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# Synthesis and hydrolytic stability of 5′-aminoacylated oligouridylic acids

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**Abstract**—Dimers and trimers of uridylic acid with a phenylalanine residue at their 5′-terminus were prepared in solution via intermediates with acid-labile protecting groups. A 5′-aminoacylated octanucleotide was prepared on solid support using a phosphoramidate linker and an allyloxycarbonyl-protected phenylalanine. 

## 1. Introduction

Aminoacylated ribonucleotides, in the form of charged tRNAs, play a pivotal role in the translation of genetic information. While tRNAs found in the ribosomal machinery are exclusively 2′/3′-esterified nucleic acids, it has recently been shown that 5′-aminoacylated ribonucleotides can be formed in ribozyme-catalyzed reactions,[1] making it desirable to develop chemical syntheses for such compounds. To our knowledge, previous synthetic work on 5′-aminoacylated RNA derivatives has been limited to the aminoacylation of nucleosides.[2] Reports on 5′-aminoacylated 2′-deoxynucloside derivatives also exist.[3] Out of an interest in non-enzymatically catalyzed acyl transfer reactions and expanding the synthetic chemistry of aminoacylated RNA,[4] we explored the chemical synthesis of 5′-aminoacylated oligoribonucleotides. Here, we report results from a study focused on phenylalanine-bearing oligouridylic acids. Both a solution phase synthesis and a synthesis on solid supports are being reported.

## 2. Results and discussion

The key difficulty in preparing aminoacylated oligonucleotides lies in the hydrolytic lability of the ester bond that makes it impossible to use conventional syntheses for oligonucleotides that involve a final deprotection/cleavage step under strongly basic conditions. To overcome this difficulty, the current synthesis was undertaken with acid-labile protecting groups for the 2′,3′- and 5′-hydroxyl groups of the ribonucleosides, and initially also for the amino group of the aminoacyl moiety. To make the approach attractive for routine syntheses, we decided to focus on a route involving commercially available RNA building blocks and supports only. This led to uridine derivative **1** with its 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp) group[5] as the phosphoramidite building block and acetonide **2** as the 3′-terminal residue (Scheme 1).

In a one-pot procedure, phosphoramidite **1**, was converted to dimer **3**. Coupling with tetrazole activation, followed by addition of oxidizer solution, and subsequent treatment with thiosulfate gave **3** in 92% yield after chromatography on silica. Initially, solid $Na_2S_2O_3$ was used in the last step of this one-pot procedure, leading to a slow reduction of the excess iodine. Later it was found that washing with a saturated thiosulfate solution during the aqueous work-up gave a more rapid conversion of the excess reagent. Dimer **3** was prepared for aminoacylation by removal of the cyanoethyl groups with triethylamine in acetonitrile (5:3 v/v) and de-dimethoxytritylation with trichloroacetic acid in $CH_2Cl_2$, giving **4** in 73% yield after precipitation and washing with diethylether.[6] The removal of the cyanoethyl groups was performed, since published work on the synthesis of peptide–DNA hybrids had shown that free diesters give higher yields in acylation reactions than triesters.[7]

Coupling of Boc-Phe-OH to **4** proceeded uneventfully when the amino acid building block was activated with

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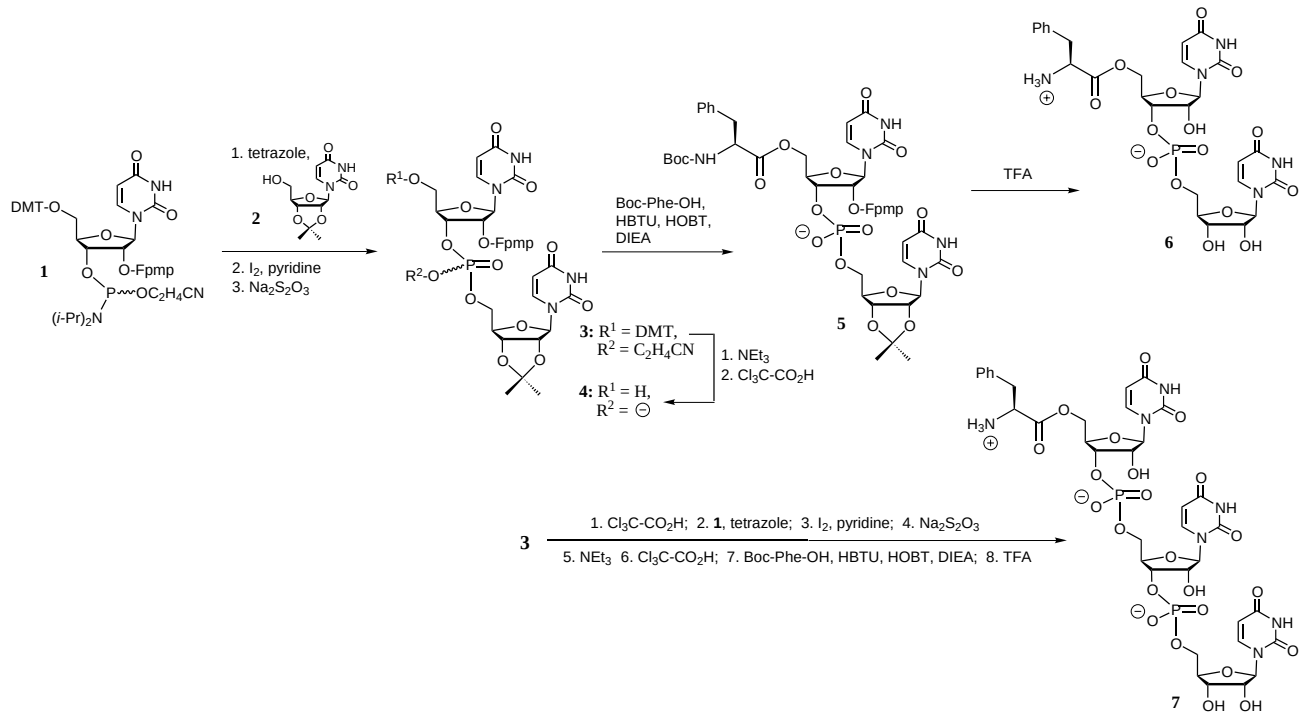
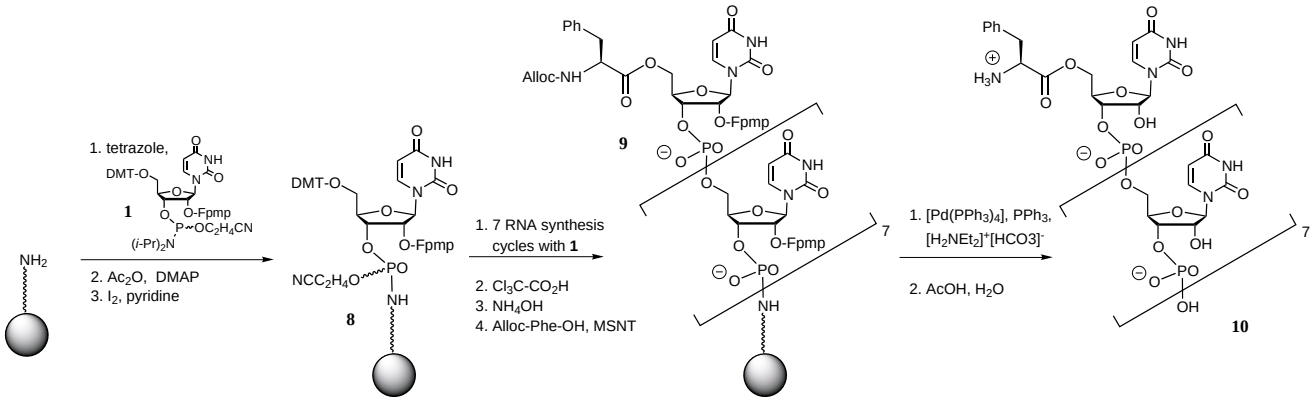



**Scheme 1.**

a mixture of the 'uronium salt' HBTU, hydroxybenzotriazole (HOBT), and Hünig's base (DIEA) in DMF. Ester **5** was obtained in 86% yield after HPLC purification, using a gradient of acetonitrile in water on a $C_{18}$-phase. In the final deprotection step, involving 80% aqueous TFA at room temperature for 30 min, the Boc, Fpmp, and isopropylidene groups were cleaved. Precipitation with ether gave aminoacylated dimer **6** in 82% yield.[8]

Extension of the solution phase method to a nucleic acid trimer was feasible, though giving a lower yield of the fully assembled target molecule (Scheme 1). Protected dimer **3** was detritylated, and phosphoramidite **1** was coupled to the newly liberated hydroxyl group, followed by oxidation with iodine/pyridine. From the resulting intermediate, thus obtained in 90% yield, the β-cyanoethyl groups were removed with triethylamine, followed by removal of the 5′-DMT group with trichloroacetic acid (66% after chromatography for the two steps). Then, Boc-protected *L*-phenylalanine was coupled under the same conditions as described above for the conversion of **4** to **5**. Final full deprotection with 80% TFA yielded aminoacylated trimer **7** in 9% overall yield. The low yield was probably due to losses during handling, since MALDI monitoring of the reaction had shown **7** as the major peak in the crude.

To allow for the synthesis of longer aminoacylated strands, a solid phase synthesis was developed. One key feature of this synthesis (Scheme 2) is the acid-labile phosphoramidate linker to the solid support, adapted from a methodology reported by Gryaznov and Letsinger for the synthesis of DNA oligomers.[9] As a




**Scheme 2.**

consequence of using this linker, the RNA oligomers prepared contained a 3′-terminal phosphate group. The derivatized support was constructed by reacting phosphoramidite **1** with solid supports bearing amino groups. The resulting immobilized phosphoramidate (**8**) was extended to an octamer using a standard protocol for phosphoramidites of ribonucleotides on an automatic synthesizer. As expected,[10] the yield of the octanucleotide, as determined via release of the DMT-cation, varied with the support. The best results were obtained with long chain alkylamine (LCAA) CPG from Sigma with pore size 500 Å and particle mesh size of 80–120, which gave the octamer in 75% overall yield. Polystyrene support from Novabiochem gave 23%, TentaGel resin from Advanced ChemTech 62%, and LCAA-CPG from CPG Inc. 64%.

After cleaving the cyanoethyl groups from the backbone phosphotriesters of the extended derivative of **8** with ammonium hydroxide, the terminal 5′-hydroxyl group of the RNA octamer was coupled to *N*α-allyloxycarbonyl (Alloc) protected phenylalanine[11] to produce **9**. An Alloc, rather than Boc, protecting group was chosen for the amino acid to introduce orthogonality, and thus allow for the assembly of oligopeptides on the RNA terminus, if necessary. Since the HBTU/HOBT coupling mixture gave only moderate yields (53%), as determined by integrating peaks in the MALDI-TOF mass spectra of crude **10**, obtained after deprotection with aqueous AcOH, a number of coupling conditions were tested. A mixture of DCC and HOBT gave 22% yield after 47 h reaction time and 8% yield after 25 h. When DCC was employed with DMAP, 25 h reaction time showed 64% of **10** in the crude, whereas DPPA, $PPh_3$/DIAD (Mitsunobu conditions),[2] cyanuric chloride, BOP-Cl, and Aldrithiol/HBTU all gave <5% of the aminoacylated octamer.

The best coupling results were obtained with 1-(2-mesitylenesulfonyl)-3-nitro-1*H*-1,2,4-triazole (MSNT) as the condensing agent. When employed with Hünig's base as the co-reagent and DMF as solvent, **10** made up 85% of the crude under optimized deprotecting conditions.[12] The optimized deprotection involved treatment of the Alloc-deprotected derivative of **9** with AcOH/water (19:1) at room temperature for 14 h, rather than with AcOH/water (4:1) for 4 h, as recommended for DNA oligomers,[9] which gave increased fragmentation. The amount of fragments was not decreased further by rigorous exclusion of possible contaminations with RNases or reducing the handling prior to sampling for MALDI-TOF MS. A MALDI mass spectrum of a typical crude product is shown in Fig. 1.

An exploratory experiment was performed to compare the stability of the 5′-ester in **10** towards hydrolysis with that of esters in similar oligoribonucleotides aminoacylated at the 2′/3′-position. The sequences CUCCACCA-Trp, and CUCCACCA-Trp-Boc prepared from the aminoacylated dinucleotides pdCpA-Trp or pdCpA-Trp-Boc[13] and 5′-CUCCAC-3′ using T4 ligase,[14] were available for this experiment from other work on acyl transfer reactions. Solutions containing the three aminoacylated species were exposed to pH 8.5, and the kinetics of the hydrolysis of the esters linkages were monitored by MALDI-TOF MS under conditions previously shown to allow quantitative detection of oligonucleotides[15] (Fig. 2).

The rates of ester hydrolysis under the pseudo-first order conditions chosen were $7.0\times10^{-4}$ $s^{-1}$ for **10**, $1.1\times10^{-3}$ $s^{-1}$ for CUCCACCA-Trp-Boc, whose amino group was protected, and $7.4\times10^{-3}$ $s^{-1}$ for CUCCACCA-Trp, translating into half-life times of $10^3$ min, $6\times10^2$ min, and 90 min, respectively. The increased hydrolytic stability of 5′-aminocylated **10** suggests that if acyl transfer from 2′/3′- to 5′-termini of RNA strands[1] did take place under prebiotic conditions, the 5′-aminoacylated strands generated would have been more long-lived than their 2′,3′-aminoacylated counterparts and therefore be potentially catalytically or metabolically useful species.[16]

In conclusion, the results from the exploratory synthetic work presented here indicate that 5′-aminoacylated

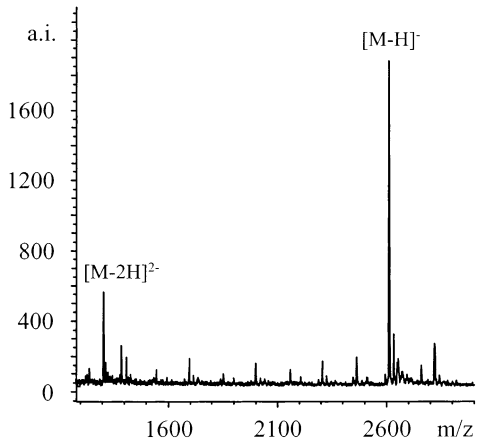



**Figure 1.** Representative MALDI-TOF spectrum of crude **10**, as obtained after deprotection with AcOH/$H_2O$ (19:1).

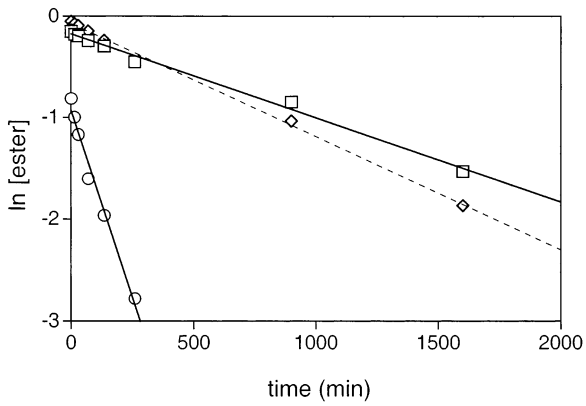



**Figure 2.** Kinetics of the hydrolysis of the ester linkages in 5′-aminoacylated **10** (squares), and 2′/3′-acylated RNA octamers CUCCACCA-Trp (circles) and CUCCACCA-Trp-Boc (diamonds) in a solution containing 300 mM LiCl, 20 mM HEPES buffer, and 0.2 mM EDTA, pH 8.5, as determined by MALDI-TOF MS.

oligomers of uridylic acid can be prepared using a methodology relying on acid-labile protecting groups and a phosphoramidate linker. The successful employment of Alloc-Phe-OH as a building block in the synthesis of **10** suggests that extension to mixed RNA sequences should be feasible, if allyl-/allyloxycarbonyl-protected phosphoramidites of adenosine, cytidine and guanosine were to be employed.[17] Finally, the synthetic methodology for ester-containing oligonucleotides might be of interest for the preparation of oligonucleotide pro-drugs bearing 5′-appendages to be removed by intracellular esterases after entry into cells.

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6. Brief protocol: A solution of **1** (0.3 g, 0.32 mmol, from Cruachem Ltd.) and **2** (98 mg, 0.62 mmol, from Sigma) in $CH_3CN$ (0.75 mL) was treated with a solution of tetrazole in $CH_3CN$ (8 mL, 0.45 M, 3.6 mmol). After 50 min, a solution of $I_2$ (12 mL, 0.2 M in water/THF/pyridine) was added. After 45 min, $Na_2S_2O_3$ (500 mg, 3.2 mmol) was added, followed by stirring for 90 min and filtration. Then, $CH_2Cl_2$ was added, the solution washed with satd $NaHCO_3$, dried, and chromatographed (silica, $CH_2Cl_2$/MeOH/$NEt_3$ 82:10:8). The product was dissolved in $CH_3CN$ (6 mL) and $NEt_3$ (10 mL) under sonication, stirred for 3.5 h, and dried. The residue was treated with $CCl_3CO_2H$ solution (2.5 mL, 0.18 M in $CH_2Cl_2$) for 3 min, followed by precipitation with $Et_2O$. The precipitate was washed with $Et_2O$, dried, and lyophilized from water. Yield of **4**: 185 mg (0.23 mmol, 73%). $^1H$ NMR ($CDCl_3$, 300 MHz): $\delta$ 1.71 (s, 6H); 1.76–1.86 (m, 4H); 2.75–2.86 (m, 4H); 2.88 (s, 3H); 3.58–3.60 (m, 2H); 3.93–3.95 (m, 2H); 4.24–4.27 (m, 1H); 4.28–4.31 (m, 1H); 4.38–4.41 (m); 4.42–4.46 (t, 1H); 4.71–4.75 (dd, 1H); 4.84–4.88 (dd, 1H); 5.60–5.63 (m, 1H), 1H); 5.76–5.79 (d, 1H); 5.92–5.95 (d, 1H); 6.92–6.97 (m, 4H); 7.53–7.56 (d, 1H); 7.74–7.77 (d, 1H); MALDI-TOF MS for $C_{33}H_{40}FN_5O_{15}P$: calcd 796.7, found 796.7.
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8. Brief protocol: A mixture of **4** (33.3 mg, 42 μmol), Boc-Phe-OH (99 mg, 374 μmol), HOBT (50 mg, 370 μmol), and HBTU (128 mg, 336 μmol) was dissolved in DMF (0.2 mL) and treated with DIEA (213 μL, 1.25 mmol). After 1 h, ammonium acetate solution (0.6 mL, 0.1 M) was added and the solution HPLC purified ($C_{18}$, $CH_3CN$ in water: 10% for 5 min, 10–50% in 30 min, elution at 23 min). Product containing fractions was dried (37.4 mg, 86%) and then resuspended in TFA (160 μL) and water (40 μL). After 30 min, $Et_2O$ (1 mL) was added. The precipitate was washed with ether, lyophilized from water, and dried. Yield of **6**: 24 mg, (34 μmol, 82%). $^1H$ NMR ($D_2O$, 300 MHz): $\delta$ 7.62 (d, 1H); 7.34 (d, 1H), 7.09 (m, 3H); 7.00 (m 2H); 5.67 (d, 1H); 5.62 (m, 2H); 5.51 (d, 1H); 4.98 (m, 1H); 4.83 (m, 1H); ca. 4.7, under HDO (m), 4.23 (m 2H); 4.01–4.02 (m, 4H); 3.91 (m, 2H); 2.90 (m, 2H); MALDI-TOF MS for $C_{27}H_{31}N_5O_{15}P$: calcd 696.5, found 696.0.
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12. Abridged protocol: Solid support **8** (30 mg) was extended to an octamer using the RNA program for ABI 381A synthesizers (1 μmol scale, trityl off mode). The assembled octamer was treated with $NH_4OH$ (1 mL) for 2 h at 55°C. The solution was aspired, the support was washed with water and $CH_3CN$, and allowed to dry. A solution of Alloc-Phe-OH (8 mg, 32 μmol), MSNT (10 mg, 33 μmol), DIEA (50 μL, 0.29 mmol), and DMF (100 μL) was added and the mixture bath-sonicated for 1 min. After 1 h the supernatant was aspired, the support washed with $CH_3CN$ and $CH_2Cl_2$, and dried. A solution of $Pd(PPh_3)_4$ (5 mg, 4.3 μmol) and diethylammonium bicarbonate (5 mg, 41 μmol) in $CH_2Cl_2$ (0.4 mL) was added, followed by sonication for 2 min. After 2 h, the CPG was washed with $CH_2Cl_2$ and $CH_3CN$ (1 mL) and dried. Then, AcOH (190 μL) and water (10 μL) were added. After 14 h, the supernant was aspired, filtered, and treated with $Et_2O$ (2 mL). The precipitation was completed at −4°C for 1 h and centrifugation. The resulting pellet gave 85–93% product, as determined by integration of the MALDI-TOF mass spectrum. MALDI-TOF MS for $C_{81}H_{98}N_{17}O_{66}P_8$: calcd 2613.5, found 2613.5.
13. Prepared using a modification of the procedure reported in Ref. 4b.
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