

Article

A Study on Synthesis and Upscaling of 2'-O-AECM-5-methyl Pyrimidine Phosphoramidites for Oligonucleotide Synthesis

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Abstract: 2'-O-(N-(Aminoethyl)carbamoyl)methyl-modified 5-methyluridine (AECM-MeU) and 5-methylcytidine (AECM-MeC) phosphoramidites are reported for the first time and prepared in multigram quantities. The syntheses of AECM-MeU and AECM-MeC nucleosides are designed for larger scales (approx. 20 g up until phosphoramidite preparation steps) using low-cost reagents and minimizing chromatographic purifications. Several steps were screened for best conditions, focusing on the most crucial steps such as N³ and/or 2'-OH alkylations, which were improved for larger scale synthesis using phase transfer catalysis (PTC). Moreover, the need of chromatographic purifications was substantially reduced by employing one-pot synthesis and improved work-up strategies.

Keywords: 2'-O-(N-(aminoethyl)carbamoyl)methyl modification; phase transfer catalysis (PTC); 5-methyluridine; 5-methylcytidine; oligonucleotides; alkylation; monoacetylation



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1. Introduction

In recent years, therapeutic oligonucleotides (ONs) have become one of the most promising classes of drug candidates for the treatment of life-threatening diseases [1]. Nevertheless, different therapeutic approaches, such as antisense oligonucleotides (ASOs) [1–3], RNA interference [4–6] and microRNA [1,7] technologies, usually face two types of limitations: oligonucleotide instability in biological fluids and problematic delivery to the site of action. Modifications of both the base and the sugar fragments of an oligonucleotide were shown to provide several advantages in the past [1]. For instance, introduction of a methyl group to the 5th position in cytosine enhances duplex thermal stability [1]. Methylation of pyrimidines also reduces the immune response [8–11]. 2'-O-Alkyl oligonucleotide modifications [12] showed several advantages like binding affinity and resistance to nucleases [13], including a relatively straightforward synthesis, allowing potentially cost-efficient manufacturing. The synthesis of 2'-aminoethoxy-modified oligonucleotides was shown to increase the binding affinity [14]. Later, using an alternative pathway, allowing introduction of different bases at a later stage into 2'-aminoethoxy-modified nucleoside monomers was introduced [15]. The 2'-O-carbamoylmethyl (CM) modification was first introduced by Grötli et al. and was shown to give substantial stabilization of duplexes [16]. Milton et al. have shown that CM-modified oligonucleotides are also highly resistant to enzymatic degradation [17]. In addition, this scaffold can be further modified by different substituents on the amide nitrogen and several research groups investigated these possibilities [18–24]. The post-synthetic modification with 2'-O-(N-(aminoethyl)carbamoyl)methyl (2'-O-AECM) was investigated by Ozaki et al. [21]. Furthermore, our group has previously demonstrated the synthesis of 2'-O-AECM modified adenosine and that this modification provides a unique combination of resistance against enzymatic degradation and

improved cell penetration properties for ONs [25]. In addition, the initial studies on 2'-O-AECM-modified ONs with all common 2'-O-AECM-modified ribonucleotides showed efficient splice-switching activity [26]. Moreover, as this modification showed promising properties for ON therapeutics [25–27], enabling its efficient synthesis, and upscaling is essential for moving oligonucleotide constructs forward towards the quantities required for animal experiments.

Herein, we report on our recent efforts to develop convenient procedures to prepare 5-methyluridine (AECM-MeU) and 5-methylcytidine (AECM-MeC) (Figure 1) monomers in larger (up to 100 g for some intermediates) scales. Furthermore, AECM-MeU and AECM-MeC are prepared as phosphoramidite derivatives that contain protecting groups (e.g., dimethoxytrityl and trifluoroacetyl on the amine) which enables them to be used as building blocks for automated solid-phase oligonucleotide synthesis.

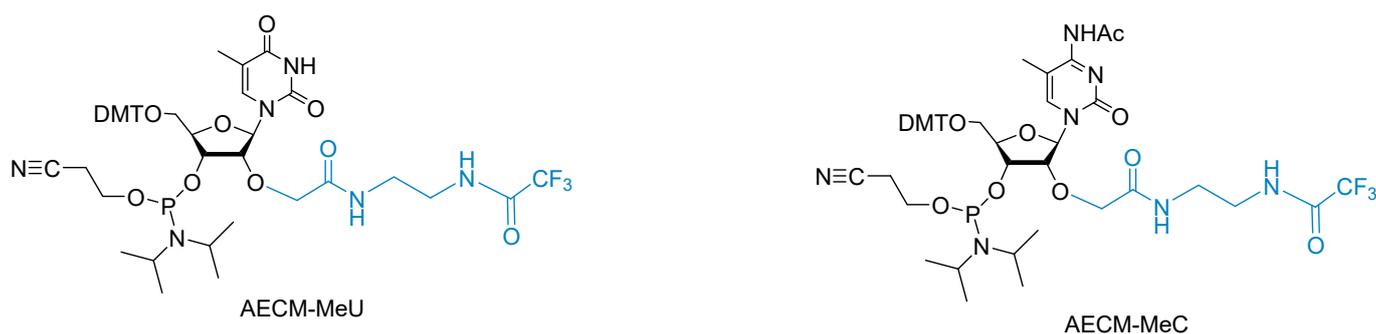


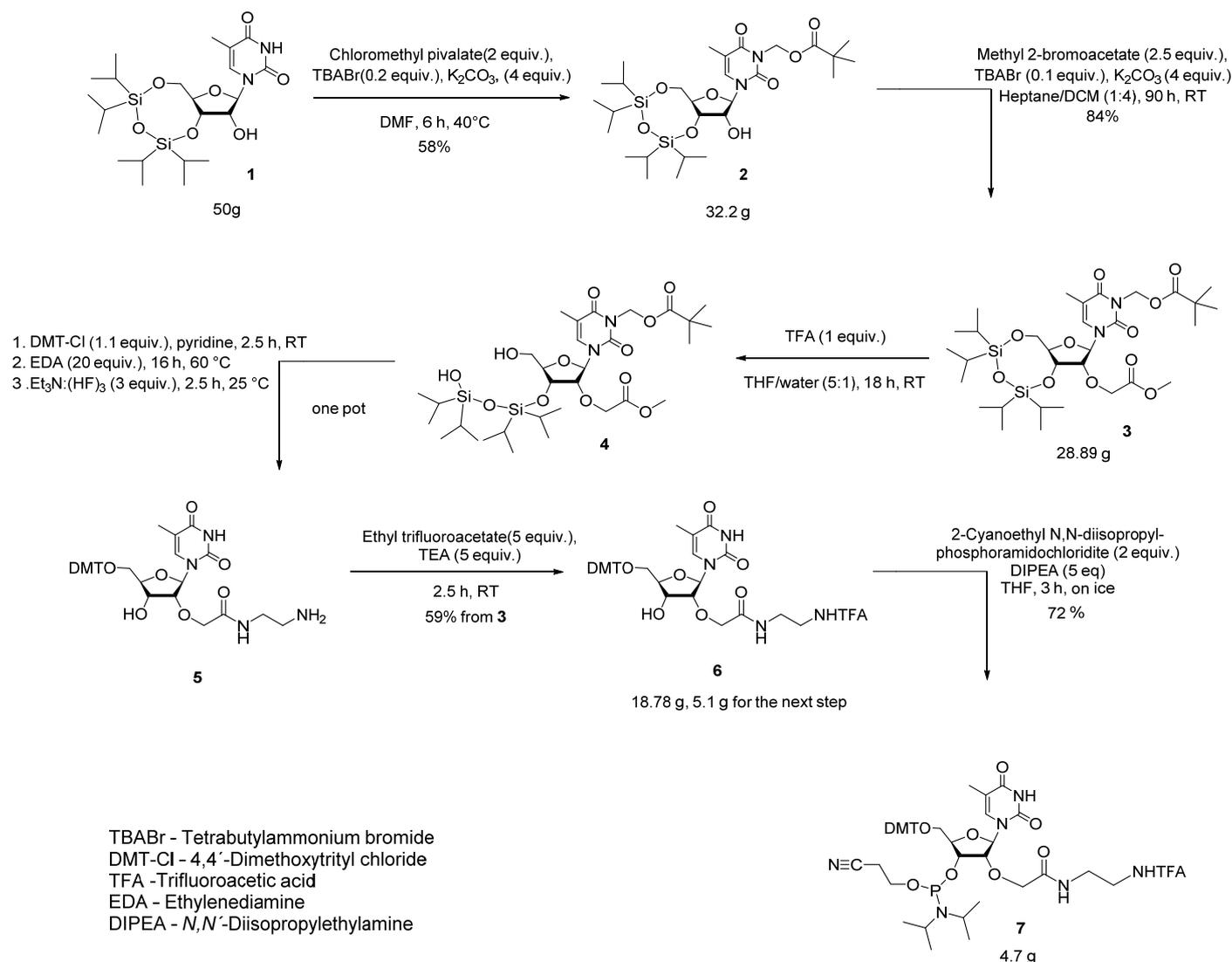
Figure 1. 2'-O-AECM modified 5-methyluridine (AECM-MeU) and 5-methylcytidine (AECM-MeC) nucleosides.

5-Methylated AECM-modified uridine and cytidine phosphoramidites (Figure 1) have not been reported before, but the synthesis of the corresponding non-methylated AECM-modified cytidine and uridine nucleosides was achieved in scales of milligrams and a few grams, respectively [26,28]. However, several steps used for the non-methylated derivatives were considerably less efficient with the 5-methyl nucleosides and gave more problems with purifications. These findings, together with a preferred use of less expensive reagent, led to the need for a complete revision of the syntheses of AECM-MeU and AECM-MeC that are also more suitable for significantly larger scales. Moreover, more efficient purification and/or work-up strategies were necessary to be able to adapt the syntheses for larger scales. Herein, the developed procedures that enabled synthetic routes with fewer chromatographic purifications required only three chromatographic purifications for the intermediates up to the AECM-MeU nucleoside preceding the phosphoramidite conversion (in eight steps of synthesis from MeU) and only one for the intermediates up to the AECM-MeC nucleoside preceding the phosphoramidite conversion (in eight steps of synthesis from MeC) was needed. This was achieved by finding suitable conditions for work-ups, forming crystalline intermediates or finding strategies to perform one-pot synthesis. However, the final phosphoramidites were chromatographed prior to oligonucleotide synthesis (AECM-MeC phosphoramidite twice) to achieve a purity needed to ensure high-quality ON.

2. Results and Discussion

2.1. Synthesis of AECM-MeU Phosphoramidite

The study started with the development of a synthetic route for AECM-MeU phosphoramidite 7 (Scheme 1). All reactions, starting with the N³ protection of 3',3',5'-O-[(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)]-5-methyluridine, except the final phosphoramidite synthesis, were either screened for optimized conditions based on quality attributes or tested in up to 1 g scale for feasibility before upscaling the reactions further. All of the screening reactions for individual steps were performed in 0.25–1 g scale and were evaluated using approximate conversions (%) from UV traces in the HPLC chromatogram (without internal standard).



Scheme 1. Complete synthetic scheme of AECM-MeU phosphoramidite using optimized conditions.

The synthesis of AECM-MeU started with preparation of 3',5'-O-[(1,1,3,3-tetraisopropyl-1,3-disiloxanediy)]-5-methyluridine (**1**) [29] in 195 g scale. Although the protection of N³ with the pivaloxymethyl (Pom) group using phase transfer catalysis (PTC) was previously described [30], the development of this step started with screening for optimized conditions for the PTC reaction (Table 1). The goal was to reduce the amounts of solvents (as more than 120 volumes in total were used in a published procedure which is not satisfactory for large-scale synthesis) as well as to decrease the amount of chloromethyl pivalate (Pom-Cl).

Evaluation of N³ protection of the AECM-MeU monomer was performed using different base–solvent combinations and tetrabutylammonium bromide (TBABr) as a PTC catalyst (Table 1). Although tetrabutylammonium hydrogen sulfate (TBAHS) was used in a published procedure, TBABr showed satisfying results in some of our initial screens and therefore we decided to continue with this PTC catalyst. In addition, some pre-screen tests showed that an increased reaction temperature could be beneficial, therefore the main screen (Table 1) was carried out at 50 °C. As one of the main goals was to evaluate if smaller amounts of Pom-Cl are sufficient for the synthesis (in a previously described procedure [30] more than 9.9 equiv. of Pom-Cl were used), the screening reactions were carried out using only a slight excess (1.5 equiv.) of this reagent (Table 1).

Table 1. Screen for suitable conditions for N³ alkylation of 5-methyl uridine intermediate.

No.	Pom-Cl, Equiv.	Solvent, 5 Vol	Base, 3 Equiv.	(PTC) Catalyst, 0.2 Equiv.	HPLC Area%, Conver., 50 °C, 1 h	HPLC Area%, Conver., 50 °C, Overnight
1.	1.5	Heptane	KHCO ₃	TBABr	Product trace	66%
2.	1.5	Heptane	NaHCO ₃	TBABr	Product trace	Product trace
3.	1.5	DCM	NaHCO ₃	TBABr	Product trace	6%
4.	1.5	Toluene	KHCO ₃	TBABr	Product trace	33%
5.	1.5	Toluene	NaHCO ₃	TBABr	Product trace	6%
6.	1.5	DMF	KHCO ₃	TBABr	23%	50%
7.	1.5	DMF	NaHCO ₃	TBABr	4%	23%
8.	1.5	DMF	K ₂ CO ₃	TBABr	53%	14% *
9.	1.5	DMF	Na ₂ CO ₃	TBABr	6%	54%

* Including degradation of the product.

The study revealed that the reaction proceeds most rapidly in DMF in the presence of K₂CO₃ (Table 1, entry 8). However, extended reaction times led to degradation which indicates that the reaction must be monitored carefully. Nonetheless, based on the screenings, final conditions for the synthesis of compound 2 were 2 equiv. of chloromethyl pivalate, 4 equiv. of K₂CO₃, 0.2 equiv. of TBABr in DMF. It was decided to increase the amount of the base in order to have a larger particle surface and 2 equiv. of Pom-Cl was used to make sure the reaction went to completion. Although HPLC analysis revealed that using these conditions, after 70–75% conversion, the amount of side products substantially increases, different bases and solvents did not seem to be more advantageous. The possibility to have a rather fast reaction as well as to decrease the amount of solvent and Pom-Cl was regarded as more attractive and the above-mentioned conditions were chosen for larger scale synthesis, which was performed in 50 g scale for 6 h. In addition, the reaction was sampled after 5.5 h to give a rather clean HPLC profile (Supplementary Figure S1).

PTC conditions were also employed for 2'-OH alkylation to obtain compound 3 and the process development to find optimal conditions of this step was performed similarly as for the N³ alkylation (Table 2). The screen was carried out using different base–solvent–PTC catalyst combinations. The results showed that a PTC catalyst is crucial for the reaction to proceed efficiently (Table 2, entry 1 and 2) with a rather minor difference between the three PTC agents tried (Table 2). As TBABr was working well for N³ alkylation and additional screens confirmed its efficiency (Supplementary Tables S1 and S2), this catalyst was chosen for the larger scale reaction. Moreover, additional screens were performed to determine suitable amounts of base and methyl 2-bromoacetate (Supplementary Table S1). Different bases (K₂CO₃ and K₃PO₄) were also compared and revealed that the alkylation reaction results in the least amount of impurities when it is performed in heptane or acetonitrile (MeCN) in the presence of K₂CO₃. To confirm these results, additional screenings (Supplementary Tables S1 and S2) were performed, which showed that a reaction in heptane was cleaner compared to MeCN (Supplementary Figures S2 and S3). However, upon a transfer of conditions to a larger scale, solubility became an issue (5 vol of heptane was used in test reactions (Table 2, entries 6 and 11)), which did not seem a problem at the time. However, larger amounts of compound 2 took longer times to dissolve in 5 vol of heptane and the alkylation product 3 can be poorly soluble in this solvent. To overcome this problem, we chose to perform the reaction in a DCM/heptane mixture (this test reaction was done prior to larger scale (Table 2, entry 14)).

Finally, after the series of screens, 5 vol DCM/heptane (1:4 (v/v)), 4 equiv. of K₂CO₃ and 0.05 equiv. of TBABr were selected for the larger scale of MeU 2'-OH alkylation. The amount of K₂CO₃ was increased for the same reason as for N³ alkylation. Nonetheless, the scaling-up of a PTC reaction using solid base can sometimes become challenging. The grinding effect of the magnet in smaller scale PTC reactions is more pronounced and hence fresh particle surface is continuously exposed. We observed that the PTC alkylation on a larger scale did not go to completion after stirring at ambient temperature for 66 h compared to the small-scale reaction which was completed after 36 h (Table 2, entry 14).

The additional added amounts of K_2CO_3 , TBABr and methyl 2-bromoacetate facilitated the reaction to go to completion. At this point, a one-pot three-step synthesis followed. The reaction sequence started with selective opening of the 5' position of compound **3** using TFA in THF:water (5:1, *v/v*).

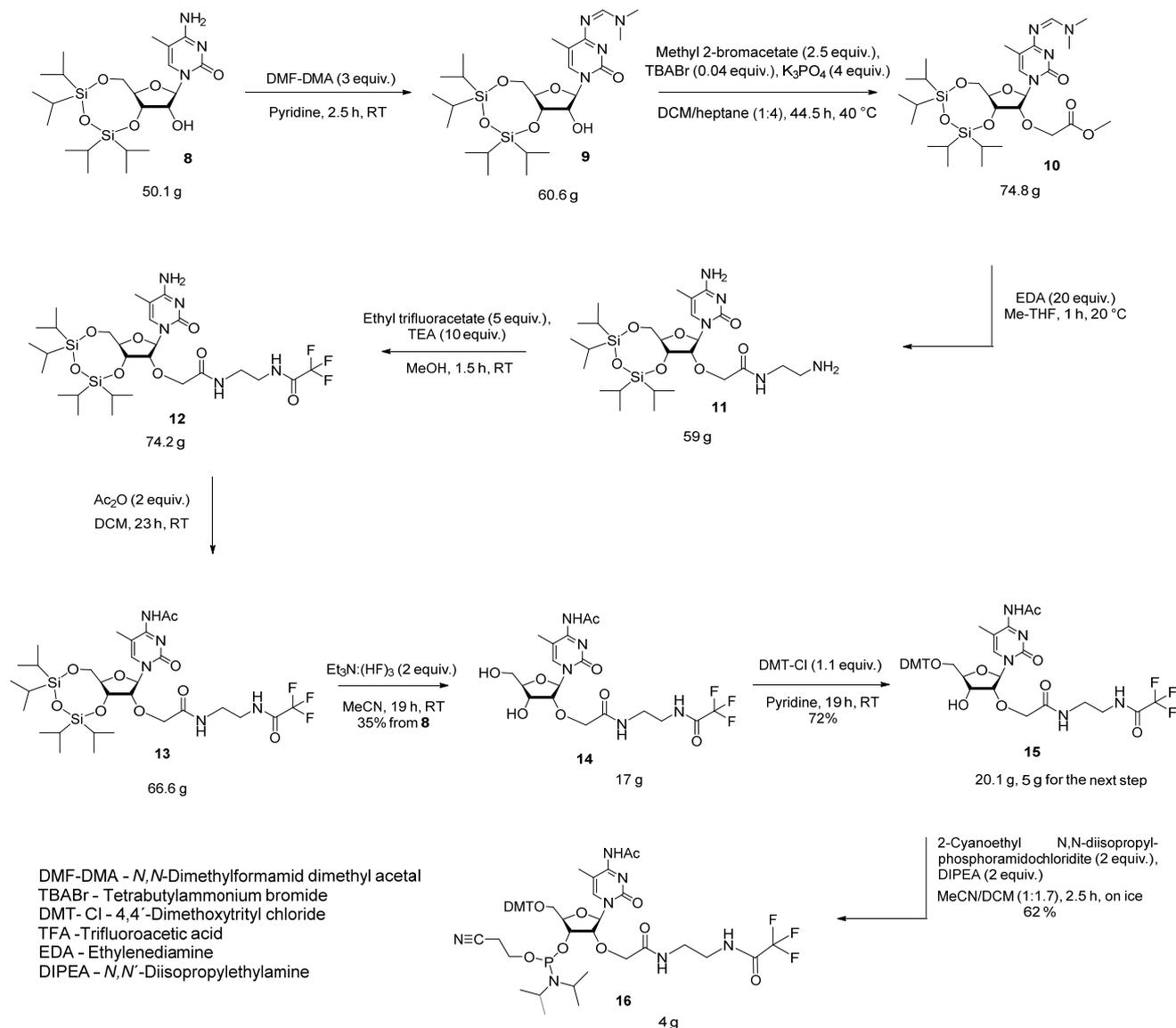
Table 2. Screen for suitable conditions for 2'-OH alkylation of 5-methyl uridine intermediate.

No.	Methyl 2-Bromoacetate	Solvent, 5 Vol	Base	(PTC) Catalyst	HPLC Area%, Conver., RT, 1 h	HPLC Area%, Conver., RT, Overnight
1.	1.2 equiv.	MeCN	K_2CO_3 , 2 equiv.	MeNOct ₃ Cl, 0.2 equiv.	7%	50%
2.	1.2 equiv.	MeCN	K_2CO_3 , 2 equiv.	-	Product trace	16%
3.	1.2 equiv.	MeCN	K_2CO_3 , 2 equiv.	Oct ₄ NBr, 0.2 equiv.	8%	54%
4.	2 equiv.	MeCN	K_2CO_3 , 2 equiv.	Oct ₄ NBr, 0.2 equiv.	15%	86%
5.	2 equiv.	Toluene	K_2CO_3 , 2 equiv.	Oct ₄ NBr, 0.2 equiv.	Product trace	11%
6.	2 equiv.	Heptane	K_2CO_3 , 2 equiv.	Oct ₄ NBr, 0.2 equiv.	5%	51%
7.	2 equiv.	DCM	K_2CO_3 , 2 equiv.	Oct ₄ NBr, 0.2 equiv.	Product trace	22%
8.	2 equiv.	DMF	K_2CO_3 , 2 equiv.	Oct ₄ NBr, 0.2 equiv.	7%	76%
9.	2 equiv.	MeCN	K_3PO_4 , 2 equiv.	Oct ₄ NBr, 0.2 equiv.	51%	83%
10.	2 equiv.	Toluene	K_3PO_4 , 2 equiv.	Oct ₄ NBr, 0.2 equiv.	4%	18%
11.	2 equiv.	Heptane	K_3PO_4 , 2 equiv.	Oct ₄ NBr, 0.2 equiv.	9%	50%
12.	2 equiv.	DCM	K_3PO_4 , 2 equiv.	Oct ₄ NBr, 0.2 equiv.	5%	37%
13.	2 equiv.	DMF	K_3PO_4 , 2 equiv.	Oct ₄ NBr, 0.2 equiv.	9%	32%
14.	2 equiv.	Heptane:DCM (4:1, <i>v/v</i>)	K_2CO_3 , 4 equiv.	TBABr, 0.05 equiv.	Product trace	76% (93% after 36 h)

Selective opening of the cyclic silyl protecting group is more advantageous for selective introduction of the 5'-O-DMT protection and subsequent steps in terms of yield and purity, as the retained silyl on the 3'-OH mitigated the risk of lactonization (2'-O-methoxyacetyl). Crude isolated product **4** was further treated with 4,4'-dimethoxytrityl chloride at ambient temperature. The reaction was then quenched with MeOH followed by addition of ethylenediamine (EDA) and the reaction mixture was then heated to 60 °C to complete aminolysis of the methyl ester and removal of the Pom protecting group from N³. After stirring overnight, TEA×3HF was added to remove the remaining silyl protecting group at the 3'-position. Following an aqueous work-up, crude **5** was acylated using ethyl trifluoroacetate and then chromatographed to afford compound **6** (59% from **3**, 29% overall yield from **1**, 84% average yield per step). Compound **6** was further phosphitylated at the 3'-position using standard conditions with 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite in the presence of DIPEA in THF to yield the AECM-MeU phosphoramidite **7**.

2.2. Synthesis of AECM-MeC Phosphoramidite

The reported synthesis procedure [26,28] of the non-methylated AECM-C monomer was used as a starting point for the preparation of AECM-MeC **16** (Scheme 2) but changes and substantial improvements were developed before the upscaling. All of the screening reactions for individual steps were also performed in 0.25–1 g scale and were evaluated using approximate conversions (%) from UV traces in the HPLC chromatogram (without internal standard).



Scheme 2. Complete synthetic scheme of AECM-MeC phosphoramidite based on optimized conditions.

The synthesis of AECM-MeC phosphoramidite **16** was carried out as shown in Scheme 2. 3',5'-O-[(1,1,3,3-Tetraisopropyl-1,3-disiloxanediyl)]-5-methylcytidine (**8**) was prepared starting from 100 g of 5-methylcytidine using a known procedure [31]. The procedure [26,28] for non-methylated AECM-C nucleoside continues with alkylation of the 2'-OH. However, for the synthesis of the AECM-MeC variant, the direct alkylation of the 2'-OH proved to be problematic due to poor solubility of compound **8** in DMF. However, it was found that conversion of the exocyclic amino group into an amidine, forming **9** [32], substantially increased the solubility, thus facilitating the 2'-O-alkylation reaction.

Based on the experience from the 2'-O-alkylation presented above to the corresponding AECM-MeU nucleoside intermediate, we assumed that similar conditions could be applied for the preparation of AECM-MeC. Nonetheless, to assess which conditions were most suitable for this substrate, a screen of different bases and solvents was performed (Table 3).

Table 3. Screen for suitable conditions for 2'-OH alkylation of 5-methyl cytidine intermediate.

No.	Methyl 2-Bromoacetate	Solvent, 5 Vol	Base	(PTC) Catalyst, 0.05 Equiv.	HPLC Area%, Conver., RT, 1 h	HPLC Area%, Conver., RT, Overnight	HPLC Area%, Conver., 50 °C, 3.5 h
1.	2 equiv.	Heptane	K ₂ CO ₃ , 4 equiv.	TBABr, 0.05 equiv.	0%	-	29% *
2.	2 equiv.	DCM	K ₂ CO ₃ , 4 equiv.	TBABr, 0.05 equiv.	0%	24%	35% *
3.	2 equiv.	MeCN	K ₂ CO ₃ , 4 equiv.	TBABr, 0.05 equiv.	8%	63%	62% *
4.	2 equiv.	Heptane	K ₃ PO ₄ , 2 equiv.	TBABr, 0.05 equiv.	0%	-	42% *
5.	2 equiv.	DCM	K ₃ PO ₄ , 2 equiv.	TBABr, 0.05 equiv.	7%	42%	56%
6.	2 equiv.	MeCN	K ₃ PO ₄ , 2 equiv.	TBABr, 0.05 equiv.	32%	89%	90%
7.	2 equiv.	DCM/heptane (5 vol), (1:4) (v/v)	K ₃ PO ₄ , 2 equiv.	TBABr, 0.02 equiv.	0%	-	89%
8.	2 equiv.	DCM/heptane (10 vol), (1:4) (v/v)	K ₃ PO ₄ , 2 equiv.	TBABr, 0.02 equiv.	0%	-	94%

* Impurities in HPLC profile.

The PTC catalyst for 2'-OH alkylation of the 5-methyl cytidine derivative remained the same. In addition, a couple of different solvents were screened (Table 3) and the results showed that both MeCN and heptane can be suitable solvents for 2'-OH alkylation of the protected MeC intermediate **9**. However, as the starting compound was only partially soluble in heptane and due to problems with the solubility of the final product, the conclusion was to try the same solvent system as for MeU (heptane/DCM mixture). Indeed, this gave a clean reaction and 94% conversion (Table 3, entry 8).

In addition, although K₂CO₃ was performing well as a base, it turned out that the reaction was even faster and cleaner when K₃PO₄ was employed as base for 5-methyl cytidine 2'-alkylation (Table 3, entries 4–8).

As K₃PO₄ and DCM/heptane proved to give the most preferred outcome in terms of yield and impurity profile, these conditions were used to upscale the reaction to 50 g of **8**. Despite increasing the temperature to 40 °C, in order to shorten the overall reaction time, the reduced grinding effect of the magnet on a larger scale gave a reaction that still required approx. 50 h to go to completion as well as the addition of additional reagents. Crude alkylated compound **10** was reacted with ethylenediamine in methyl-THF to give intermediate **11** (including removal of the exocyclic amine protecting group) followed by washes using NH₄Cl (aq.) to remove the excess of ethylenediamine. Then, crude **11** was subsequently treated with ethyl trifluoroacetate in methanol to afford compound **12**. The exocyclic amino group was acetylated to give **13**. However, this step, following a procedure for the corresponding AECM-C using acetic anhydride in pyridine [26,28], proved to require more attention as we observed around 20% of bis acetylated side product. Reduced added amounts of Ac₂O did not improve the outcome as we noticed that the bis acetylated product forms in parallel with the desired mono acetylated compound. Due to this, we performed another screen to test different conditions for acetylation (different solvents, different amounts of Ac₂O, different reaction times as well as the possibility to use TEA as a base (Supplementary Table S3)). However, this screen was unsuccessful in finding conditions for monoacetylation. Further evaluation of literature procedures revealed that in order to avoid bis acetylation, the reaction can be successfully carried out without the presence of any base [33]. Indeed, the slow addition of acetic anhydride without the presence of any base gave the monoacetylated derivative **13**. The crude product **13** was then treated with triethylamine trishydrofluoride in MeCN to give crystalline base-protected compound **14**. After stirring the reaction mixture overnight, tert-butyl methyl ether (TBME) was used as an anti-solvent to ensure a full precipitation of compound **14**, which was further purified by re-slurrying compound **14** in DCM. Compound **14** was then converted to 5'-O-4,4'-dimethoxytrityl derivative **15** using standard dimethoxytritylating conditions followed by flash column chromatography to obtain pure **15** (25% overall yield from **8**, 82% average yield per step). Tritylated compound **15** was then phosphitylated using standard conditions at the 3'-position to give the AECM-MeC phosphoramidite **16**.

3. Materials and Methods

3.1. General Information

Unless otherwise noted, all reagents and solvents used in chemical synthesis were of commercial grade.

HPLC was performed on an UltiMate 3000 HPLC system (Thermo Fisher Scientific, Germering, Germany) with UV detection at 254 nm. Reversed-phase (RP) HPLC was performed on a XBridge[®] Oligonucleotide BEH C18 (4.6 × 50 mm) column (Waters, Dublin, Ireland) with 1 mL/min flow rate.

Chromatographic separations were performed on a CombiFlash[®] Rf 200 by Teledyne ISCO system (Lincoln, NE, USA) using Biotage[®] normal or reversed-phase (RP) silica columns (Uppsala, Sweden).

Mass spectrometry analysis was performed with Waters Xevo QToF G2-XS (Dublin, Ireland).

NMR spectra were recorded using a Bruker AV 500 MHz (500.13 MHz in ¹H, 125.76 MHz in ¹³C and 202.47 MHz in ³¹P, 470.56 MHz in ¹⁹F) spectrometer (Fällanden, Switzerland) using deuterated solvent signal as an internal standard. 1,2,4,5-Tetrachloro-3-nitrobenzene was used as an internal standard for quantitative NMRs (assay). Chemical shifts (δ scale) are reported in parts per million (ppm). Coupling constants (J values) are given in Hertz (Hz).

3.2. Synthesis of Compounds 2–7

*N*³-Pivaloyloxymethyl-3',5'-O-[(tetraisopropylidisiloxan-1,3-diyl)]-5-methyl-uridine (2).

Fifty grams (91 mmol) of 3',5'-O-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)-5-methyluridine (1) (91% assay by NMR, Supplementary Figure S4) was dissolved in 250 mL dimethylformamide. K₂CO₃ (55.2 g, 399 mmol) was added to the solution followed by the addition of tetrabutylammonium bromide (6.44 g, 20.0 mmol). Then, chloromethyl pivalate (30.1 g, 200 mmol) was added dropwise to the reaction mixture under nitrogen atmosphere at ambient temperature. Then, the temperature of the reaction mixture was increased to 40 °C and left to stir for 6 h. K₂CO₃ was filtered off, and volatiles were removed under reduced pressure. The remaining crude material was re-dissolved in dichloromethane, organic phase was washed with saturated aq. NaHCO₃ twice, dried over Na₂SO₄, filtered and evaporated under reduced pressure. The crude product was subjected to a column and chromatographed using 0 to 20% ethyl acetate in heptane. Fractions containing the product were concentrated and the isolated product was co-evaporated once with dichloromethane to give compound 2 (32.2 g, 52.4 mmol, 57.6%) as a white foam.

¹H NMR (500 MHz, CDCl₃): δ = 7.36 (d, *J* = 1.3 Hz, 1H), 6.00–5.91 (m, 2H), 5.73 (d, *J* = 1.1 Hz, 1H), 4.44 (dd, *J* = 8.4, 5.3 Hz, 1H), 4.20–4.14 (m, 2H), 4.08–3.97 (m, 2H), 2.88 (s, 1H), 1.94 (s, 3H), 1.19 (s, 9H), 1.13–0.96 (m, 28H). ¹³C NMR (202.47 MHz, CD₃OD): 177.65, 162.62, 150.11, 134.93, 110.01, 91.68, 82.15, 77.73, 75.18, 69.60, 65.13, 60.71, 38.97, 27.17, 17.56, 17.51, 17.42, 17.39, 17.20, 17.13, 17.07, 17.00, 13.54, 13.34, 13.12, 12.85, 12.71 ppm. ES-MS calc. for C₂₈H₅₀N₂O₉Si₂ [M+H]⁺ 615.3133, found 615.3139.

2'-O-(O-Methylcarboxymethyl)-*N*³-pivaloyloxymethyl-3',5'-O-[(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)methyl]-5-methyl-uridine (3).

Compound (2) (30.68 g, 49.9 mmol) was dissolved in a mixture of 123 mL heptane and 31 mL dichloromethane and the obtained solution was subsequently flushed with nitrogen. Methyl 2-bromoacetate (9.5 mL, 100 mmol) was slowly added to the stirred solution followed by the addition of tetrabutylammonium bromide (800 mg, 2.5 mmol) and K₂CO₃ (28 g, 200 mmol). The resulting suspension was stirred at ambient temperature for 66 h. As the reaction was not completed, additional methyl 2-bromoacetate (2.4 mL, 25 mmol), K₂CO₃ (13.8 g, 100 mmol) and tetrabutylammonium bromide (800 mg, 2.5 mmol) were added and the reaction was left to stir at ambient temperature for an additional 24 h. Then, 200 mL of water was added to the reaction mixture and phases were separated. Organic phase was washed with a 200 mL acetonitrile–water mix (1:1 *v/v*) which led to

the formation of three phases. The phases containing product (middle and upper) were collected, dried over Na_2SO_4 , filtered and evaporated to dryness in vacuo. The crude product was purified via flash chromatography using 0 to 100% ethyl acetate in heptane as eluent to afford compound **3** as a colorless oil (28.89 g, 42.06 mmol, 84.3%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ = 7.53 (s, 1H), 5.83–5.74 (m, 2H), 5.70 (s, 1H), 4.47 (d, J = 16.6 Hz, 1H), 4.36 (d, J = 16.5 Hz, 1H), 4.30 (dd, J = 9.2, 4.9 Hz, 1H), 4.22 (d, J = 5.0 Hz, 1H), 4.14 (dd, J = 13.5, 2.0 Hz, 1H), 4.03–3.98 (m, 1H), 3.92 (dd, J = 13.5, 2.6 Hz, 1H), 3.63 (s, 3H), 1.82 (d, J = 1.2 Hz, 3H), 1.10 (s, 9H), 1.08–0.95 (m, 28H). ^{13}C NMR (202.47 MHz, $\text{DMSO}-d_6$): δ = 178.37, 170.07, 161.71, 149.73, 136.57, 110.17, 90.87, 83.49, 83.01, 70.23, 68.57, 66.10, 60.42, 52.34, 39.87, 33.07, 30.18, 27.41, 23.34, 18.04, 17.95, 17.77, 17.60, 17.50, 17.36, 14.72, 14.47, 14.26, 14.05, 13.92, 13.49 ppm. ES-MS calcd. for $\text{C}_{31}\text{H}_{54}\text{N}_2\text{O}_{11}\text{Si}_2$ [$\text{M} + \text{H}$] $^+$ 687.3344, found 687.3337.

5'-O-(4,4'-Dimethoxytrityl)-2'-O-[(*N*-(trifluoroacetamidoethyl)carbamoyl)methyl]-5-methyl-uridine (**6**).

Compound **3** (28.89 g, 42.06 mmol) was dissolved in 289 mL THF and 58 mL water. Trifluoroacetic acid (3.2 mL, 42 mmol) was slowly added to the resulting solution and the reaction mixture was stirred at ambient temperature for 18 h. Reaction was quenched with 144 mL pyridine and volatiles were evaporated under reduced pressure. The residue was co-evaporated with pyridine to remove residual water, re-dissolved (pyridine, 300 mL) and half of the solvent volume was evaporated. 4,4'-Dimethoxytrityl chloride (15.7 g, 46 mmol) was added to the resulting mixture and stirred for 2.5 h at ambient temperature. Then, the reaction was quenched with MeOH (150 mL) followed by the addition of ethylenediamine (56 mL, 840 mmol) to the resulting solution, and the temperature of the reaction was set to 60 °C and was left to stir for 16 h. $\text{Et}_3\text{N}(\text{HF})_3$ (20.6 mL, 126 mmol) was added to the (60 °C) reaction mixture, stirred for a few minutes and then the reaction was cooled down to 25 °C and stirred for 2.5 h. Volatiles were evaporated in vacuo, the residue partitioned between saturated aq. NH_4Cl and dichloromethane (250 mL each), phases separated, organic phase washed with saturated aq. NH_4Cl and water (250 mL each), dried over Na_2SO_4 , filtered and evaporated under reduced pressure. The crude obtained material was dissolved in dichloromethane (139 mL), and triethylamine (30.0 mL, 215 mmol) was added to the resulting solution followed by the addition of ethyl trifluoroacetate (25.0 mL, 210 mmol). Reaction was stirred for 2.5 h at ambient temperature, then washed with saturated aq. NaHCO_2 , phases were separated, organic phase was dried over Na_2SO_4 , filtered and evaporated under reduced pressure. The crude product was subjected to a silica column and chromatographed using 0 to 20% methanol in dichloromethane containing 1% triethylamine. The obtained material had to be re-chromatographed and was subjected to a second silica column and chromatographed using EtOAc/MeOH (95:5 *v/v*) containing 1% triethylamine as eluent to give compound **6** as a white foam (18.78 g, 59% overall yield after five steps, 95% assay according to NMR). ^1H NMR (500 MHz, CD_3OD): δ = 7.74 (d, J = 1.3 Hz, 1H), 7.46–7.43 (m, 2H), 7.36–7.28 (m, 6H), 7.28–7.22 (m, 1H), 6.93–6.81 (m, 4H), 5.93 (d, J = 2.4 Hz, 1H), 4.51 (dd, J = 7.5, 5.2 Hz, 1H), 4.34–4.22 (m, 2H), 4.14 (dt, J = 7.6, 2.6 Hz, 1H), 4.10–4.07 (m, 1H), 3.78 (s, 6H), 3.54 (dd, J = 11.0, 2.1 Hz, 1H), 3.46–3.42 (m, 5H), 1.33 (d, J = 1.2 Hz, 3H). ^{13}C NMR (202.47 MHz, CD_3OD): δ = 172.29, 165.87, 159.85, 159.00, 151.76, 145.45, 136.47, 136.38, 136.21, 130.91, 128.96, 128.46, 127.62, 118.11, 117.39, 115.83, 113.74, 110.92, 89.16, 87.53, 84.31, 83.56, 70.35, 69.52, 62.49, 55.22, 39.85, 38.59, 11.66, 0.26. ^{19}F NMR (470.56 MHz, CD_3OD): δ = -77.38 ppm. ES-MS calc. for $\text{C}_{37}\text{H}_{39}\text{F}_3\text{N}_4\text{O}_{10}$ [$\text{M} + \text{Na}$] $^+$ 779.2516, found 779.2514.

3'-O-(*N,N*-Diisopropylamino-(2-cyanoethoxy)phosphinyl)-5'-O-(4-methoxytrityl)-2'-O-[(*N*-(trifluoroacetamidoethyl)carbamoyl)methyl]-5-methyl-uridine (**7**).

Compound **6** (5.1 g, 6.7 mmol) was dissolved in 52 mL of anhydrous THF. The solution was cooled in an ice bath and *N,N'*-diisopropylethylamine (5.9 mL, 34 mmol) was added under nitrogen atmosphere followed by the addition of 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite (3.0 mL, 13 mmol) and the reaction mixture was

stirred for another 3 h. The reaction mixture was quenched with 300 μ L MeOH and filtered. The filtrate was concentrated under reduced pressure and the residue partitioned between DCM and saturated aq. NaHCO₃. Phases were separated and aqueous phase was again extracted with DCM. Organic phases were pooled together and dried over Na₂SO₄. The crude product was purified via flash column chromatography using 0 to 15% acetonitrile in ethyl acetate containing 1% triethylamine as eluent. Fractions were evaporated and yielded a white foam which was re-slurried in heptane to give the final compound **7** (4.7 g, 4.91 mmol, 72.5%). ³¹P NMR (202.47 MHz, CDCl₃): δ = 149.6, 148.4 ppm. ¹H, ¹³C and ¹⁹F NMRs are presented in the Supplementary Materials (Supplementary Figures S13–S15). ES–MS calc. for C₄₆H₅₆F₃N₆O₁₁P[M][−] 955.3624, found 955.3601.

3.3. Synthesis of Compounds 9–16

*N*⁴-Dimethylformamidono-3',5'-O-[(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)]-5-methyl-cytidine (**9**).

In 600 mL pyridine, 50.1 g (100 mmol) of 3',5'-O-[(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)]-5-methyl-cytidine (**8**) was dissolved, and 350 mL of the solvent was evaporated prior to addition of *N,N*-dimethylformamide dimethyl acetal (40 mL, 301.1 mmol). After stirring at ambient temperature for 2.5 h, the solvent was evaporated under reduced pressure, the obtained crude material was co-evaporated with toluene (\times 3) and dried in vacuum to give 60.6 g of crude product **9**. ¹H and ¹³C NMR data are included in the Supplementary Materials (Supplementary Figures S17–S19). ES-MS calc. for C₂₅H₄₆N₄O₆Si₂ [M+H]⁺ 555.3034, found 555.3043.

*N*⁴-Dimethylformamidino-2'-O-(*O*-methylcarboxymethyl)-3',5'-O-[(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)]-5-methyl-cytidine (**10**).

Crude compound **9** (58.6 g) was dissolved in a mixture of dichloromethane (120 mL) and heptane (470 mL). Methyl 2-bromoacetate (20 mL, 211.3 mmol) was slowly added to the stirred solution followed by the addition of tetrabutylammonium bromide (680 mg, 2.1 mmol) and K₃PO₄ (44.8 g, 210.5 mmol). The resulting suspension was stirred at 40 °C for 26 h and then additional methyl 2-bromoacetate (5.0 mL, 53 mmol), K₃PO₄ (44.8 g, 210.5 mmol) and TBABr (680 mg, 2.1 mmol) were added. The reaction was left to stir at 40 °C. After 18.5 h, one more portion of K₃PO₄ (44.8 g, 210.5 mmol) was added, stirred for an additional 8 h at 40 °C and then 500 mL of water was added to the reaction mixture. Phases were separated, organic phase washed with MeCN:water (1:1) and then water, dried over Na₂SO₄, filtered and evaporated. Obtained crude oil **10** (74.8 g) was used for the next step without any further purification. ES-MS calc. for C₂₈H₅₀N₄O₈Si₂ [M+H]⁺ 627.3245, found 627.3232.

2'-O-(*N*-(Ethyl)carbamoyl)methyl-3',5'-O-[(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)]-5-methyl-cytidine (**11**).

Crude compound **10** (74.2 g) was dissolved in 750 mL Me-THF. One hundred and sixty milliliters (2.4 mol) of ethylenediamine was added to the reaction mixture. It was stirred at ambient temperature for 1 h and then washed twice with 370 mL sat. NH₄Cl followed by water (2 \times 370 mL). The organic phase was evaporated under reduced pressure. As the water washes were found to contain some product, it was extracted with dichloromethane (\times 2). The combined dichloromethane phases were dried over Na₂SO₄, filtered and evaporated. Dissolving the residues from the organic phases in DCM and re-evaporation gave compound **11** (59 g) as a pale yellow foam which was used in the next step without any further purification. ¹H and ¹³C NMRs are presented in the the Supplementary Materials (Supplementary Figures S18 and S19). ES-MS calc. for C₂₆H₄₉N₅O₇Si₂ [M+H]⁺ 600.3249, found 600.3255.

2'-O-(*N*-(Trifluoroacetamidoethyl)carbamoyl)methyl-3',5'-O-[(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)]-5-methyl-cytidine (**12**).

Crude compound **11** (59 g) was dissolved in 400 mL methanol. Ethyl trifluoroacetate (60 mL, 504.2 mmol) was slowly added to the reaction mixture followed by the addition of triethylamine (136 mL, 975.7 mmol). Reaction was stirred at ambient temperature for 1.5 h, volatiles were evaporated under reduced pressure, residue was co-evaporated with toluene ($\times 3$) and the obtained crude product **12** (74.2 g) was used for the next step without any further purification. ^1H and ^{13}C NMRs are presented in the Supplementary Materials (Supplementary Figures S20 and S21). ES-MS calc. for $\text{C}_{28}\text{H}_{48}\text{F}_3\text{N}_5\text{O}_8\text{Si}_2$ $[\text{M}+\text{H}]^+$ 696.3072, found 696.3059.

N^4 -Acetyl-2'-O-[(N-(trifluoroacetamidoethyl)carbamoyl)methyl-3',5'-O-[(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)]-5-methyl-cytidine (**13**).

Crude compound **12** (74.2 g) was dissolved in 371 mL dichloromethane. Acetic anhydride (20.1 mL, 212.6 mmol) was slowly added to the reaction mixture and left to stir at ambient temperature for 23 h. Then, 100 mL of water was added to the reaction mixture and stirred for 7 min followed by the addition of 400 mL sat. NaHCO_3 . After stirring for a few minutes, the reaction mixture was transferred to a separating funnel, phases separated and organic phase washed ($\times 2$) with sat. aq. NaHCO_3 and water. Organic phase was dried over Na_2SO_4 , filtered and concentrated under reduced pressure. Obtained crude light orange foam **13** (66.6 g) was used for the next step without any further purification. ^1H NMR is presented in the Supplementary Materials (Supplementary Figure S22). ES-MS calc. for $\text{C}_{30}\text{H}_{50}\text{F}_3\text{N}_5\text{O}_9\text{Si}_2$ $[\text{M}+\text{H}]^+$ 738.3177, found 738.3159.

N^4 -Acetyl-2'-O-[(N-(trifluoroacetamidoethyl)carbamoyl)methyl]-5-methyl-cytidine (**14**).

Crude compound **13** (65.8 g) was dissolved in 329 mL of acetonitrile, triethylamine trihydrofluoride (28 mL, 170 mmol) was added and the reaction mixture was stirred at ambient temperature for 19 h. Precipitated product was filtered off, 400 mL of tert-butyl methyl ether was added to the filtrate as a co-solvent, left to stir over a weekend and filtered again. Products from both precipitations were pooled together, re-slurried in dichloromethane and left to stir overnight. The slurry was filtered to give 17 g of the product **14** (35% from **8**). ^1H NMR (500 MHz, CD_3OD): δ = 8.45 (s, 1H), 5.93 (s, 1H), 4.42–4.28 (m, 2H), 4.25 (s, 1H), 4.10 (d, J = 8.5 Hz, 1H), 4.03 (d, J = 12.5 Hz, 1H), 3.98 (d, J = 5.0 Hz, 1H), 3.83 (d, J = 12.5 Hz, 1H), 3.53–3.37 (m, 4H), 2.39 (s, 3H), 2.05 (s, 3H). ^{13}C NMR (202.47 MHz, CD_3OD): δ = 170.96, 156.75, 143.91, 117.97, 115.31, 93.87, 89.35, 84.31, 70.94, 69.49, 67.38, 62.64, 59.10, 40.40, 37.43, 26.59, 24.79, 11.17. ^{19}F NMR (470.56 MHz, CD_3OD): δ = -77.36 ppm. ES-MS calc. for $\text{C}_{18}\text{H}_{24}\text{F}_3\text{N}_5\text{O}_8$ $[\text{M}+\text{H}]^+$ 496.1655, found 496.1653.

N^4 -Acetyl-5'-O-(4,4'-dimethoxytrityl)-2'-O-[(N-(trifluoroacetamidoethyl)carbamoyl)methyl]-5-methyl-cytidine (**15**).

Compound **14** (17.4 g, 35.12 mmol) was dried by co-evaporation with pyridine ($\times 3$), re-dissolved in pyridine (87 mL), cooled in an ice bath and 4,4'-dimethoxytrityl chloride (12.5 g, 36.9 mmol) was added as a solid to the reaction solution. After stirring the reaction mixture for 3 h in an ice bath, an additional portion of 4,4'-dimethoxytrityl chloride (0.59 g, 1.8 mmol) was added and left to stir at RT for 16 h. Reaction was quenched with 200 μL of MeOH, then the reaction mixture was diluted with sat. aq. NaHCO_3 and extracted with ethyl acetate. The organic phase was washed with sat. aq. NaHCO_3 ($\times 2$), water and brine, dried over Na_2SO_4 and evaporated. Obtained crude pale foam was purified via flash chromatography (pre-equilibrated with 1% TEA in DCM) using a gradient from 0 to 25% MeOH in DCM in the presence of 1% TEA. Compound **15** was obtained as a pale yellow foam after evaporation of pure fractions. The foam was re-slurried in heptane to give compound **15** as a white powder (20.1 g, 72%, 92% NMR assay, residual solvents present). ^1H NMR (500 MHz, CD_3OD): δ = 8.19 (s, 1H), 7.45 (d, J = 7.1 Hz, 2H), 7.36–7.17 (m, 7H), 6.84 (d, J = 9.0 Hz, 4H), 5.89 (d, J = 0.9 Hz, 1H), 4.57 (dd, J = 9.1, 5.0 Hz, 1H), 4.44–4.34 (m, 2H), 4.22 (d, J = 11.4 Hz, 1H), 4.06 (d, J = 5.4 Hz, 1H), 3.74 (s, 6H), 3.60 (d, J = 11.2 Hz, 1H), 3.44 (s, 5H), 2.36 (s, 3H), 1.45 (s, 3H). ^{13}C NMR (202.47 MHz, CD_3OD): δ = 173.29, 172.98, 163.90, 160.35, 159.48, 159.19, 157.06, 142.91, 136.95, 136.76, 131.45, 129.53, 129.08, 128.22,

118.66, 116.38, 114.36, 107.82, 91.42, 88.03, 85.19, 83.78, 71.03, 62.33, 55.81, 13.73, 13.21. ^{19}F NMR (470.56 MHz, CD_3OD): $\delta = -77.18$ ppm. ES-MS calc. for $\text{C}_{39}\text{H}_{42}\text{F}_3\text{N}_5\text{O}_{10}$ $[\text{M}+\text{H}]^+$ 798.2962, found 798.2963.

*N*⁴-Acetyl-3'-*O*-(*N,N*-diisopropylamino-(2-cyanoethoxy)phosphinyl)-5'-*O*-(4-methoxytrityl)-2'-*O*-[(*N*-(trifluoroacetamidoethyl)carbamoyl)methyl]methyl-cytidine (16).

Compound **15** (5.0 g, 6.5 mmol) was dissolved in acetonitrile (15 mL) and dichloromethane (25 mL). The solution was cooled in an ice bath and *N,N*-diisopropylethylamine (2.2 mL, 13 mmol) was added under nitrogen atmosphere. Then, 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite (2.8 mL, 13 mmol) was added dropwise and the reaction mixture was stirred on ice for 1 h, the ice bath was removed and reaction stirred at ambient temperature for an additional 1.5 h. The reaction was quenched with 260 μL MeOH, volatiles were evaporated, residue dissolved in ethyl acetate and washed with sat. aq. NaHCO_3 ($\times 2$) and brine ($\times 1$). Organic phase was dried over Na_2SO_4 , evaporated and purified on a silica gel column (1% triethylamine in DCM pre-equilibrated) using 0 to 20% MeOH in DCM with 1% triethylamine in both solvents. Obtained white foam was re-slurried in heptane to give a mix (5.6 g) of a final compound **16** (72%) and a hydrolysis product of 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite (28%) (Supplementary Figure S29) as a white powder (estimated final yield of the product **16** from ^{31}P NMR: 4.0 g, 4 mmol, 62%). The impurity was successfully removed on an RP column using 25 to 100% MeCN in H_2O (with 1% TEA) (Supplementary Figure S30). ^{31}P NMR (202.47 MHz, CD_3CN): $\delta = 151.05, 148.4$ ppm. ^1H , ^{13}C and ^{19}F NMRs are presented in the Supplementary Materials (Supplementary Figures S31–S33). ES-MS calc. for $\text{C}_{48}\text{H}_{59}\text{F}_3\text{N}_7\text{O}_{11}\text{P}$ $[\text{M}]^-$ 996.3890, found 996.3871.

4. Conclusions

This study reports on procedures to prepare AECM-modified 5-methyluridine and 5-methylcytidine monomers in multigram scales aimed for the use in automated solid-phase oligonucleotide synthesis. The developed synthetic route for AECM-MeU monomer requires few purification steps. 2'-*O*-Alkylation of compound **2** was substantially improved by using PTC conditions which enable the possibility to use a low-cost and more readily available reagent. Additionally, reduced volumes of solvents as well as amounts of reagents for syntheses of compounds **2** and **6** were achieved compared to reported syntheses for related compounds. Moreover, selective opening of 5'-position of compound **3** unlocks the possibility to do three steps in one pot, and four steps in total without chromatographic purification to give 59% yield of compound **6** over five steps. The synthesis of the AECM-MeC monomer is clearly more optimally developed than for the preparation of AECM-C [26,28]. Using other work-up strategies, introduction of an amidine protecting group (compound **9**) and the possibility to crystallize compound **14** allowed us to avoid chromatography throughout the synthesis until the last phosphoramidite forming step. PTC conditions were also used to alkylate compound **9** at the 2'-OH position which reduced the cost of the synthesis by exchanging the P1-*t*-Bu-tris(tetramethylene) phosphazene base (BTTP) with the much lower cost reagent K_2CO_3 . Moreover, syntheses of 5-methylated AECM-modified uridine and cytidine monomers are reported for the first time, which now enable their incorporation into oligonucleotides. In addition, syntheses were performed at larger scales to reduce cost and time for making these monomers for further use in biological evaluation of AECM-containing oligonucleotides.

Supplementary Materials: The following are available online, Tables S1 and S2: Screen for different conditions for 2'-OH alkylation of compound 3, Table S3: Screen for different conditions for N⁴ acetylation of compound 13, Figure S1: HPLC profile of crude compound 2 in 50 g scale synthesis, Figures S2 and S3: HPLC profiles of screen for different conditions for 2'-OH alkylation of compound 3, Figures S4–S15: ¹H NMR, ¹³C NMR, ¹⁹F NMR and ³¹P NMR spectra for MeU intermediates, Figures S16–S33: ¹H NMR, ¹³C NMR, ¹⁹F NMR and ³¹P NMR spectra for MeC intermediates, Figures S34–S37: HPLC profiles of MeU intermediates, Figures S38–S45: HPLC profiles of MeC intermediates, Figures S46–S57: ESI-TOF mass spectra for MeU and MeC intermediates.

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