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A Robust Strategy for Introducing Amino-Modifiers in Nucleic Acids: Enabling Novel Amino Tandem Oligonucleotide Synthesis in DNA and RNA

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Amino-modifiers are pivotal chemical modifications in nucleic acid scaffolds, serving applications ranging from (bio)conjugation to probing the origins of life. We report a simple, efficient, and cost-effective methodology for the introduction of amino-modifiers into DNA and RNA. This approach leverages a commercially available sulfonyl-containing solid support, which is first converted into a mixed *N*-hydroxysuccinimide carbonate, enabling robust conjugation with primary and secondary amines whether nucleosidic or non-nucleosidic. Oligonucleotides are synthesized via solid-phase synthesis and purified using standard methods, with little to no modification. Building on this framework, we introduce a novel amino-containing tandem oligonucleotide synthesis (aTOS) methodology, which facilitates the introduction of multiple terminal amino (or monophosphate) groups across two oligonucleotide strands. This innovative method broadens the toolkit for the introduction of amino modifications in nucleic acids, for applications in nucleic acid (bio)chemistry and biotechnology.

1. Introduction

Solid-phase synthesis (SPS) is the conventional methodology for the preparation of short oligonucleotide sequences. The fully synthetic process allows for the introduction of various chemical modifications in nucleic acid scaffolds, so long as they are inherently compatible with the solid-phase synthesis cycle. Altering the solid-phase cycle is possible but can be laborious, expensive and time-consuming. Such chemical modifications are typically introduced to improve, enhance and/or impart function to that nucleic acid sequence.[1,2]

The introduction of amines into oligonucleotides is particularly useful and has found many applications in the literature, including post-synthetic conjugation strategies.[3–10] The aliphatic amines are inherently more nucleophilic than other nucleophiles found in nucleic acids allowing an orthogonal means of conjugation. Applications ranging from conjugating fluorescent moieties to the incorporation of other desirable ligands like azides, chelators, and sugars have been reported. Recently, we reported a strategy to perform on-column post-synthetic conjugation of oligomers containing amino modifiers with ligands containing a monophosphate or carboxylate.[3]

Amino functionalities in nucleic acids have been introduced in oligomers internally and at both the 3′- and 5′-ends. The 3′-end typically poses more difficulties, especially when using standard 3′→5′ synthesis, as the solid-support needs to be derivatized with the amine. The reverse synthesis pathway (5′ → 3′) is possible but requires expensive materials and a corresponding (typically custom) amino-containing phosphoramidite building block.[11] A myriad of solid-supports have been derivatized to support the introduction of amino groups, many of which require significant synthetic efforts (see Figure 1).[12–14] Common cleavage conditions include base treatment (ammonium hydroxide), reduction in basic conditions, and photolysis. The Richert group developed elegant chemistry employing a hexafluoroglutaric linker to functionalize 3′-amino-2′,3′-dideoxynucleosides.[15] Certain amino-modifier functionalized solid-supports are now commercialized (e.g. 3′-amino-3′-deoxythymidine), but the list is not exhaustive and alternate amino modifiers (e.g. 3′-amino-2′,3′-dideoxy-(cytidine/adenosine/guanosine)) require different strategies. In our hands,[3] obtaining DNA/RNA strands containing a 3′-amino-2′,3′-dideoxy unit at the 3′-end was possible using the Richert methodology,[15] but the process was fairly laborious and time-consuming. In addition, we noted that the functionalization was highly dependent on water content, with certain hygroscopic conjugators yielding low loading supports.

Here, we report a fast, economical, and versatile on-column methodology to incorporate terminal amines to oligonucleotides. The procedure makes use of commercially available 2,2′-sulfonyl diethylene derivatized control pore glass (so-called 3′-Phosphate-ON CPG). We showcase the introduction of linear alkylene amines, nucleoside amines, and disulfide amines as prime examples. We use this facile approach to demonstrate, for the first time to the extent of our knowledge, tandem

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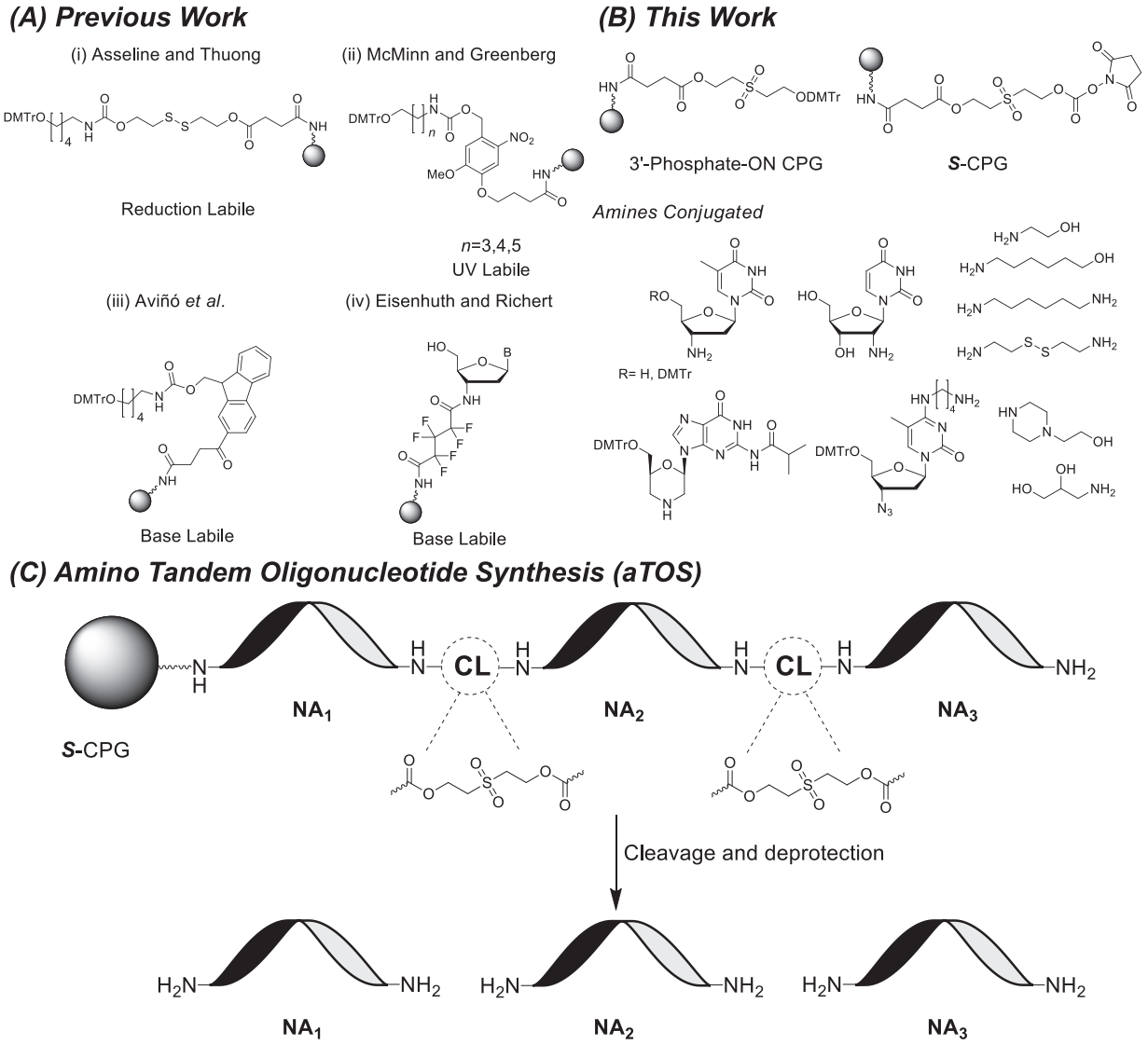


Figure 1. (A) Selected examples of solid-supports for 3′-amino modifier incorporation.[12–15] (B) Our solid support functionalization to prepare oligonucleotides containing an amino modifier using ***S***-CPG. (C) Schematic representation of amino tandem oligonucleotide synthesis (aTOS) before, and after, solid support cleavage and deprotection. Nucleic acids (NA) are assembled linearly and are separated by a base labile cleavable carbamate-containing linker (CL).

oligonucleotide synthesis (TOS) of terminally amino-modified nucleic acid sequences (Figure 1C, aTOS). TOS is a strategy to enable the synthesis of multiple shorter oligonucleotides in a single strand, each separated by a cleavable linker, resulting in a mixture of oligomers after cleavage and deprotection off of solid-supports.[16] This is particularly attractive in applications where more than one oligonucleotide is required such as studies involving PCR and/or short interfering RNA (siRNA). Advantages include cutting cost (e.g. amount of solid support), labor (e.g. pre- and post-handling manipulations), and potentially increasing overall yield (e.g. synthesizing multiple copies of the same oligomer by TOS). We,[17] and others,[18–25] have developed pathways for achieving TOS. In our recently reported study, we utilized a commercially available sulfonyl-containing cleavable linker phosphoramidite to achieve TOS both in DNA and RNA. In light of this previous work, we now showcase the synthesis of various pairs of oligomers either containing terminal amino or monophosphate groups. We then employ a commercially available linker as a branching agent to produce an assembly of three strands, two of which have an identical sequence identity. The latter finds direct application in primer extension studies, that typically have an excess of the template strand relative to the primer strand.

2. Results and Discussion

2.1. Solid-Support Functionalization and Amino-Modified Oligonucleotide Synthesis

We first developed a carbamate-mediated approach to easily functionalize CPG with an amino-modifier. Carbamate moieties are relatively stable in acid and base and can be easily accessed

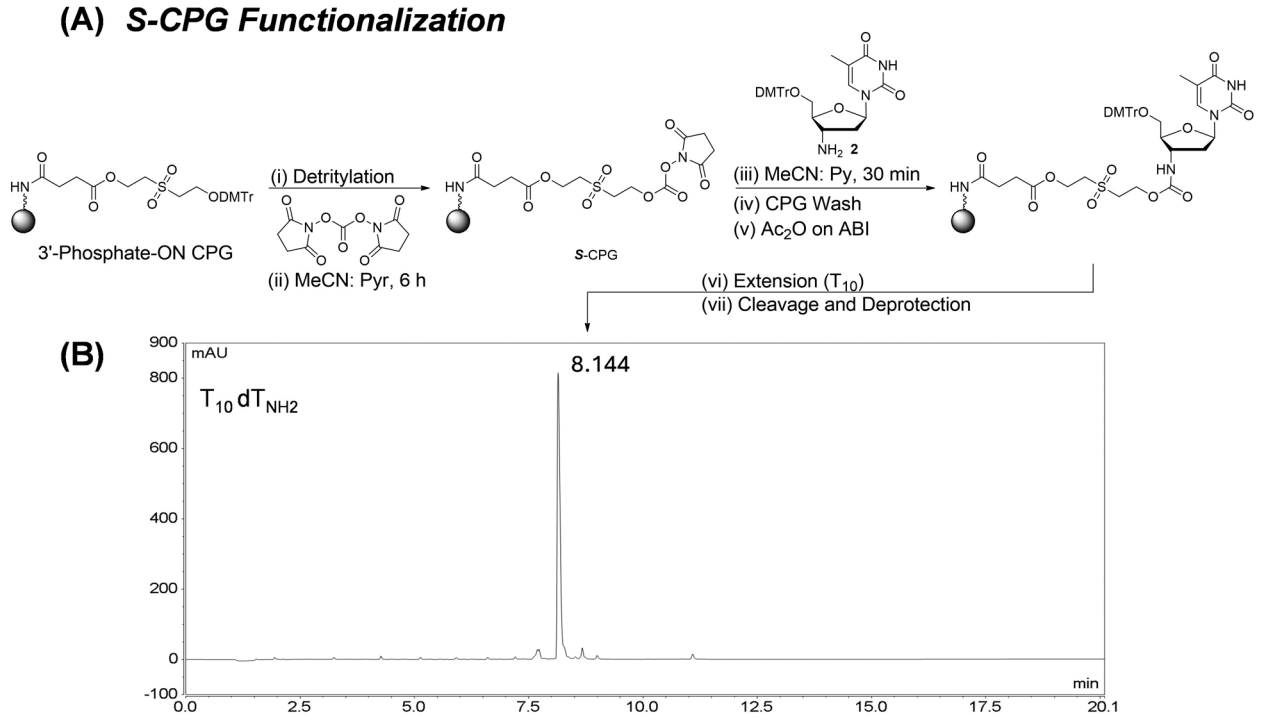
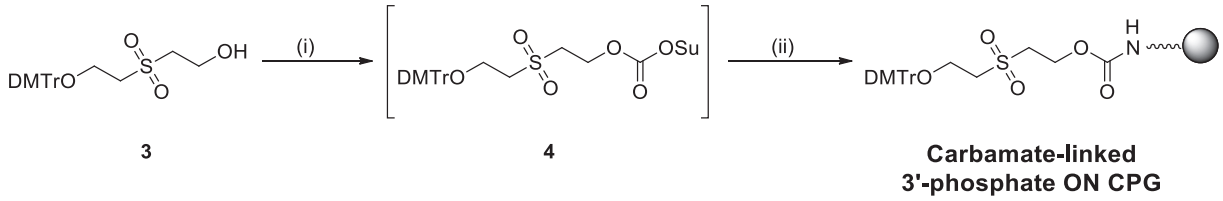


Figure 2. (A) On column functionalization of 3′-Phosphate-ON CPG for amino modifier incorporation. (i) Detritylation on ABI 394 using manufacturer's protocols. (ii) Saturated solution of *N*,*N*′-disuccinimidyl carbonate (DSC) in 1:1 (v/v) MeCN: Py (500 µL) for 6 h. (iii) Coupling of 5′-*O*-(4,4′-dimethoxytrityl)-3′-amino-3′-deoxythymidine **2** (10 mg) in a 1:1 (v/v) MeCN: Py (500 µL, 27 mM) solution for 30 min. (iv) Washing of the CPG using MeCN (10 mL). (v) Capping of the CPG on ABI 394 using Ac_2O. (vi) Extension of the strand by 10 thymidinyl units, followed by (vii) standard cleavage and deprotection in NH_4OH at 55 °C for 17 h. (B) SAX-HPLC trace of the crude oligonucleotide strand containing a terminal 3′-amino-3′-deoxythymidine (dT_{NH2}). Retention time (min) of the desired material is reported directly on the chromatogram.

using an *N*-hydroxysuccinimide mixed carbonate or chloroformate reagents. We opted for the *N*-hydroxysuccinimide mixed carbonate (Figure 1B and ***S***-CPG) as these are selective to non-aromatic amines in the presence of alcohols and/or anilines, as well as being compatible with aqueous conditions. This potential strategy demonstrates a clear advantage given that many amino-modifiers are hygroscopic, and/or are water soluble. We used 5′-protected 3′-amino-3′-deoxythymidine **2** as our model amino-modifiers as this compound is readily accessible (e.g. 4,4′-dimethoxytritylation of 3′-azido-3′-deoxythymidine (AZT) to produce 5′-DMTr-3′-azido-3′-deoxythymidine **1**, followed by conventional reduction of the azido group to the amine[15,26]). As previously devised for carbamate-linked oligonucleotides, a spacer capable of immolating the carbamate is required as the carbamate is relatively stable in standard base-mediated cleavage and deprotection. We chose the 3′-Phosphate-ON CPG (Figure 2) as our immolative handle, based on our recent successful application with TOS.[17] We envisioned that the β-elimination of the sulfonyl linker produces an intermediary carbamate anion that spontaneously undergoes decarboxylation (as observed with many carbamate protecting groups like Fmoc and Boc). An aliquot of support (roughly 20 mg, 1 µmol) was detritylated on an automated oligonucleotide synthesizer (ABI 394), and manually reacted with a saturated solution of *N*,*N*′-disuccinimidyl carbonate (DSC) in 1:1 (v/v) acetonitrile (MeCN): pyridine (Py) (500 µL) for 6 h. The corresponding activated solid-support was then reacted with a solution of 5′-*O*-(4,4′-dimethoxytrityl)-3′-amino-3′-deoxythymidine **2** (10 mg) in 1:1 (v/v) MeCN: Py (500 µL) for 30 min. The functionalized support was capped on the oligonucleotide synthesizer, followed by SPS. We were pleased to observe the bright orange solution of the 4,4′-dimethoxytrityl (DMTr) cation on the synthesizer for the initial detritylation, suggesting successful conjugation of compound **2** to the pre-activated solid-support. The monomer unit was extended with ten couplings to produce a 5′-TTT TTT TTT TdT_{NH2} (T_{10}-dT_{NH2}). Standard deprotection conditions (either NH_4OH at 55 °C for 17 h, or AMA at 65 °C for 1 h) furnished a single major product by strong anion-exchange high-pressure liquid chromatography (SAX-HPLC) analysis. Mass spectrometry of the purified oligonucleotide was consistent with the expected mass/charge ratio (*m*/*z*), confirming the development of a successful methodology. A time trial for DSC-derivatization of the 3′-Phosphate-ON CPG was conducted indirectly by tracking the trityl counts[27] from the dT monomer **2** conjugation to ***S***-CPG, as well as recovery yields of sequences post-elongation (i.e. T_9-dT_{NH2} recovery relative to the control T_{10p}, see Supporting Information for details). Results indicated that DSC functionalization between 2–6 h was optimal, with a seemingly decrease in overall yield for an initial 24 h DSC treatment.

As the 3′-amino group should be inherently more nucleophilic than the 5′-hydroxyl group, we repeated our functionalization and T_{10}-dT_{NH2} oligonucleotide assembly using the unprotected 3′-amino-3′-deoxythymidine and found comparable results as those depicted in Figure 2 (Figure S15, Supporting



Scheme 1. Synthesis of the crude carbonate **4** for CPG functionalization reactions. Reagents and conditions: (i) DSC (3.0 equiv.), MeCN: Py (3:1); (ii) Native Amino 500 Å LCAA (0.04 equiv.), MeCN: Py (1:1).

Information). Next, we repeated the DNA synthesis of a mixed sequence containing the 3′-amino-3′-deoxythymidine terminal, showcasing that our methodology is compatible with sequence contexts other than poly-T (Supporting Information Figure S17, Supporting Information). Reverse-synthesis was done successfully, by first attaching a 5′-amino-5′-deoxythymidine unit to the 3′-Phosphate-ON CPG, followed by extension using 3′-*O*-DMTr-nucleoside 5′-*O*-phosphoramidites (so-called reverse amidites) (Figure S19, Supporting Information). Finally, RNA synthesis was also accomplished with a 3′-amino-3′-deoxythymidine terminal, using standard cleavage and deprotection protocols (Figure S21, Supporting Information). We were pleased to observe efficient synthesis as well as mass spectral analysis in agreement with our expected *m/z* values. Subsequent trials with a protected cytidyl analogue were successful (Figures S23 and S25, Supporting Information).

Realizing the elevated cost of the 3′-Phosphate-ON CPG relative to standard native amino CPG, we developed a straightforward means of preparing a carbamate-linked 2,2′-sulfonyl diethylene derivatized CPG (**Scheme 1**). Here the mixed carbonate species **4** was prepared by reacting the commercially available DMTr-protected linker **3** with DSC in the presence of Py and MeCN at room temperature. After 4 h, TLC analysis indicated reaction completion. The solvent was removed and the resulting crude mixed carbonate **4** was solely worked up and used for the CPG derivatization without further purification. Loading of the CPG was estimated to be 98 µmol per gram of solid support, using trityl analysis,[27] and the quality of the product strands were comparable to the commercially available 3′-Phosphate-ON CPG (see Supporting Information for more details).

Next, we evaluated a non-nucleosidic linker containing an amine and alcohol group. We tested our strategy with two linkers of different lengths, 1,2-ethanolamine and 1,6-hexanolamine, with the latter sold commercially as a protected phosphoramidite. As with the unprotected 3′-amino-3′-deoxythymidine, a single major product was observed by SAX-HPLC analysis from these syntheses, with *m/z* in agreement with the expected product (Figures S27 and S29, Supporting Information). Chromatograms revealed notable quantities of byproduct species eluting later relative to the desired product (data not shown). The issue was remedied by extensive washing of the solid-support after the coupling of the amino-ol linker using DMF, acetone, followed by MeCN. We suspect that perhaps the reacting linker could have insolubility issues causing the presence of impurities on the resulting solid support that ultimately undergoes oligonucleotide synthesis.

We wondered whether *bis*-amino linkers, such as 1,6-hexadiamine and cystamine, could be compatible with our functionalization pathway (Figure 3). In this iteration, the unprotected *bis*-amino linker was coupled to the ***S***-CPG, resulting in a solid-support containing a new terminal amino group. The support was then treated with 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(2,5-dioxo-1-pyrrolidinyloxycarbonyl)-thymidine **6** (Figure 3), to generate a stable non-immolative carbamate linked support with the first nucleoside attached. The sequence was extended by SPS, cleaved and deprotected, and analyzed by LC to reveal a major product consistent with the desired modification (Figure 3). Mass spectral analysis confirmed the presence of the hexyldiamino group or cystaminyl within the oligomers. The cystaminyl group is of particular interest given its ability to form reversible crosslinks or oligonucleotide-protein conjugates.[28–30] In addition, a mixed sequence context was synthesized via hexyldiamine group (Figures S35 and S36, Supporting Information) to verify sequence generalizability. Generating the small molecule conjugate between thymidine and either 1,6-hexanediamine or cystamine (See Supporting Information for synthetic details), followed by conjugation to ***S***-CPG yielded comparable product DNA strands to the on-column carbamate construction (See Figure S37 and S39, Supporting Information). The hexyldiamino group was also conjugated in aqueous conditions (solution of 100 mM HEPES pH 8.0 containing 50% (v/v) DMF), with the major species corresponding to the desired sequence, demonstrating aqueous compatibility of this methodology (Figure S41, Supporting Information). This is of course attractive for compounds that have improved solubility in the presence of water, and/or for ligands that are hygroscopic. Notably, examples with free terminal amines are susceptible to derivatization by reacting with electrophilic species that are formed during the cleavage and deprotection step (as revealed by SAX-HPLC analysis in Supporting Information). To expand versatility, a cystaminyl-containing 2′-deoxycytidyl terminated strand was synthesized in a similar fashion as described above (i.e. using 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(2,5-dioxo-1-pyrrolidinyloxycarbonyl)-N^4-acetyl-2′-deoxy-cytidine **9** with cystamine derivatized solid-support, or using the small molecule conjugate **10** already containing the cytamine moiety, see Supporting Information for synthetic details). In both cases, one major product was observed by LC, with MS analysis in agreement with calculated *m/z* values (see LC traces in Figures S43 and S45, Supporting Information, and MS in Figures S44 and S46, Supporting Information).

Morpholino oligonucleotides have been investigated for various applications in bioconjugation,[31] therapeutics,[32] and

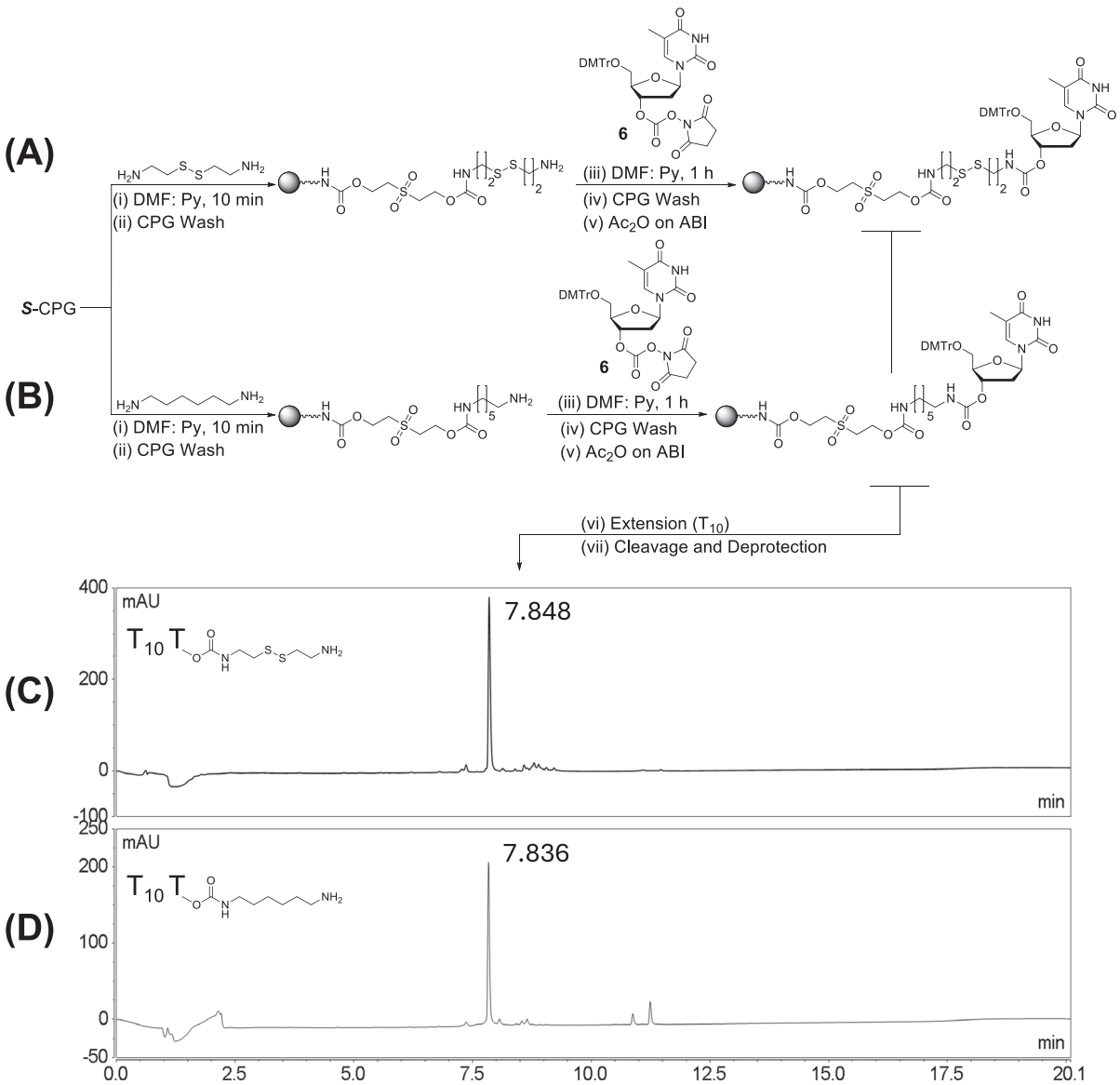


Figure 3. (A) On column functionalization of ***S***-CPG for di-amino modifier incorporation. (A) (i) Coupling of a 50 mM solution of cystamine in 1:1 (v/v) DMSO: Py (500 µL) solution for 10 min. (ii) Washing of the CPG using DMSO, DMF, acetone and MeCN (10 mL each). (iii) Coupling of the crude carbonate **6** in 1:1 (v/v) DMF: Py (100 mM, 1 mL) for 1 h. (iv) Washing of the CPG using DMF, DCM, acetone, hexanes and MeCN (10 mL each). (v) Capping of the CPG on ABI 394 using Ac_2O. (vi) Extension of the strand by 10 thymidinyl units, followed by (vii) standard cleavage and deprotection in AMA at 65 °C for 1 h. (B) Same as (A) except with 1,6-hexadiamine as the di-amino modifier. (C) SAX-HPLC trace of the crude oligonucleotide strand containing a carbamate linked 3′-cystamine amino modifier. (D) SAX-HPLC trace of the crude oligonucleotide strand containing a carbamate linked 3′-(1,6-hexadiamine) amino modifier. Retention time (min) of the desired material is reported directly on the chromatogram.

origins of life[33] Oligomers containing a 3′-terminal morpholino modification can be accessed post-synthetically using reductive amination[34,35], but this precludes the ability of having any other vicinal diols within the scaffold. We sought to use our developed methodology for the preparation of nucleic acids directly containing a 3′-terminated morpholino unit. This also served as an example of secondary amine-conjugation. We used either protected morpholino-U **11** or morpholino-G **12**, both of which are commercially available, as representative pyrimidine or purine examples. We were pleased to observe excellent synthesis in both cases (Figure 4), with *m/z* consistent with expected values. A mixed sequence was also successfully synthesized from morpholino-U derivatized solid-support (Figures S51 and S52, Supporting Information). Additionally, the feasibility of our methodology was tested with 1-(2-hydroxyethyl) piperazine as a non-nucleosidic example (Figure S53, Supporting Information), which demonstrated good-to-excellent synthesis.

Recognizing that amines are more nucleophilic than alcohol functionality, we attempted to develop an orthogonal means of performing SPS of oligomers containing a 2′-amino-2′-deoxyuridine insert at the 3′-terminal (Figure 5). We expected the amino group to conjugate efficiently to ***S***-CPG, as shown by numerous examples above. However, the presence of two free hydroxyl functionalities would inevitably cause branching during SPS. To mitigate this issue, we attempted to protect the 5′-hydroxyl selectively using the bulky DMTr group, as

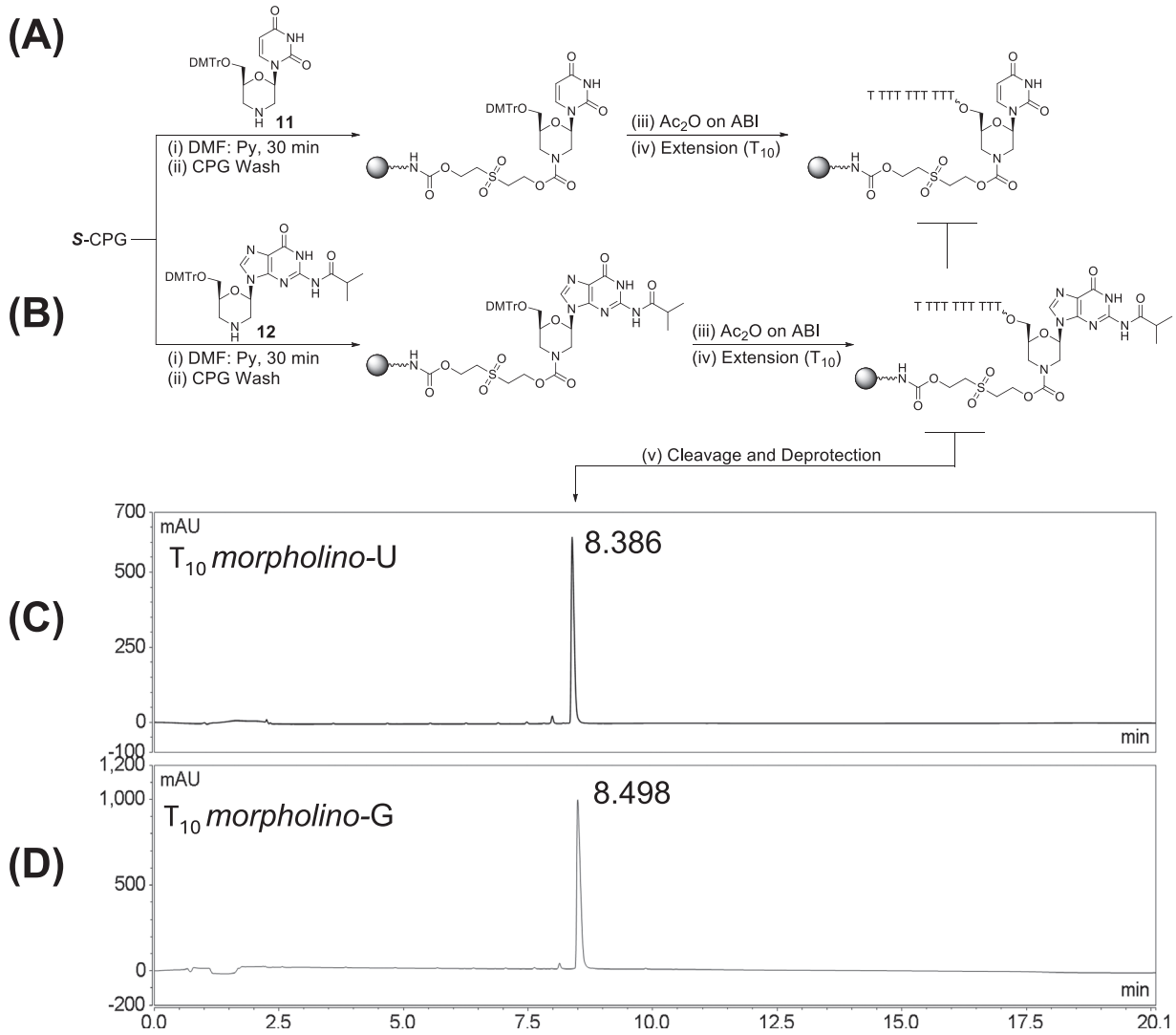


Figure 4. (A) On-column functionalization of ***S***-CPG for morpholino incorporation. (A) (i) Coupling of a 25 mM solution of morpholino-U **11** in 1:1 (v/v) DMF: Py (500 µL) solution for 30 min. (ii) Washing of the CPG using DMF, acetone and MeCN (10 mL each). (iii) Capping of the CPG on ABI 394 using Ac_2O. (iv) Extension of the strand by 10 thymidinyl units, followed by (v) standard cleavage and deprotection in NH_4OH at 55 °C for 17 h. (B) Same as (A) except with morpholino-G **12** as the amino modifier. (C) SAX-HPLC trace of the crude oligonucleotide strand containing the terminal 3′-morpholino-U. (D) SAX-HPLC trace of the crude oligonucleotide strand containing the terminal 3′-morpholino-G. Retention time (min) of the desired material is reported directly on the chromatogram.

performed on standard nucleosides. We treated the support with DMTr-Cl in pyridine for 16 h at room temperature. The nucleoside-bound CPG was then capped on the synthesizer using the manufacturer's protocols. We opted for the more-labile Pac_2O capping reagent, instead of the standard acetic anhydride, as we feared that the migration of the 3′-*O* protecting group to the 2′-amino group could result in a stable amide linkage.[36] We were pleased to observe a single major product from the resulting synthesis. Purification was readily achievable, and the desired product was confirmed by MS. As a control, the same sequence was synthesized via the commercial 2′-amino-2′-deoxyuridine amidite and co-injected post purification to confirm extension from the desired 5′-position (see Figure S57, Supporting Information). Our strategy opens new and exciting practical avenues for solid-supported protecting group chemistry.

As a final example, we prepared a nucleobase amino-modified linker (compound **13**, Scheme S4, Supporting Information) using a convenient convertible nucleoside approach.[37–39] 5′-*O*-DMTr-3′-azido-3′-deoxythymidine **1** was reacted with triazole and phosphorus oxychloride in the presence of excess triethylamine. The triazoyl intermediate was worked up and reacted with putrescine to furnish compound **13** in good yield (see Supporting Information for synthetic details). Compound **13** was coupled to the 3′-Phosphate-ON CPG using the procedure reported above. As with other examples, we were pleased to observe one major product by SAX-HPLC (Figure S59, Supporting Information). Notably, this example is of particular interest given that the resulting oligomer contains two orthogonal conjugation handles, a primary amino and an azido functional group. For instance, one could envision a copper-catalyzed [3 + 2] cycloaddition reaction with an alkyne containing ligand,[40] followed

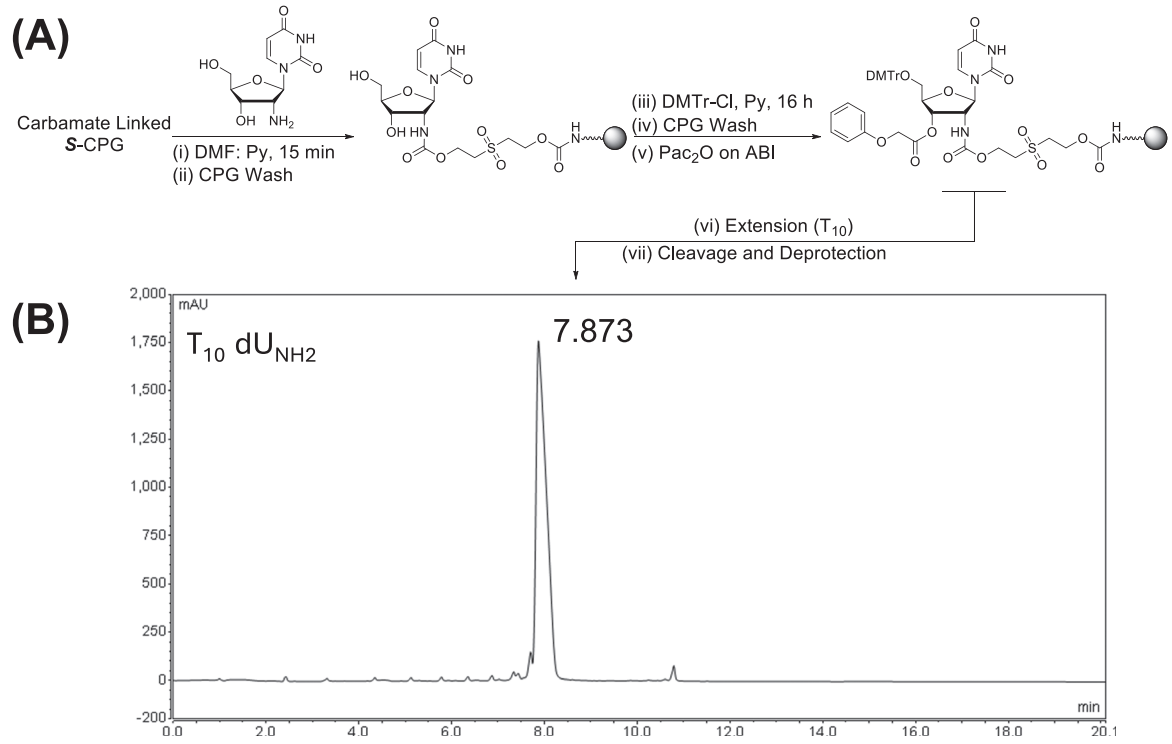


Figure 5. (A) On-column functionalization of ***S***-CPG for 2′-amino-2′-deoxyuridine incorporation. (A) (i) Coupling of a 50 mM solution of 2′-NH_2-dU in 1:1 (v/v) DMF: Py (500 µL) solution for 15 min. (ii) Washing of the CPG using DMF, acetone and MeCN (10 mL each). (iii) On-column DMTr protection using 1.00 mL of a 100 mM solution of DMTr-Cl in pyridine for 16 h. (iv) Washing of the CPG with DCM, hexanes, acetone and MeCN (10 mL each). (v) Capping of the CPG on ABI 394 using Pac_2O. (vi) Extension of the strand by 10 thymidinyl units, followed by (vii) standard cleavage and deprotection in AMA at 65 °C for 1 h. (B) SAX-HPLC trace of the crude oligonucleotide strand containing the terminal 2′- amino-2′-deoxyuridine. Retention time (min) of the desired material is reported directly on the chromatogram.

by the reaction with the *N*-hydroxysuccinimide ester-containing ligand.

2.2. Amino-modified Tandem Oligonucleotide Synthesis (aTOS)

TOS is an effective methodology for preparing multiple oligonucleotide strands within a single sequence, each separated by a cleavable linker. In our recently reported work, we used the commercially available sulfonyl-containing ***X***-amidite (chemical structure illustrated in Figure **8B**) to perform TOS on DNA, RNA, and modified oligonucleotides.[17] Given the clear applications of amino-modifiers within nucleic acids, and the similarities between the ***X***-amidite and compound **4** (**Scheme 1**), we asked whether we could perform TOS on amino-modified oligomers. This would serve as the first example, to the extent of our knowledge, of TOS with amino-modifiers.

To answer this question, we sought to synthesize a DNA duplex with all four terminal positions modified with an amino group (Figure **6A** for schematic representation). We selected a model set of strands (poly-d*T* strand and a mixed d*T*/d*A* strand) that differed in length to be able to monitor via SAX-HPLC, and that could potentially hybridize once in native conditions (after cleavage and deprotection). Here, 5′-*O*-(4,4′-dimethethoxytrityl)-3′-amino-3′-deoxythymidine **2** was conjugated to ***S***-CPG, followed by extension and the incorporation of the commercially available 5′-NH-MMTr-thymidine 3′-*O*-phosphoramidite at the 5′-terminal. Detritylation was achievable with twice the standard deblock protocol, whereby the sequence was coupled with mixed carbonate linker **4** (100 mM) in a 1:1 (v/v) MeCN: Py solution (800 µL, 80 equiv.) at room temperature for 1 h. The liquid was then removed, the support washed with MeCN, and another aliquot was added for 1 h. After the second coupling, the support was thoroughly washed with DMF, acetone and MeCN. The support-bound sequence was then capped and detritylated on the synthesizer and subsequently treated with a saturated solution of DSC in 1:1 (v/v) MeCN: Py solution (500 µL) for 8 h at room temperature. The pre-activated support-bound sequence was treated with compound **2** again (50 mM) in 1:1 (v/v) DMF: Py solution (500 µL, 25 equiv.) for 15 min. It was then thoroughly washed, as previously described and capped on the ABI 394. Finally, the strand was elongated once more on the synthesizer using standard procedures. The cleavage and deprotection step was performed in ammonium hydroxide, followed by SAX-HPLC analysis, which revealed the presence of two major species (Figure **6C**). Purification of the two major species from the aTOS experiment and their MS analysis revealed the presence of two oligomers with *m/z* consistent with our expected products. We expect that these constructs could undergo effective ligation using reagents such as 1,1′-carbonyldiimidazole or squaramide, as previously reported by the Brown group.[41]

We tested alternative aTOS permutations that would enable substituting certain terminal amino groups with either terminal

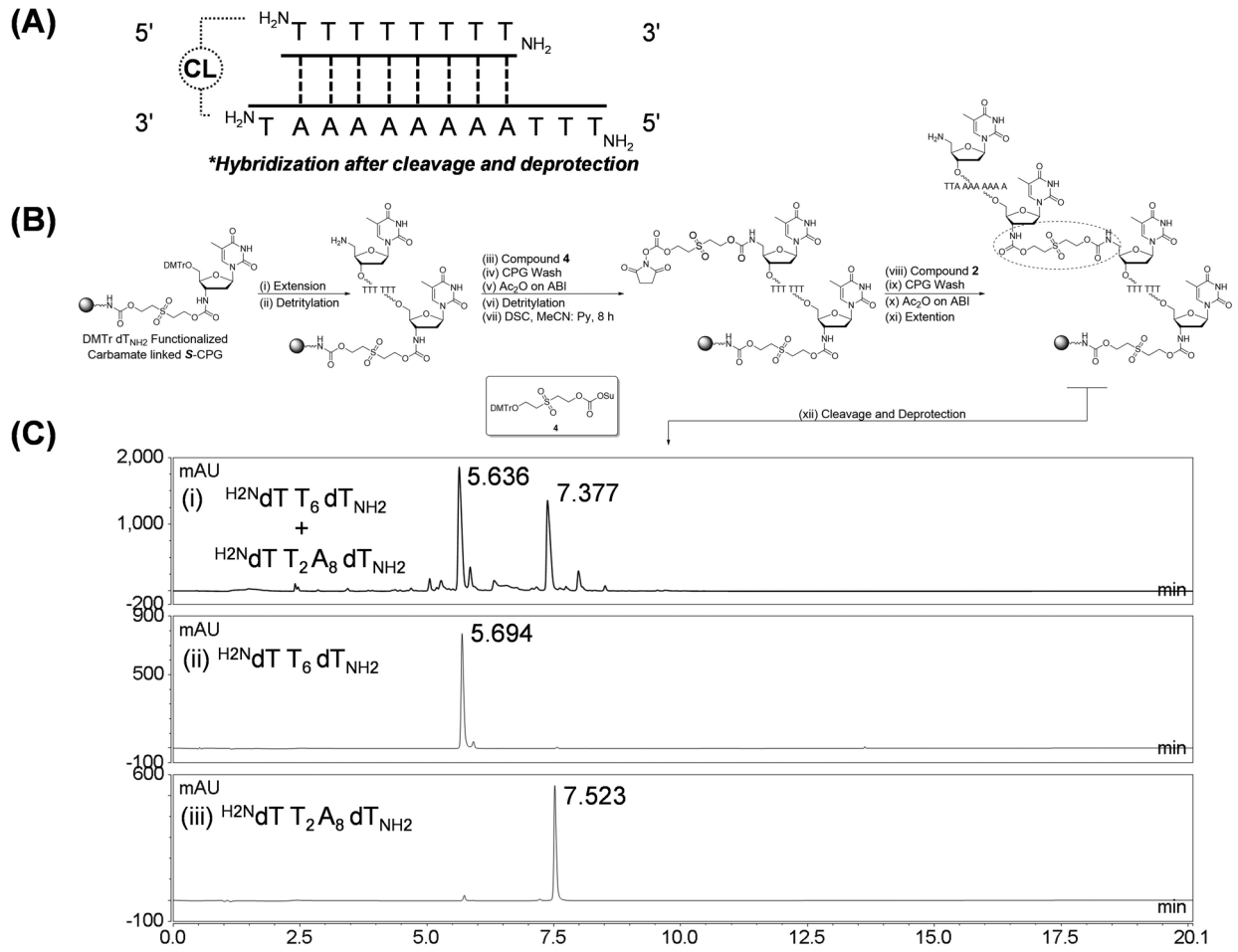


Figure 6. (A) Schematic representation of amino tandem oligonucleotide synthesis (aTOS) duplex, containing four terminal amines. To be noted, potential hybridization would only occur after cleavage and deprotection in native conditions (purification may be necessary). (B) On-column functionalization of DMTr-dT_{NH2} coupled, carbamate linked ***S***-CPG for aTOS containing 4 terminal amines. (i) Extension of the 5′-NH_2 dT containing strand one and (ii) detritylation of MMTr. (iii) Coupling of the crude carbonate **4** in 1:1 (v/v) MeCN: Py (800 µL, 100 mM) for 1 h. Washing of the CPG with MeCN (10 mL). Second round of coupling of compound 4 in 1:1 (v/v) MeCN: Py (1.00 mL, 100 mm) for 1 h. (iv) Washing of the CPG using DMF, acetone and MeCN (10 mL each). (v) Capping of the CPG on ABI 394 using Ac_2O. (vi) Detritylation of DMTr and subsequent (vii) carbonate reaction using a saturated solution of DSC (500 µL, 1:1, MeCN: Py) for 8 h. (viii) Coupling of 5′-*O*-(4,4′-dimethoxytrityl)-3′-amino-3′-deoxythymidine **2** in 1:1 (v/v) DMF: Py (500 µL, 50 mM) solution for 15 min. (ix) Washing of the CPG using DMF, acetone and MeCN (10 mL each). (x) Capping of the CPG on ABI 394 using Ac_2O. (xi) Extension of the second strand followed by (xii) standard cleavage and deprotection in NH_4OH at 55 °C for 17 h. (C) SAX-HPLC trace of the crude aTOS mixture (i), purified shorter duplex strand (ii) and purified longer duplex strand (iii). "^{NH2}dT", "dT_{NH2}", "CL" denote 5′-amino-5′-deoxythymidine, 3′-amino-3′-deoxythymidine and base labile cleavable carbamate-containing linker, respectively. Retention time (min) of the desired material(s) is reported directly on the chromatogram.

hydroxyl or phosphate groups, thereby enhancing the versatility of aTOS. As a first proof-of-concept, we devised an experiment that would generate a pair of strands containing two 3′-terminal amino groups and two terminal 5′-hydroxyl (Figure 7). This array could be handy in post-synthetic ligation experiments or simultaneously labeling the 3′-positions of the two strands (e.g. using NHS-ester containing ligands), without compromising the ability of enzymes from acting on the 5′-hydroxyl position. Standard UnyLinker CPG (1000 Å) was extended to a desired sequence length using reverse T amidites, with the last position being 3′-NH-MMTr-thymidine 5′-*O*-phosphoramidite.[11] Deblocking the MMTr group, followed by coupling of the mixed carbonate **4** furnished an intermediate that could be detritylated on the synthesizer. The terminal hydroxyl was activated by treatment with DSC as described above, and protected 3′-amino-3′-deoxythymidine **2** was then conjugated. Extension was achieved using standard amidites to prepare the full-length sequence, which was then subject to standard cleavage and deprotection conditions. As with the previous aTOS experiment, SAX-HPLC and LR-ESI-MS analysis confirmed the identity of the desired materials. Of course, the inverted example where two strand containing 5′-terminal amino groups, and 3′-terminal hydroxyl group, is theoretically possible with slight modification to the latter strategy described.

In a final proof-of-concept variation of the aTOS experiment, we hypothesized that we could create a duplex containing two amino and two phosphate terminal groups, such that each end of the duplex features one phosphate and one amino group (Figure 8). Starting with compound **2** attached to ***S***-CPG, we coupled eight additional thymidine units, followed by coupling of the ***X***-amidite. After detritylation, the sulfonyl linker was reacted with DSC (8 h) and then treated with compound **2**. The

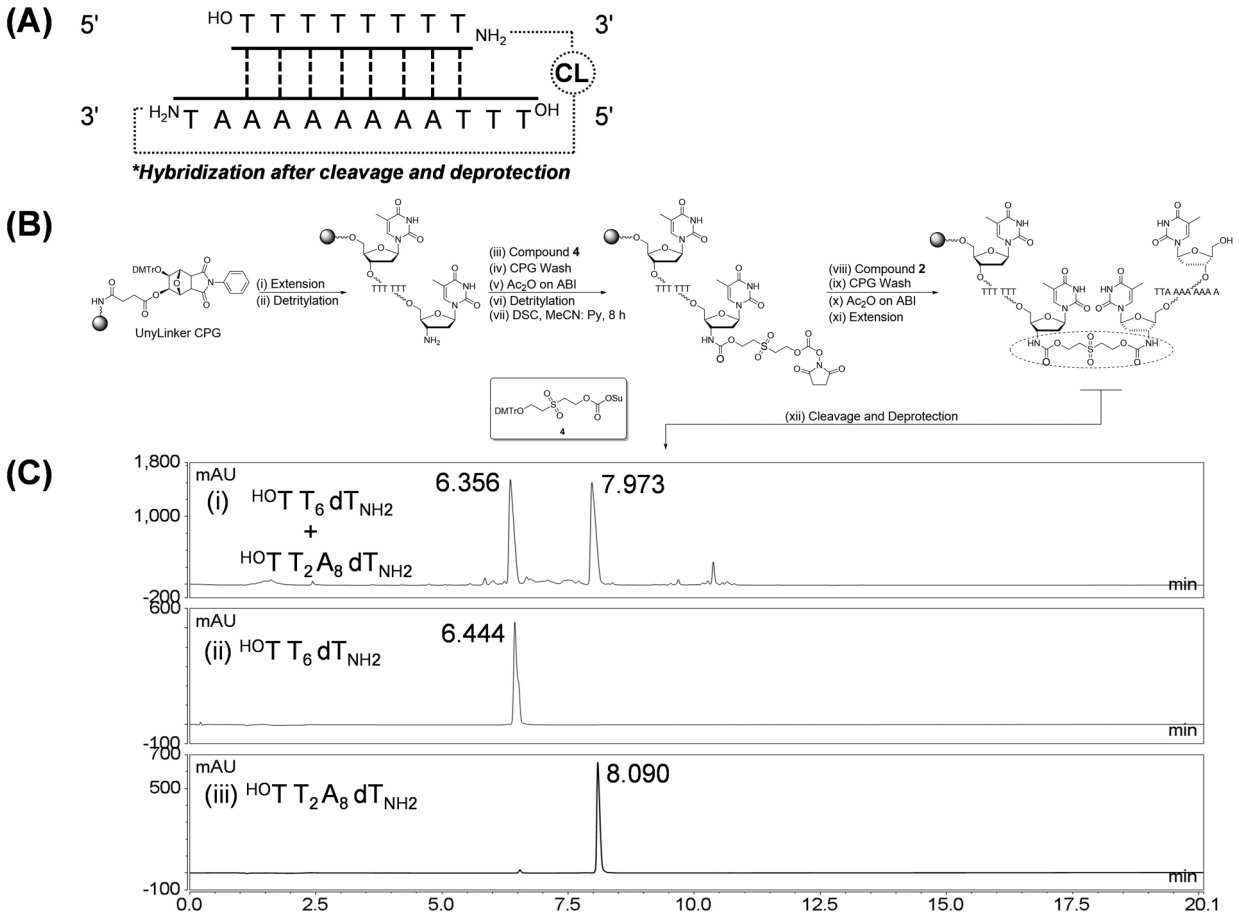


Figure 7. (A) Schematic representation of amino tandem oligonucleotide synthesis (aTOS) duplex, containing two terminal amines and two terminal hydroxyls. To be noted, potential hybridization would only occur after cleavage and deprotection in native conditions (purification may be necessary). (B) On-column functionalization of UnyLinker for aTOS containing 2 terminal amines and 2 hydroxyls. (i) Extension of 3′-NH_2 dT containing strand one using reverse amidites from UnyLinker CPG and (ii) detritylation of MMTr. (iii) Coupling of compound **4** in 1:1 (v/v) MeCN: Py (1.00 mL, 100 mm) for 1 h. Washing of the CPG with MeCN (10 mL). Second round of coupling of compound 4 in 1:1 (v/v) MeCN: Py (1.00 mL, 100 mm) for 1 h. (iv) Washing of the CPG using DMF, acetone and MeCN (10 mL each). (v) Capping of the CPG on ABI 394 using Ac_2O. (vi) Detritylation of DMTr and subsequent (vii) carbonate reaction using a saturated solution of DSC (500 µL, 1:1, MeCN: Py) for 8 h. (viii) Coupling of 5′-*O*-(4,4′-dimethoxytrityl)-3′-amino-3′-deoxythymidine **2** in a 1:1 (v/v) DMF: Py (500 µL, 50 mm) solution for 15 min. (ix) Washing of the CPG using DMF, acetone and MeCN (10 mL each). (x) Capping of the CPG on ABI 394 using Ac_2O. (xi) Extension of the second strand followed by (xii) standard cleavage and deprotection in NH_4OH at 55 °C for 17 h. (C) SAX-HPLC trace of the crude aTOS mixture (i), purified shorter duplex strand (ii) and purified longer duplex strand (iii). "dT_{NH2}", "CL" denote 3′-amino-3′-deoxythymidine and base labile cleavable carbamate-containing linker, respectively. Retention time (min) of the desired material(s) is reported directly on the chromatogram.

resulting sequence was extended once more and terminated with a final coupling of the ***X***-amidite. Cleavage and deprotection furnished the desired duplex. We also repeated a similar experiment in RNA to verify generalizability and obtained comparable results to our DNA constructs (Figure 9). We are currently investigating whether these constructs, containing amino terminals, can undergo nonenzymatic ligation to form small circular oligomers, as previously investigated for DNA oligomers by the Kool group.[42,43]

With the successful demonstration of the latter aTOS experiments, an array of different strategies could be employed to generate sequences with either an amino, a hydroxyl, or a phosphate group. This, like many TOS methodologies, is not without limitations. As described, the aTOS method must include at least 2 terminal amino functionalities, or 1 terminal amino and 1 terminal phosphate functionality. We have not yet been able to make a strand with only one terminal amine and three terminal hydroxyls. Although the coupling of the amine to a mixed carbonate is relatively fast (~15 min) under our conditions and the on-column preparation of the mixed carbonate can be shortened in time (2 h), the incorporation of the primary amines results in inevitable loss of yield (primarily due to the liability of the sulfonyl linker to basic conditions). Albeit the introduction of a stronger base, like triethylamine, enhances the reaction rates of the DSC coupling, a significant amount of linker degradation occurs under these more aggressive conditions (data not shown). Presently, aTOS is not fully automated and does require experimenter intervention/manipulation of the support-bound sequences, which may be tedious for high-throughput synthesis applications. We hypothesize that minor adjustments to the automated oligonucleotide synthesizers will allow for a fully automated aTOS methodology. A potential limitation arises when an automated oligonucleotide synthesizer operates suboptimally, as the

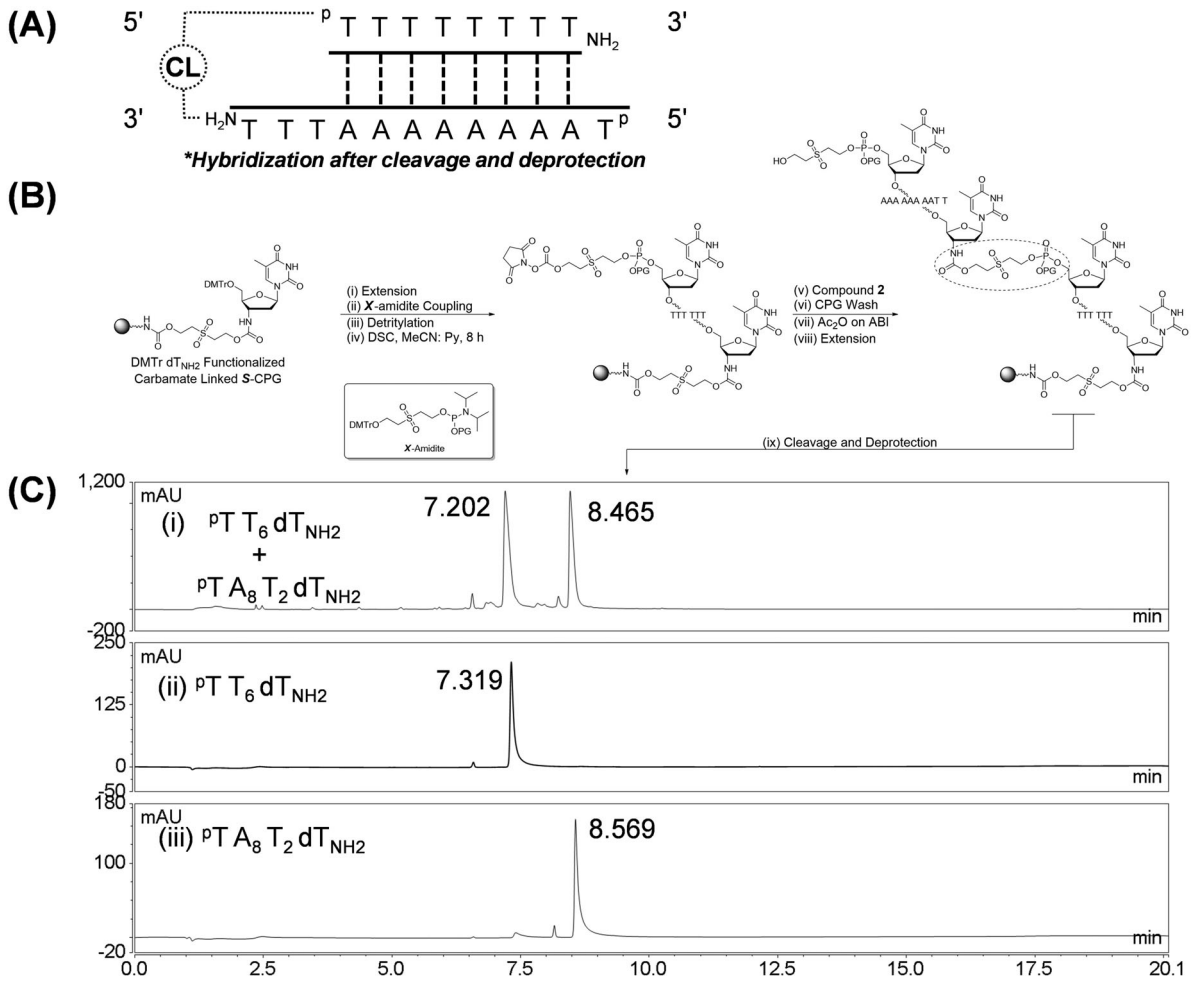


Figure 8. (A) Schematic representation of amino tandem oligonucleotide synthesis (aTOS) duplex, containing two terminal amines and two terminal phosphates. To be noted, potential hybridization would only occur after cleavage and deprotection in native conditions (purification may be necessary). (B) On-column functionalization of DMTr-dT_{NH2} coupled, carbamate linked ***S***-CPG for aTOS containing 2 terminal amines and 2 phosphates. (i) Extension of the strand and (ii) coupling of the ***X***-amidite (50 mm) using manufacturer's protocols. (iii) Detritylation of CPG on ABI 394 using manufacturer's protocols. (iv) Carbonate reaction using a saturated solution of DSC (500 µL, 1:1, MeCN: Py) for 8 h. (v) Coupling of 5′-*O*-(4,4′-dimethoxytrityl)-3′-amino-3′-deoxythymidine **2** in a 1:1 (v/v) DMF: Py (500 µL, 50 mm) solution for 15 min. (vi) Washing of the CPG using DMF, acetone and MeCN (10 mL each). (vii) Capping of the CPG on ABI 394 using Ac_2O. (viii) Extension of the second strand followed by (ix) standard cleavage and deprotection in NH_4OH at 55 °C for 17 h. (C) SAX-HPLC trace of the crude aTOS mixture (i), purified shorter duplex strand (ii) and purified longer duplex strand (iii). "dT_{NH2}", "P", "CL", "PG" denotes 3′-amino-3′-deoxythymidine, phosphate group, base labile cleavable carbamate-containing linker and cyanoethyl protecting group, respectively. Retention time (min) of the desired material(s) is reported directly on the chromatogram.

purification of the oligomer mixture can become challenging. This difficulty is influenced by factors such as sequence context, particularly in cases where strong hybridization occurs between sequences.

We envisioned the use of a new methodology for nonenzymatic primer extension experiments as these are useful models for origins of life studies. In these experiments, a shorter (primer) strand is hybridized to a longer (template) strand. Typically, the template strand is in higher excess relative to the primer strand to ensure that all of the primer strand is hybridized. In the current TOS methodology, the yield of the second synthesized strand segment is reduced relative to the first because the SPS cycle does not achieve 100% yield and the coupling of the downstream linker **4** is roughly 70–80% yield. As a result, we developed a branched TOS approach for this particular application where an excess of one strand is desired over another (Figure 10). An initial strand was prepared containing a 3′-amino group and coupled to the ***X***-amidite. The pre-activated strand was treated with a 2-amino-1,3-propanediol (serinol) spacer. As already established in literature for branching amidites, every subsequent amidite was doubly coupled with extended wait times for each coupling (10 min each; 20 min total),[23] beginning with ***X***-amidite unit incorporation. We isolated the two strands by SAX-HPLC, characterized by MS, and quantified using estimated molar extinction coefficients of the model primer and template strands. We were pleased to observe a larger proportion of the template relative to the primer strand (roughly a 1: 1.2 primer/template ratio), which is suitable for primer extension experiments. We suspect that other branching agents, such as 1,3-diamino-2-propanol, would be amenable to branched aTOS, enabling the preparation of three orthogonal strands. In lieu of serinol, 2′-amino-2′-deoxyuridine was also conjugated as a

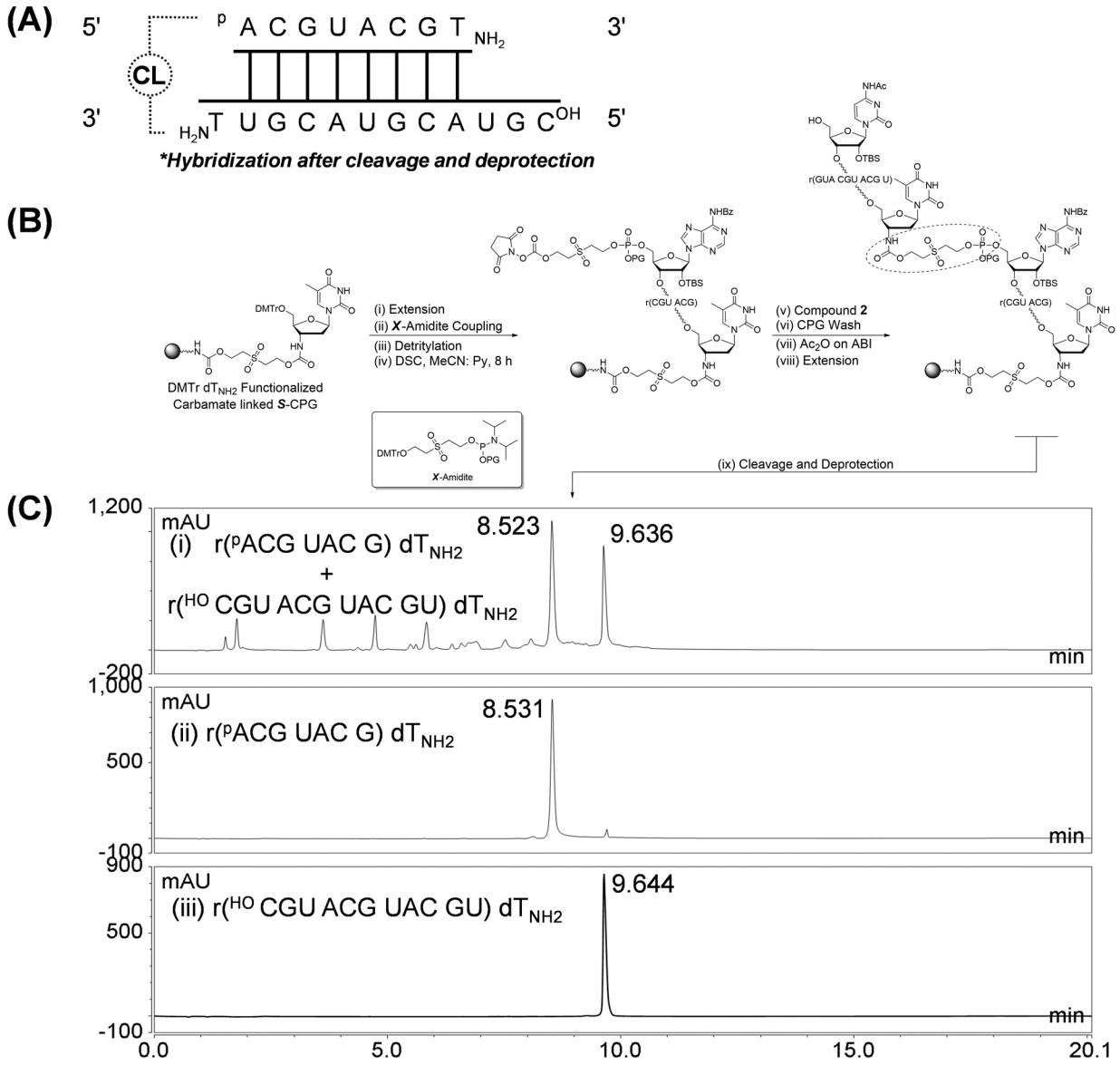


Figure 9. (A) Schematic representation of amino tandem oligonucleotide synthesis (aTOS) duplex on RNA, containing two terminal amines, one terminal phosphate and one terminal alcohol. To be noted, potential hybridization would only occur after cleavage and deprotection in native conditions (purification may be necessary). (B) On-column functionalization of DMTr-dT_{NH2} coupled, carbamate linked ***S***-CPG for aTOS containing 2 terminal amines, 1 terminal phosphate and 1 terminal alcohol. (i) Extension of the strand and (ii) coupling of the ***X***-amidite (50 mm) using manufacturer's protocols. (iii) Detritylation of the CPG on ABI 394 using manufacturer's protocols. (iv) Carbonate reaction using a saturated solution of DSC (500 µL, 1:1, MeCN: Py) for 8 h. (v) Coupling of 5′-*O*-(4,4′-dimethoxytrityl)-3′-amino-3′-deoxythymidine **2** in a 1:1 (v/v) DMF: Py (500 mL, 50 mm) solution for 15 min. (vi) Washing of the CPG using DMF, acetone and MeCN (10 mL each). (vii) Capping of the CPG on ABI 394 using Ac_2O. (viii) Extension of the second strand followed by (ix) standard cleavage and deprotection (see Supporting Information for more details). (C) SAX-HPLC trace of the crude aTOS mixture (i), purified shorter duplex strand (ii) and purified longer duplex strand (iii). "dT_{NH2}", "P", "CL", "PG" denote 3′-amino-3′-deoxythymidine, phosphate group, base labile cleavable carbamate-containing linker and cyanoethyl protecting group, respectively. Retention time (min) of the desired material(s) is reported directly on the chromatogram.

branch point to success, albeit a lower template to primer ratio (data not shown).

3. Conclusions

Our group strives to make the preparation of modified oligonucleotides more accessible to researchers who lack the infrastructure or expertise in organic chemistry. There is elevated utility for the introduction of amino modifiers into oligonucleotides in conjugation applications. Perhaps one of the most notable examples include oligonucleotide therapeutics, with the recent FDA-approval of four GalNAc-siRNA conjugations and many more in the clinics.[44] The Szostak group and the Richert group have used the 3′-amino-2′,3′-dideoxyriboside terminated RNA (or DNA) primers for origins of life research on nonenzymatic primer extension reactions.[45–50] Such primers undergo efficient template-directed nonenzymatic synthesis

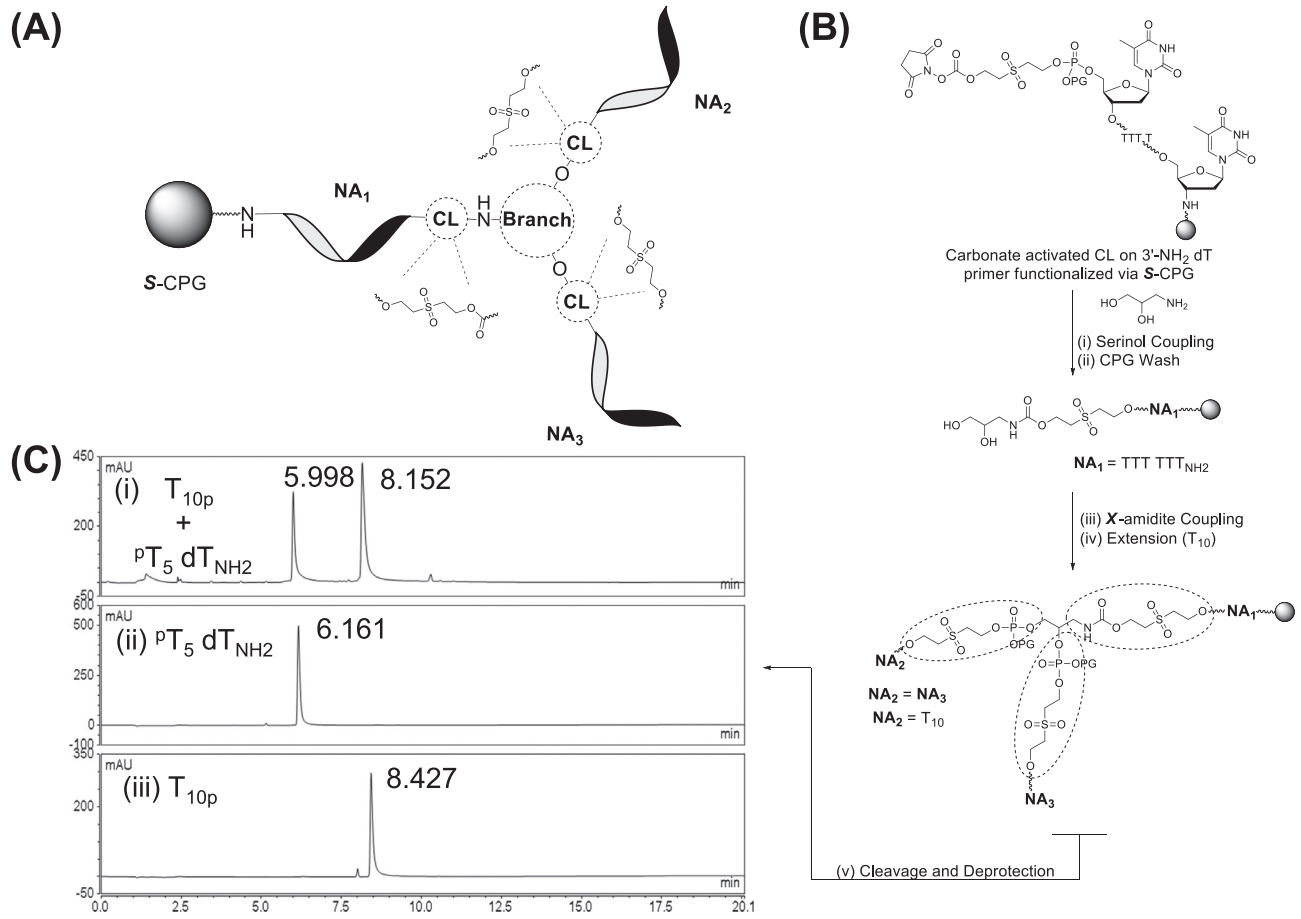


Figure 10. (A) Schematic representation of branched tandem oligonucleotide synthesis (bTOS). Note that in this example NA_2 is the same as NA_3. (B) On column functionalization of DMTr-dT_{NH2} **2** coupled ***S***-CPG for bTOS containing 1 primer and 2 template strands. (i) Serinol coupling to the activated carbonate strand 1 in 1:1 DMSO: Py (50 mM, 500 µL, 10 min) and (ii) washing of the solid-support with DMSO, DMF, acetone, MeCN (10 mL each). (iii) Double coupling of the ***X***-amidite (50 mM) was done at an extended time (10 min each). (iv) Extension of the two strands was done using 100 mM amidite, doubly coupled and with extended time (10 min each). (v) Standard cleavage and deprotection in NH_4OH at 55 °C for 17 h. (C) SAX-HPLC trace of the crude aTOS mixture (i), purified shorter duplex strand (ii) and purified longer duplex strand (iii). Retention time (min) of the desired material(s) is reported directly on the chromatogram.

with amino-ribonucleoside 5′-monophosphates activated with a good leaving group (e.g. 2-methylimidazole, 2-aminoimidazole, and 1-hydroxy-7-azabenzotriazole) to produce phosphoramidate substrates.

We have developed a robust and generalizable methodology for amino modifiers using commercially available (or easily synthesized) feedstocks. Our strategy makes use of a 2,2′-sulfonyldiethylene moiety as a cost-effective and readily available cleavable linker. Primary and secondary amines, nucleosidic or non-nucleosidic, handles are rapidly conjugated to the commercially available 3′-Phosphate-ON CPG, once preactivated with DSC. Standard DNA or RNA synthesis, including cleavage and deprotection, protocols are compatible with our method. To the extent of our knowledge, we report the first example of amino-modifier TOS (aTOS). Building on our previous work, we showcase a variety of different flavors of aTOS, which produces sequences containing amino-modifiers at all or some terminal positions. Moreover, our strategy is amenable to generating substrate pairs containing terminal amino or phosphate groups. This added flexibility can be valuable for bioconjugation and/or ligation purposes. We are currently exploring novel linkers and methodologies to improve the compatibility and high-throughput synthesis of chemically modified oligonucleotides.

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Conflict of Interests

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: amino-modified DNA/RNA · base-labile linker · solid-phase Synthesis · solid-support Chemistry · tandem Oligonucleotide Chemistry

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