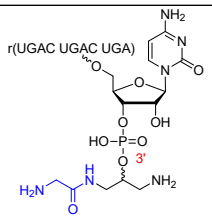
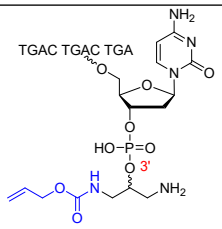
Supporting Table S1- Loading of functionalized ***R***-CPG solid supports used in this study, determined *via* quantitative trityl assay.[6]

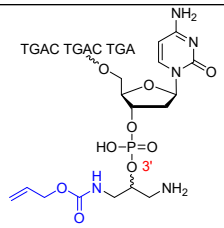
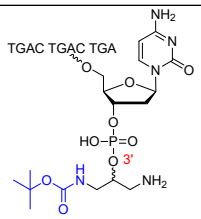
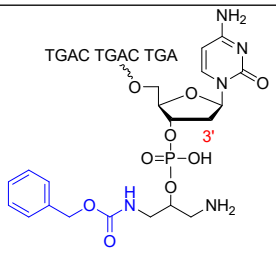
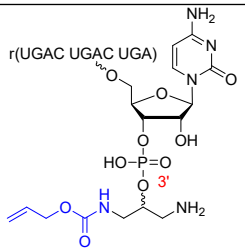
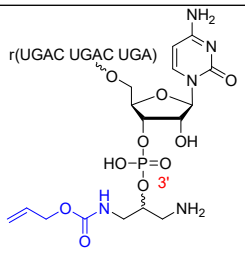
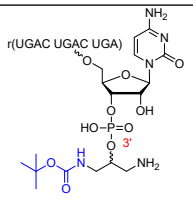
Monomer	CPG Loading (µmol/g)
	33.5
	38.9
	23.2

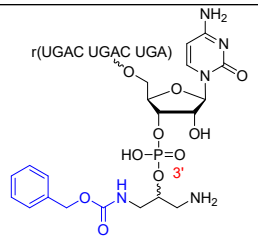
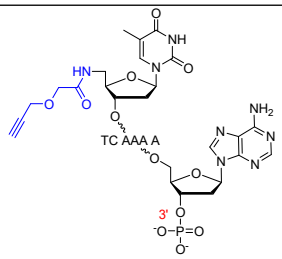
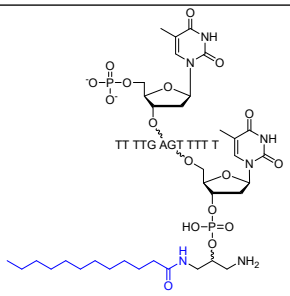
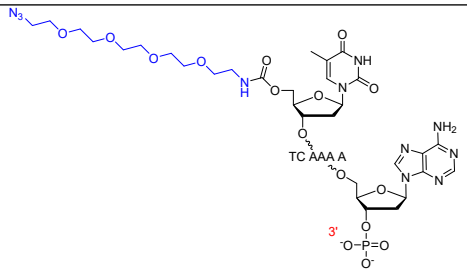
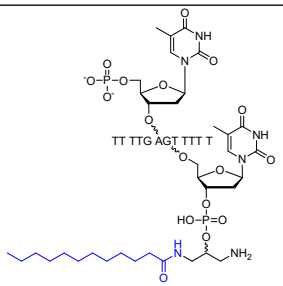
Supporting Table S2- Oligonucleotide isolated yields after SAX-HPLC purification and standard desalting procedures (unless otherwise indicated).

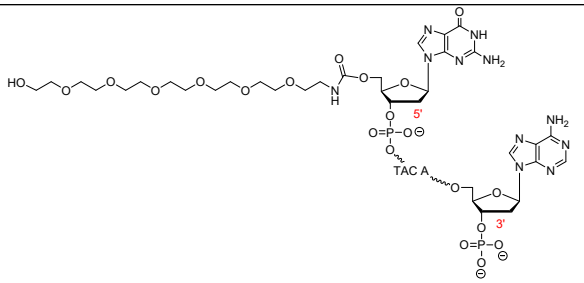
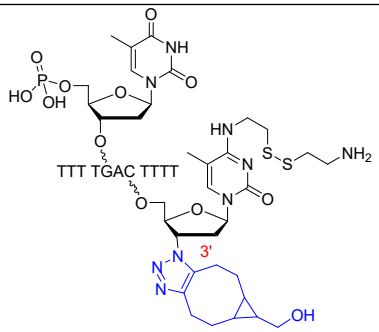
Oligonucleotide	Isolated Yields
	0.01 µmol (6%) (via ***S***-CPG)
	0.02 µmol (10%) (via ***R***-CPG)
	0.11 µmol (11%)

	0.14 µmol (34%)
	0.16 µmol (40%)
	0.18 µmol (21%)
	0.08 µmol (15%)
	0.18 µmol (21%)
	0.15 µmol (32%)

	0.31 µmol (47%)
	0.16 µmol (37%)
	0.16 µmol (41%)
	0.05 µmol (7%)
	0.13 µmol (17%)
	0.16 µmol (31%). Aqueous conjugation conditions.

	0.04 µmol (28%). Organic conjugation conditions.
	0.05 µmol (24%)
	0.22 µmol (39%)
	0.11 µmol (19%). Aqueous conjugation conditions.
	0.01 µmol (5%). Organic conjugation conditions.
	0.01 µmol (10%).

	0.10 µmol (19%)
	0.03 µmol (12%)
	0.04 µmol (16%)
	0.04 µmol (34%)
	0.05 µmol (37%)

	0.07 µmol (19%)
	0.06 µmol (19%)

Supporting References

(1) Saraya, J. S.; O'Flaherty, D. K. A Facile and General Tandem Oligonucleotide Synthesis Methodology for DNA and RNA. *ChemBioChem* **2024**, *25* (6), e202300870. https://doi.org/10.1002/cbic.202300870.

(2) Lelyveld, V. S.; O'Flaherty, D. K.; Zhou, L.; Izgu, E. C.; Szostak, J. W. DNA Polymerase Activity on Synthetic N3'→P5' Phosphoramidate DNA Templates. *Nucleic Acids Research* **2019**, *47* (17), 8941–8949. https://doi.org/10.1093/nar/gkz707.

(3) Horton, N. G.; Saraya, J. S.; O'Flaherty, D. K. Novel Disulfide-Containing Linkages of Pyrimidine Nucleobases to Solid Supports: A Versatile Platform for Oligonucleotide Conjugation. *Bioconjugate Chem.* **2025**, *36* (10), 2237–2246. https://doi.org/10.1021/acs.bioconjchem.5c00326.

(4) Saraya, J. S.; Sammons, S. R.; O'Flaherty, D. K. Aqueous Compatible Post-Synthetic On-Column Conjugation of Nucleic Acids Using Amino-Modifiers. *ChemBioChem* **2025**, *26* (1), e202400643. https://doi.org/10.1002/cbic.202400643.

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(6) Guzaev, A. P.; Pon, R. T. Attachment of Nucleosides and Other Linkers to Solid-Phase Supports for Oligonucleotide Synthesis. *CP Nucleic Acid Chemistry* **2013**, *52* (1). https://doi.org/10.1002/0471142700.nc0302s52.