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Synthesis and Stability of a 2′-*O*-[*N*-(Aminoethyl)carbamoyl]methyladenosine-Containing Dinucleotide

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Working towards the synthesis of 2′-*O*-[*N*-(aminoethyl)-carbamoyl]methyl-modified di- and oligonucleotides, we have synthesised a protected 2′-*O*-[*N*-(aminoethyl)carbamoyl]methyl-modified adenosine where the modification is introduced in a convenient one-pot three-step procedure. The corresponding H-phosphonate building block was also synthesised, and from this intermediate, a 2′-*O*-[*N*-(aminoethyl)carbamoyl]methyl-containing dinucleotide could be made. We also performed studies on the chemical and enzymatic stability of this dinucleotide. The dinucleotide was subjected to different ammonolysis and other basic conditions, and HPLC analysis showed that the modification was intact to most conditions, but that there was some minor hydrolysis when NH_3 (concd. aq.) was used at 55 °C. Under several other sets of conditions, including saturated NH_3 in methanol, and ethylenediamine, the amide remained intact. Treatment of the dinucleotide with Phosphodiesterase I from *Crotalus adamanteus* venom and Phosphodiesterase II from bovine spleen showed that the *N*-(aminoethyl)carbamoylmethyl moiety gives the phosphodiester linkage substantial protection against enzymatic degradation; the phosphodiester was not degraded by PDE II at all after seven days.

Introduction

Synthetic nucleic acids have been and still are crucial for the development of life science research, and modified oligonucleotides are developed as means to treat patients with genetic diseases. Most oligonucleotide therapies, including siRNA[1] (short interfering RNA) and antisense technologies,[2] including splice-switching,[3] are limited by e.g., the lability of oligonucleotides in biological fluids, and poor delivery to the site of action. Efficiency in the regulation of gene expression is more readily achieved if turnover of the target RNA is obtained. This can occur if native enzymes (e.g., RNAse H for antisense, and RISC complex for siRNA) can recognise the relevant oligonucleotide complex. An alternative approach, independent of native enzymes, is the use of oligonucleotide-based artificial nucleases (OBANs)[4,5] with the aim of producing artificial enzymes that can cleave mRNA sequences arising from genetic or viral diseases. This allows modifications that give stable oligonucleotides that are not degraded by host enzymes. The approaches of splice-correction or splice-switching,[3] interest in which is currently growing, are typically independent of recognition by native enzymes, and therefore more modifications are allowed. A large number of modifications of oligonucleotides have been explored in the development of oligonucleotides for biotechnology or therapy. Important features of such modifications are affinity for the target molecule, preferably a low-cost manufacturing process, stability against enzymatic degradation, and uptake into cells. In particular, 2′-*O*-alkyloligoribonucleotides[6] are a class of modified oligonucleotides that has attracted great interest. To modify the 2′-position has several advantages, including the fact that low-cost starting materials can be used. In contrast to 2′-deoxynucleosides, the electron-withdrawing groups in the 2′-position affect the pseudoaromatic character of the nucleobase,[7] the conformational equilibrium in the ribose moiety is also pushed towards the north (C3′-*endo*) conformations, as found in the A-form geometry of RNA duplexes, which typically leads to more stable duplexes with the target RNA.[8] A number of 2′-*O*-alkyloligoribonucleotide modifications have, in contrast to results with DNA, been shown to give increased stability of duplexes with RNA.[9] The 2′-*O*-carbamoylmethyl (CM) modification[10] caught our attention, and we have recently shown that this is highly resistant to enzymatic degradation.[11] Another interesting feature of the 2′-*O*-carbamoylmethyl group is that, depending on the synthetic route used, it can be readily functionalised with different substituents on the amide nitrogen.[12] An interesting substitution of the CM that should give even higher hydrogen-bonding potential (e.g., with the hydration network), as well as open-

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ing the possibility for electrostatic interactions, is a 2′-*O*-[*N*-(aminoethyl)carbamoyl]methyl (AECM) modification (Figure 1).

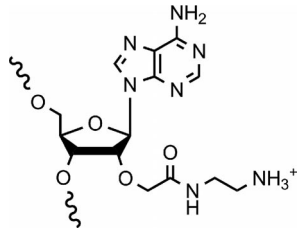


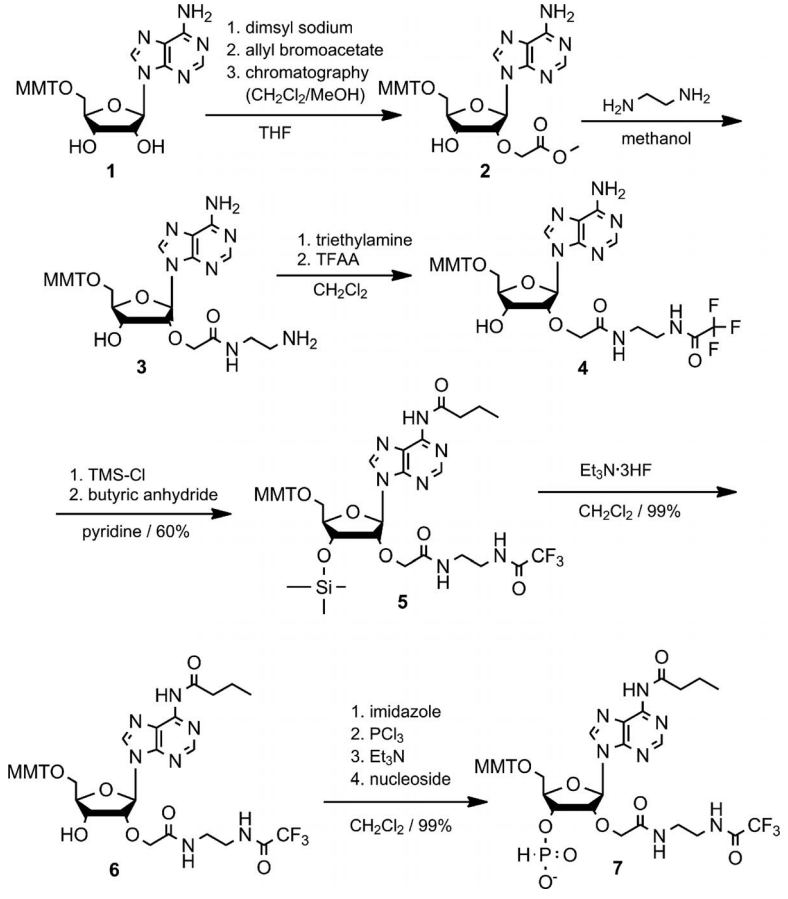
Figure 1. The 2′-*O*-[*N*-(aminoethyl)carbamoyl]methyl modification.

The fact that 2′-*O*-aminopropyl-RNA carrying a positively charged ammonium group is resistant to nuclease degradation is of additional interest.[13] As the uncharged CM modification already results in substantial stabilisation, the combination of CM with a charged ammonium group in AECM seemed to be worth testing. While we were pursuing our studies on the AECM modification, it was reported that oligonucleotides containing a single 2′-*O*-[*N*-(aminoethyl)carbamoyl]uridine moiety gave a substantial decrease in the melting point of duplexes with both DNA and RNA.[14] We found this result unexpected, surprising, and unconvincing so we were not discouraged from pursuing our investigation of the AECM modification further, starting with the modification of adenosine.

In this paper, we report the synthesis of 2′-*O*-[*N*-(aminoethyl)carbamoyl]methyladenosine building blocks, and also the synthesis of a AECM-A-containing dinucleotide that is used as a model compound for further studies. In a previous study of a CM-modified dinucleotide, we found that treatment with concentrated aqueous ammonia resulted in partial hydrolysis of the carbamoylmethyl group.[10] The stability of the 2′-*O*-AECM group under several different sets of basic conditions that are often used for the final deprotection of oligonucleotides was therefore investigated. In addition, we also subjected the AECM-containing dinucleotide to spleen phosphodiesterase II and snake-venom phosphodiesterase I to see whether this modification rendered the dinucleotide even more stable than the CM modification.

Results and Discussion

2′-*O*-Alkyladenosines have been obtained by direct alkylation of unprotected adenosine, e.g., using sodium hydride and an alkyl halide in DMF.[15,16] We chose to alkylate 5′-*O*-(4-monomethoxytrityl)adenosine (**1**; Scheme 1) due to its higher solubility in organic solvents and also to the somewhat higher selectivity for the 2′-*O*-alkylated product over

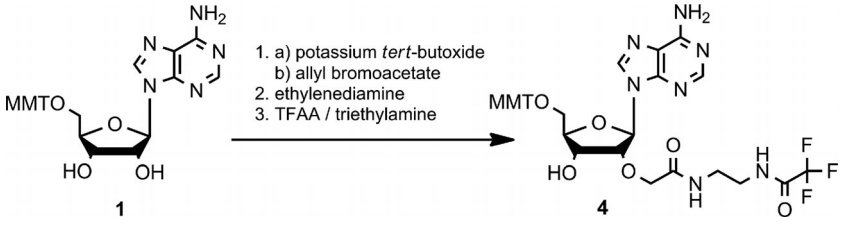


Scheme 1. Synthesis of 2′-*O*-[*N*-(aminoethyl)carbamoyl]methyl-adenosine H-phosphonate building block **7**.

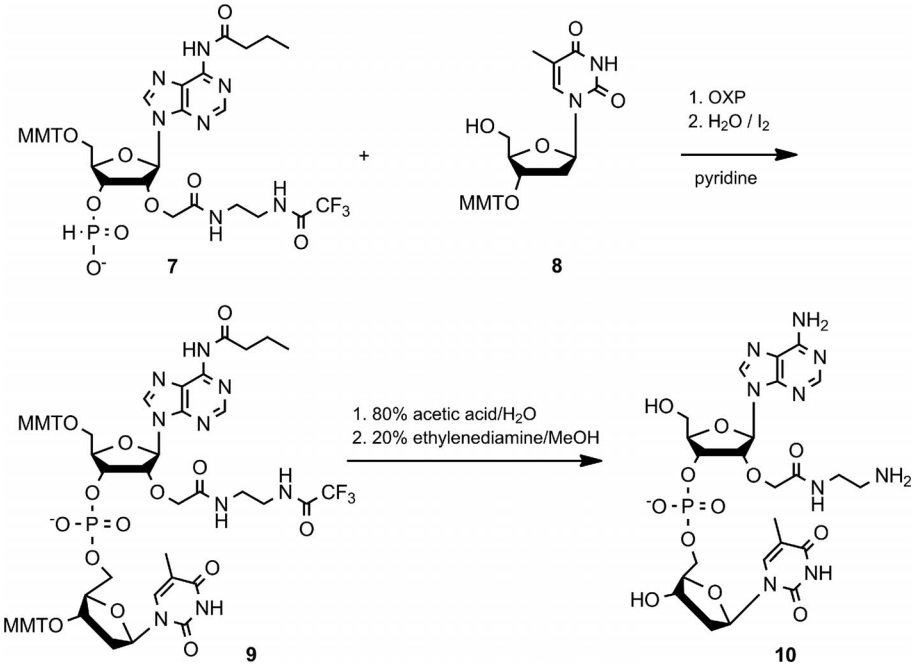
the 3′-*O*-alkylated compound compared to when unprotected adenosine is used. Compound **1** was dissolved in THF and treated with dimsyl sodium[17] as a soluble base in THF to give the corresponding 2′-*O*-alkoxide.[18] Allyl bromoacetate was then added, and after isolation, methyl ester **2** (transesterification with methanol apparently occurred during chromatography in CH_2Cl_2/methanol, possibly via the 2′,3′-lactone) was obtained (Scheme 1; substitution of the isolated compound on O-2′ was confirmed by ^1H–^{13}C correlated NMR spectroscopy, see Supporting Information). Aminolysis of **2** was performed with ethylenediamine in methanol to give compound **3**. Further treatment of **3** with trifluoroacetic anhydride (TFAA) gave compound **4**, and after protection of the remaining hydroxy group with trimethylsilyl chloride (TMSCl), protection of N-6 by treatment with butyric anhydride gave **5** (Scheme 1). Removal of the 3′-*O*-TMS group gave **6**, and subsequent phosphonylation using PCl_3/imidazole[19] gave H-phosphonate building block **7** (Scheme 1).

An improved three-step one-pot synthesis of compound **4** was also developed: 1) Treatment with potassium *tert*-butoxide, a soluble and commercially available base, in THF, followed by allyl bromoacetate (or the commercially available methyl bromoacetate); 2) Subsequent treatment of the product with ethylenediamine; 3) Treatment with TFAA after evaporation of the excess ethylenediamine. The product was then subjected to chromatography on silica to give compound **4** in 77% isolated yield (Scheme 2; the 2′-substitution was confirmed by 2D NMR spectroscopy, see Supporting Information). This procedure was more convenient and gave a somewhat higher overall yield than the sequence with isolation after each step. Following a similar one-pot procedure, a substrate for Cu-catalysed 1,3-dipolar cycloaddition ("click chemistry"),[20] the corresponding 2′-*O*-[*N*-(azidoethyl)carbamoyl]methyl nucleoside, can be made conveniently from **1** and azidoethylamine.[21,22]

To investigate the stability of the 2′-*O*-AECM functionality to different sets of basic reaction conditions with a vicinal phosphodiester group, as well as to find out the effect of the modification on the degradation by phosphate-cleaving enzymes, a 2′-*O*-AECM dinucleotide was synthesised using H-phosphonate chemistry.[23] AECM-modified H-phosphonate **7** was coupled to 3′-*O*-MMT-thymidine **8** (MMT = monomethoxytrityl) using bis(2-oxo-3-oxazolidinyl) phosphinic chloride (OXP),[24] and this was followed by oxidation with iodine/pyridine/H_2O. Fully protected dimer **9** was then subjected to treatment with acetic acid (80% aq.) to remove the MMT-groups. Final removal of the acyl



Scheme 2. Improved three-step one-pot synthesis of trifluoroacetyl-protected 2′-*O*-(*N*-aminoethylcarbamoyl)methyl-modified adenosine **4**.



Scheme 3. Synthesis of 2′-*O*-[*N*-(aminoethyl)carbamoyl]methyl-adenosine-containing dinucleotide **10**.

protection was performed with ethylenediamine (20% in methanol). The crude material was purified using reverse-phase (RP) HPLC chromatography to give 2′-*O*-AECM-modified dinucleotide **10** (Scheme 3).

The isolated dinucleotide (i.e., **10**; AECM-AT) gave a clean HPLC-profile (Figure 2, a), with a retention time different from the previously studied 2′-*O*-carbamoylmethyladenosine 3′-(thymidine 5′-phosphate)[10] (CMAT, Figure 2, c) and the hydrolysis product, i.e., 2′-*O*-carboxymethyladenosine 3′-(thymidine 5′-phosphate)[10] (COMAT, Figure 2, b).

2′-*O*-AECM dinucleotide **10** was then subjected to different conditions for ammonolysis and aminolysis with ethylenediamine. As was seen in the previously reported study of the CMAT, AECM-dinucleotide **10** was not completely stable under standard deprotection conditions for oligonucleotides, i.e., concentrated aqueous ammonia at 55 °C. The degree of hydrolysis of the 2′-*O*-carboxymethyladenosine 3′-(thymidine 5′-phosphate) moiety was less pronounced than for CMAT,[10] and amounted to 1% degradation after 24 h (Figure 2, d) and 2% after 48 h (Figure 2, e) upon treatment with ammonia (30% aq.) at 55 °C.

Treatment of **10** at room temperature with ethylenediamine (20% in methanol) or with saturated methanolic ammonia, on the other hand, resulted in a clean product for which the HPLC chromatogram did not reveal any traces of degradation, even after treatment for 48 h (Figure 2f and g). Although the retention times are different for CMAT and AECM-AT (Figure 2a and c), we considered that peak overlap could mask the presence of CMAT in the AECM-AT after treatment with methanolic ammonia. To see whether we could detect any contamination by CMAT, we also analysed the samples by MS, and we could not detect any mass peaks corresponding to CMAT but only from the

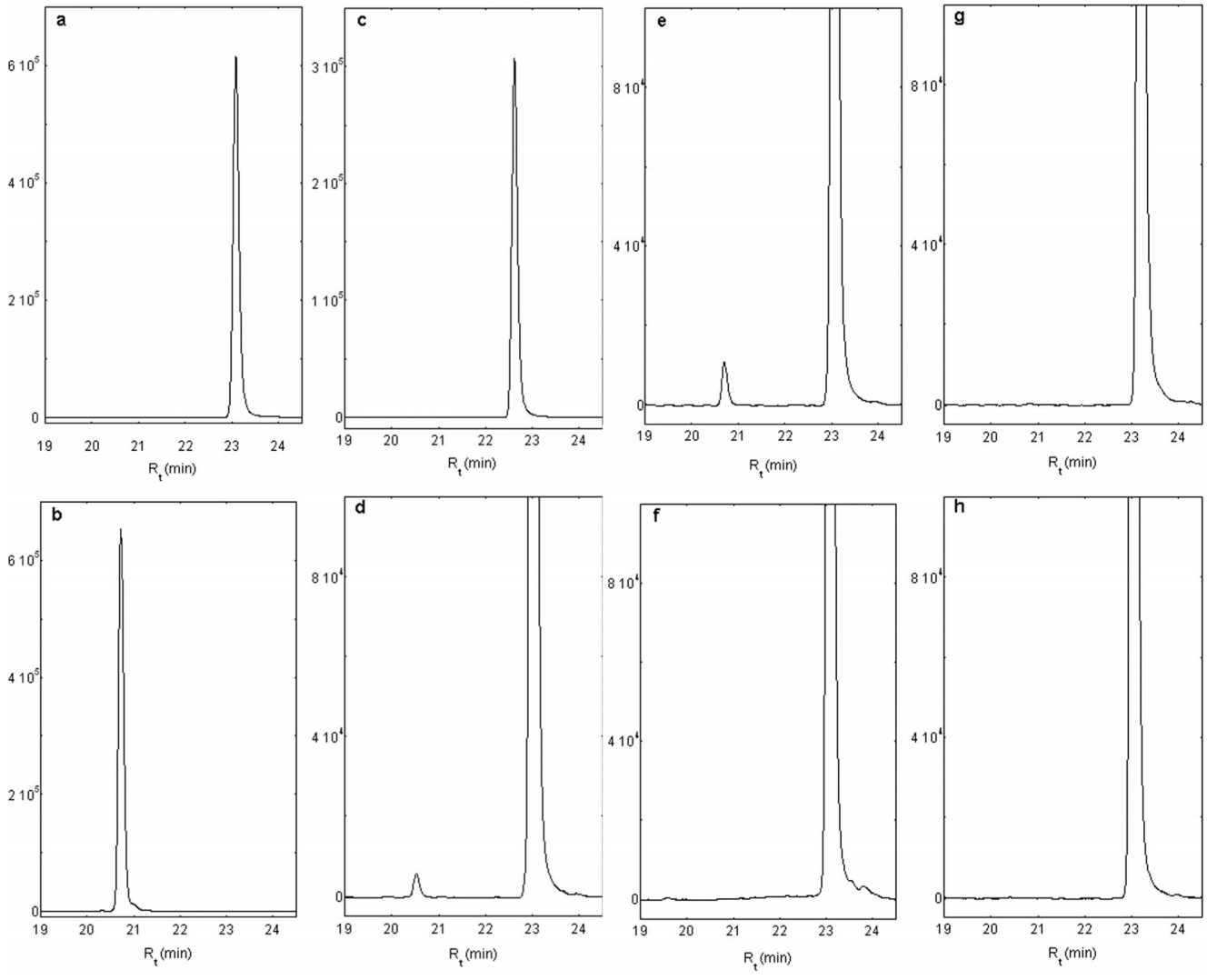


Figure 2. RP-HPLC analysis of a) 2′-*O*-(*N*-aminoethylcarbamoyl)-methyladenosine 3′-(thymidine 5′-phosphate) **10**; b) 2′-*O*-carboxymethyladenosine 3′-(thymidine 5′-phosphate); c) 2′-*O*-carbamoylmethyladenosine 3′-(thymidine 5′-phosphate); crude mixtures after treatment of **10** (enlarged chromatograms) with different aqueous or alcoholic ammonia or ethylenediamine solutions: d) NH_3 (concd. aq.) at 55 °C, 24 h; e) NH_3 (concd. aq.) at 55 °C, 48 h; f) ethylenediamine (20% in methanol), room temp., 48 h; g) methanol saturated with NH_3, room temp., 48 h; h) NH_3 (aq.)/ethanol (3:1), room temp., 24 h.

AECM-AT. Thus, there is no detectable conversion from AECM-AT into CMAT in methanolic ammonia after 48 h. Another set of reaction conditions that are sometimes used for the deprotection of oligonucleotides,[25] concentrated ammonia (30% aq.)/ethanol (3:1) at room temperature, also caused no degradation (Figure 2, h).

To see to what extent the *N*-(aminoethyl)carbamoylmethyl moiety would cause retardation of enzyme-catalysed hydrolysis of the vicinal phosphodiester groups was also of interest. Thus 2′-*O*-[*N*-(aminoethyl)carbamoyl]methyladenosine 3′-(thymidine 5′-phosphate) **10** was treated with the enzymes Phosphodiesterase I (PDE I) from *Crotalus adamanteus* venom and Phosphodiesterase II (PDE II) from bovine spleen, and the mixtures were analysed by RP-HPLC. For comparison, an unmodified dApT dimer was also subjected to similar treatment.

Aminoethylcarbamoylmethyl-modified dinucleotide **10** is highly resistant to enzyme-catalysed cleavage by snake-venom PDE I. Under conditions where the unmodified dimer was completely cleaved in about 2 h, there was still ca. 75% of **10** remaining, even after 7 d (Figure 3, upper part). When treated with spleen PDE II, modified dimer **10** was not cleaved at all after 7 d, under conditions where dApT was completely cleaved in a few hours (Figure 3, lower part). The resistance to enzymatic degradation is remarkable, and is even greater than that of the stable CMAT dinucleotide,[10] which lacks the aminoethyl substituent.

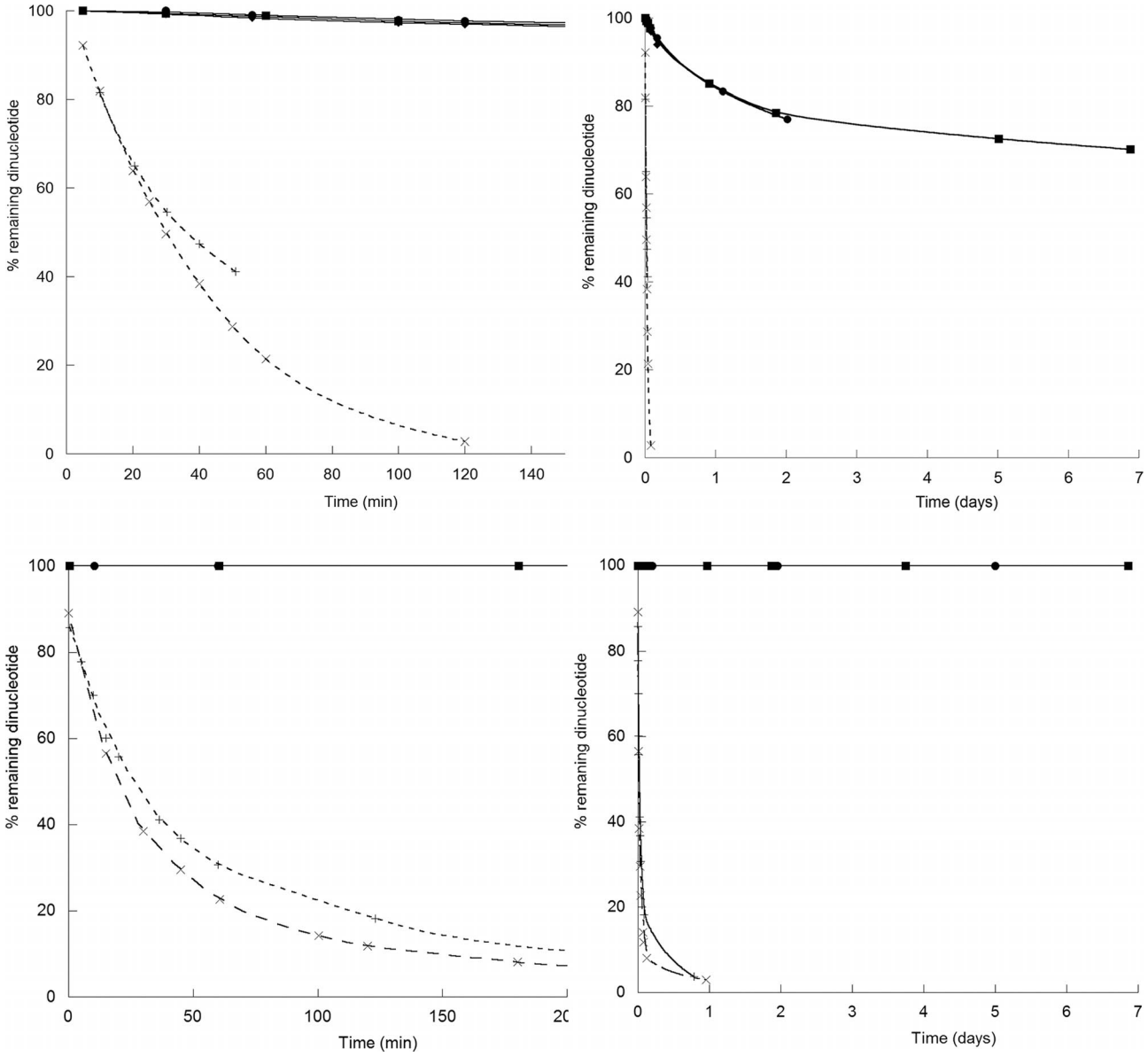


Figure 3. Graphs over different timescales showing percentage remaining of 2′-*O*-[*N*-(aminoethyl)carbamoyl]methyladenosine 3′-(thymidine 5′-phosphate) **10** (filled symbols and solid lines) after treatment with snake-venom PDE I (upper graphs) or spleen PDE II (lower graphs). Cleavage of the reference compound dApT under similar conditions is represented by × and + symbols (and dashed lines).

Conclusions

We have described the synthesis of 2′-*O*-[*N*-(aminoethyl)-carbamoyl]methyladenosine (AECM-A) building blocks as well as an AECM-A-containing dinucleotide (AECM-AT). The preferred method for the crucial introduction of the 2′-modification is the convenient and efficient one-pot three-step procedure shown in Scheme 2. Synthesis of an AECM-A H-phosphonate then enabled the preparation of AECM-AT dinucleotide **10**.

These results show that the 2′-*O*-[*N*-(aminoethyl)carbamoyl]methyl modification vicinal to a phosphodiester can be hydrolysed by concentrated aqueous ammonia, conditions that are commonly used for the complete deprotection of oligonucleotides. The degradation only occurs to a minor extent, but a degradation of even 1–2% is undesirable, especially for a fully modified oligonucleotide. However, this degradation can be avoided by the use of ethylenediamine or methanolic ammonia for deprotection, as under these conditions, the aminoethylcarbamoylmethyl group is stable.

We also showed that the 2′-*O*-aminoethylcarbamoyl (AECM) moiety, to an even greater extent than the carbamoylmethyl modification,[10] protected the dinucleotide against enzyme-catalysed degradation by snake-venom phosphodiesterase I, and made it completely resistant to degradation by spleen phosphodiesterase II. This makes the AECM an interesting modification to use in constructs for biotechnological and therapeutic applications such as oligonucleotide-based artificial nucleases and antisense oligonucleotides. It would be worth investigating fully or partially AECM-modified oligonucleotides to see how the modification affects duplex stability and biological activity.

Experimental Section

General Materials and Methods: Allyl bromoacetate was synthesised accordingly to a reported procedure.[26] Compound **1** (5′-MMT-A) was prepared following a standard procedure[27] (see also Supporting Information), and compound **8** (3′-MMT-T) was prepared as reported.[28] Other reagents were of commercial grade, and solvents and concentrated ammonia were of commercial analytical grade. Solvents were dried over 4 Å (pyridine and CH_2Cl_2) or 3 Å (methanol, ethanol, and acetonitrile) molecular sieves. THF and dioxane were distilled at atmospheric pressure from $LiAlH_4$, prior to use. Toluene and diethyl ether were dried with sodium wire. Ethylenediamine and triethylamine were distilled from calcium hydride. Silica gel column chromatography was generally carried out using Matrex silica, 60 Å (35–70 μM, Amicron). Solutions were concentrated under reduced pressure at temperatures not exceeding 40 °C. Ammonia (g) for the preparation of an ammonia–methanol solution was from Aga Gas, Sweden. The 2′-*O*-carbamoylmethyladenosine 3′-(thymidine 5′-phosphate) (CMAT) and 2′-*O*-carboxymethyladenosine 3′-(thymidine 5′-phosphate) (COMAT) used as HPLC references were prepared as reported.[10] The dApT dimer used for comparison in the enzymatic study was purchased from Cybergene AB and then further purified by HPLC using the same gradient as was for the analysis (see below). Both Phosphodiesterase enzymes were purchased from Sigma. TLC analysis was carried out on pre-coated plates, Silica Gel 60 F_{254} (Merck), with detection by UV light and/or by charring with sulfuric acid (8% in methanol). Reverse-phase HPLC was carried out with a Jasco apparatus. For the analysis of the chemical stability reactions, the HPLC apparatus was equipped with a Reprosil Pur-C18 column (3 × 250 mm). A 30 min linear gradient from 50 mM triethylammonium acetate (pH 6.5) to 10% of 50 mM triethylammonium acetate in acetonitrile was applied at room temperature, with a flow rate of 0.43 mL/min. UV detection was carried out at 254 nm. In the enzymatic degradation study, the column and buffers used for the HPLC analysis were the same, but a different gradient was used (0–10% for 20 min, and then 10–35% for a further 10 min). All NMR spectra were recorded using a Bruker AVANCE DRX-400 instrument (400.13 MHz for ^1H, 162.00 MHz for ^{31}P, and 100.62 MHz for ^{13}C) in the solvents specified. ^{13}C and ^1H chemical shifts are given in ppm. Most ^1H NMR assignments were made by standard ^1H–^1H-correlation spectroscopy (COSY). Mass spectrometric analysis was carried out with a Micromass LCT ESI-TOF mass spectrometer, using leucine enkephaline as an internal mass standard. Dinucleotides were analysed in negative mode in acetonitrile/water (1:1 v/v) solutions.

5′-*O*-(4-*O*-Monomethoxytrityl)-2′-*O*-(methoxycarbonyl)-methyladenosine (2): Compound **1** (2.0 g, 3.7 mmol) was dried by evaporation of added dry MeCN (50 mL). The dried material was dissolved in distilled THF (200 mL), and dimsyl sodium (2 M solution in DMSO; 4.44 mmol) was slowly added to the mixture. The mixture was stirred for 30 min, and then allyl bromoacetate (4.8 mmol) was added. After 1.5 h, ca. 175 mL of the THF was evaporated under reduced pressure. CH_2Cl_2 (400 mL) was added, and the organic phase was washed with a mixture of brine (300 mL) and H_2O (100 mL). The organic phase was dried with $MgSO_4$ and concentrated. The crude product was purified by chromatography on a silica gel column (CH_2Cl_2/methanol, 20:1 containing 0.05% triethylamine). During the chromatography and/or subsequent concentration, transesterification occurred so that methyl ester **2** (1.45 g, 68%) was obtained. ^1H NMR (400 MHz, $CDCl_3$): δ = 3.47 (dd, J = 10.3 and 4.4 Hz, 1 H, 5′-Ha), 3.53 (dd, J = 10.3 and 3.2 Hz, 1 H, 5′-Hb), 3.75 (s, 3 H, CH_3-trityl), 3.80 (s, 3 H, *O*Me-carbamoyl), 4.25–4.35 (m, 2 H, CH_2-carbamoyl, 4′-H), 4.42–4.49 (m, 2 H, CH_2-carbamoyl, 3′-H), 4.75 (t, J = 4.7 Hz, 1 H, 2′-H), 6.16 (d, J = 6.7 Hz, 1 H, 1′-H), 6.30 (br. s, 1 H, 3′-OH), 6.82 (d, J = 8.9 Hz, 2 H, trityl), 7.20–7.50 (m, 12 H, trityl), 8.07 (s, 1 H, H2-base), 8.25 (s, 1 H, H8-base) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 51.9, 54.7, 63.0, 67.9, 69.6, 83.5, 83.6, 86.5, 86.7, 112.8, 119.7, 126.7, 127.8, 128.0, 130.4, 134.9, 143.7, 149.4, 155.4, 159.2, 171.2 ppm. HMQC and HMBC NMR spectra are included in Supporting Information.

5′-*O*-(4-*O*-Monomethoxytrityl)-2′-*O*-[*N*-(*N*-trifluoroacetamido)-ethyl]carbamoylmethyladenosine (4)

Method A: Ethylenediamine (25 mL) was added to a solution of compound **2** (15 g, 24.5 mmol) in EtOH (99.5% dried with 3 Å molecular sieves; 150 mL) at room temperature. The reaction mixture was left overnight at room tempeature. The solvent was evaporated under reduced pressure. Pyridine (50 mL) was added and then evaporated three times, and then toluene (50 mL) was added and then evaporated three times. During this process, nitrogen (gas) was used to equalise the pressure in between each round of evaporation. The crude material (i.e., **3**) was used without further purification.

Crude **3** was then dissolved in CH_2Cl_2 (500 mL), and triethylamine (17.49 mL, 125.5 mmol) was added. TFAA (5 mL, 35.97 mmol) was added over 3 h, and the reaction mixture was left for a further 1 h, after which TLC analysis showed that the reaction was complete. The crude mixture was concentrated under reduced pressure until 100 mL of the CH_2Cl_2 remained. The mixture was extracted with ethyl acetate (350 mL) and washed twice with a mixture of

H_2O (100 mL), brine (100 mL), and $NaHCO_3$ (saturated aq.; 100 mL). Then the organic phase was dried with Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (CH_2Cl_2/MeOH, 10:1 with 0.05% triethylamine) to give **4** (16.24 g, 90% from **2**) as a white foam.

Method B – One-Pot Procedure: Compound **1** (2.50 g, 4.77 mmol) was dried by evaporation of added dry THF (distilled from $LiAlH_4$), and then the dried material was dissolved in dry THF (200 mL). Potassium *tert*-butoxide (0.696 g, 6.20 mmol) was added, and after 15 min, allyl bromoacetate (1.12 g, 6.20 mmol) was added. The reaction mixture was stirred for 2 h, after which time TLC showed that all of compound **1** had been consumed. The THF was evaporated under reduced pressure, and EtOH (99.8% dried with 3 Å molecular sieves; 100 mL) and ethylenediamine (2.87 g, 47.7 mmol) were added. The reaction mixture was stirred for 2 h, and then dry THF (100 mL) was added and the reaction mixture was left overnight at room temperature. TLC showed complete conversion of the alkylated products. The solvent was evaporated, and the excess ethylenediamine was removed by co-evaporation with added dioxane four times (4× 15 mL). The solid material was dissolved in dry CH_2Cl_2 (200 mL), and triethylamine (1.45 g, 14.31 mmol) and TFAA (1.65 g, 7.85 mmol) were added. TLC showed that the reaction was complete after 2 h at room temperature. The reaction mixture was washed with a mixture of water and brine (1:1; 400 mL). The organic phase was dried with $MgSO_4$ and concentrated. The crude product was purified by column chromatography on silica using CH_2Cl_2/methanol (15:1 with 0.005% triethylamine) as eluent to give **4** (2.7 g, 77%) as a white foam. (A slightly modified version of this reaction was performed using methyl bromoacetate instead of the allyl reagent, with the difference that the ethylenediamine was dried twice by evaporation of added *n*-butanol and then three times with dioxane. Compound **4** was then obtained in a yield of 72%.) ^{1}H NMR (400 MHz, [D_6]-DMSO): δ = 3.14–3.30 (m, 6 H, CH_2-ethylene, 5′-Ha, 5′-Hb), 3.72 (s, 3 H, CH_3-trityl), 4.02–4.18 (m, 3 H, CH_2-carbamoyl, 4′-H), 4.50–4.63 (m, 2 H, 2′-H, 3′-H), 5.48 (d, J = 6.4 Hz, 1 H, 3′-OH), 6.13 (d, J = 3.1 Hz, 1 H, 1′-H), 6.80 (d, J = 9.2 Hz, 2 H, trityl), 7.15–7.38 (m, 12 H, trityl), 8.12 (s, 1 H, H2-base), 8.25 (s, 1 H, H8-base), 9.48 (br. s, 1 H, NH) ppm. ^{13}C NMR (100.6 MHz, [D_6]-DMSO): δ = 37.2, 39.0, 55.1, 63.5, 69.4, 69.5, 81.8, 82.5, 86.0, 86.3, 113.3, 116.1 (q, J = 285 Hz), 118.3, 127.0, 127.9, 128.0, 130.1, 135.3, 144.5, 149.2, 153.0, 156.5, 156.8 (q, J = 39 Hz), 158.4, 169.5 ppm. Additional NMR spectroscopic data, including HMQC and HMBC analysis, is given in the Supporting Information. $C_{36}H_{36}N_7O_7F_3$: calcd. C 58.77, H 4.93, N 13.33; found C 58.57, H 5.03, N 13.16.

***N*^{6}-Butyryl-5′-*O*-(4-*O*-monomethoxytrityl)-2′-*O*-[*N*-(trifluoroacetamidoethyl)carbamoyl]methyl-3′-*O*-trimethylsilyladenosine (5):** Compound **4** (235 mg, 0.32 mmol) was dried by evaporation of added pyridine (dried with 4 Å molecular sieves), and the dried material was dissolved in dry pyridine (3 mL). Trimethylsilyl chloride (TMSCl; 123 μL, 0.96 mmol) was then added. The reaction mixture was stirred for 1 h at room temperature. The solution was then cooled to –10 °C (ice–salt bath), and butyric anhydride (64 μL, 0.39 mmol) was added. The reaction mixture was left overnight. The solvent was removed under reduced pressure to give the crude product, which was purified by chromatography on silica gel using CH_2Cl_2/methanol (20:1) as eluent to give **5** (169 mg, 60%) as a white foam. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.05 (s, 9 H, Si-Me), 1.03 (t, J = 7.4 Hz, 3 H, CH_3-Bu), 1.73–1.82 (m, 2 H, CH_2-Bu), 2.89 (t, J = 7.4 Hz, 2 H, CH_2-Bu), 3.27 (dd, J = 10.8 and 4.0 Hz, 1 H, 5′-Ha), 3.40 (m, 4 H, CH_2-ethylene), 3.56 (dd, J = 10.8 and 3.4 Hz, 1 H, 5′-Hb), 3.75 (s, 3 H, CH_3-trityl), 4.10–4.27 (m, 3 H, CH_2-carbamoyl, 4′-H), 4.48–4.60 (m, 2 H, 2′-H, 3′-H), 6.15 (d, J = 3.3 Hz, 1 H, 1′-H), 6.77 (d, J = 8.8 Hz, 2 H, trityl), 7.15–7.50 (m, 12 H, trityl), 8.25 (s, 1 H, H2-base), 8.65 (s, 1 H, H8-base) ppm.

***N*^{6}-Butyryl-5′-*O*-(4-*O*-monomethoxytrityl)-2′-*O*-[*N*-(trifluoroacetamidoethyl)carbamoyl]methyladenosine (6):** Compound **5** (0.085 mmol, 75 mg) was dissolved in CH_2Cl_2 (dried with 4 Å molecular sieves; 5 mL), and triethylamine-trihydrofluoride (three drops) was added to the solution whilst stirring. After 20 min, TLC showed that the reaction was complete. The solution was washed with brine (whose pH had been adjusted to 8 with saturated $NaHCO_3$; 5 mL). The solvent was removed from the organic phase under reduced pressure to give product **6** (68 mg, 99%) as a white foam that appeared to be pure by NMR spectroscopic analysis. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.04 (t, J = 7.4 Hz, 3 H, CH_3-Bu), 1.75–1.85 (m, 2 H, CH_2-Bu), 2.81–2.89 (t, J = 7.4 Hz, 2 H, CH_2-Bu), 3.30–3.55 (m, 6 H, 5′-Ha, 5′-Hb, CH_2-ethylene), 3.75 (s, 3 H, CH_3-trityl), 4.24 (ABq, J = 15.2 Hz, 2 H, CH_2-carbamoyl), 4.33–4.39 (m, 1 H, 4′-H), 4.50–4.75 (m, 2 H, 2′-H, 3′-H), 6.20 (d, J = 3.5 Hz, 1 H, 1′-H), 6.75 (d, J = 8.9 Hz, 2 H, trityl), 7.20–7.50 (m, 12 H, trityl), 8.25 (s, 1 H, H2-base), 8.65 (s, 1 H, H8-base) ppm.

***N*^{6}-Butyryl-5′-*O*-(4-*O*-monomethoxytrityl)-2′-*O*-[*N*-(trifluoroacetamidoethyl)carbamoyl]methyladenosine 3′-H-Phosphonate Triethylammonium Salt (7):** Imidazole (265 mg, 3.95 mmol) was dissolved in CH_2Cl_2 (dried with 4 Å molecular sieves; 35 mL). The solution was chilled to –10 °C, and PCl_3 (116 μL, 1.26 mmol) and then triethylamine (560 μL, 4.04 mmol) were added. The solution was cooled down further to –78 °C. Compound **6** (285 mg, 0.359 mmol) was dissolved in dry CH_2Cl_2 (3 mL). The solution containing compound **6** was then slowly added by syringe through a septum into the chilled PCl_3/imidazole solution. The mixture was then left for 2 h, during which time it was allowed to reach room temperature. The mixture was then extracted with CH_2Cl_2 (15 mL). The organic phase was washed with triethylammonium hydrogen carbonate (2 M aq.; 2× 30 mL) and dried with Na_2SO_4. The solvent was removed under reduced pressure to give **7** (345 mg, 99%) as a white foam, and NMR spectroscopic analysis showed that no further purification was needed before the next step. ^{1}H NMR (400 MHz, $CDCl_3$ plus Et_3N): δ = 1.05 (t, J = 7.4 Hz, 3 H, CH_3-Bu), 1.20 (CH_3 from Et_3N), 1.77–1.86 (m, 2 H, CH_2-Bu), 2.82–2.91 (m, 2 H, CH_2-Bu plus CH_2 from Et_3N), 3.35–3.65 (m, 6 H, 5′-Ha, 5′-Hb, CH_2-ethylene), 3.77 (s, 3 H, CH_3-trityl), 4.03 (ABq, J = 15.8 Hz, 2 H, CH_2-carbamoyl), 4.31–4.40 (m, 1 H, 4′-H), 4.87–4.93 (m, 1 H, 2′-H), 5.00–5.12 (m, 1 H, 3′-H), 6.94 (d, J = 640 Hz, 1 H, P-H), 6.23 (d, J = 5.5 Hz, 1 H, H′-1), 6.82 (d, J = 8.8 Hz, 2 H, trityl), 7.20–7.48 (m, 12 H, trityl), 8.12 (s, 1 H, H2-base), 8.60 (s, 1 H, H8-base) ppm. ^{31}P NMR: δ = 3.5 ppm. ES-TOF: m/ z calculated for M-, 868.2683, found 868.2664.

2′-*O*-[*N*-(Aminoethyl)carbamoyl]methyladenosine 3′-(Thymidine 5′-Phosphate) (10): H-phosphonate **7** (200 mg, 0.21 mmol) and 3′-*O*-MMT-thymidine **8** (114 mg, 0.21 mmol) were dissolved in pyridine (10 mL). Bis(2-oxo-3-oxazolidinyl) phosphinic chloride (OXP; 107 mg, 0.42 mmol) was added, and the solution was then stirred for 1 h at room temperature. TLC analysis suggested that the formation of **9** was complete. H_2O (0.10 mL) and iodine (100 mg) were added, and the mixture was stirred for a further 35 min. EtOAc (250 mL) was then added, and the solution was washed with a sulfite solution [brine/Na_2SO_3 (1 M)/H_2O, 1:1:1; 2× 50 mL]. The organic phase was dried with Na_2SO_4 and concentrated under reduced pressure. Traces of pyridine were removed by the evaporation of added THF (distilled from $LiAlH_4$). The solid material was then

suspended in aqueous acetic acid (80%; 10 mL), and the mixture was kept for 4 h. The solution was partioned between diethyl ether (30 mL) and H_2O (20 mL). The aqueous phase was evaporated under reduced pressure, ethylenediamine (20% in MeOH dried with 3 Å molecular sieves; 8 mL) was added, and the resulting reaction mixture was left for 21 h at room temperature. The solution was concentrated under reduced pressure, and then ethanol (99.5%; 10 mL) was added and then evaporated. This was repeated three times. Ammonium acetate (10 mg) was added followed by ethanol, and then the liquid was evaporated. The crude product (i.e., **10**; 82 mg, 0.12 mmol, 57%) was of 97% purity (as judged by HPLC), but to obtain very pure material for the studies of the dimer, a 60 mg portion of the material was further purified by reverse-phase HPLC to give **10** (40 mg). [Reprosil Pur C-18 column (20 × 250 mm) using a 30 min. linear gradient from 50 mM aqueous triethylammonium acetate (TEAA, pH 6.5) to 50mM TEAA in 20% acetonitrile, flow rate of 10 mL/min]. ^{1}H NMR (400 MHz, D_2O): δ = 1.77 (s, 3 H, CH_3-Thym), 2.28–2.40 (m, 2 H, 2′-Ha-Thym, 2′-Hb-Thym), 3.15 (t, J = 5.8 Hz, 2 H, CH_2-ethylene), 3.46–3.55 (m, 2 H, CH_2-ethylene), 3.82 (dd, J = 13.0 and 3.0 Hz, 1 H, 5′-Ha-Ad), 3.95 (dd, J = 13.0 and 2.2 Hz, 1 H, 5′-Hb-Ad), 4.05–4.20 (m, 3 H, 5′-Ha-Thym, 5′-Hb-Thym, 4′-H-Thym), 4.25 (ABq, J = 15.3 Hz, 2 H, 2′-*O*-CH_2-carbonyl), 4.46–4.51 (m, 2 H, 4′-H-Ad, 3′-H-Thym), 4.72 (t, J = 4.8 Hz, 1 H, 2′-H-Ad), 4.84–4.90 (m, 1 H, 3′-H-Ad), 6.15–6.20 (m, 2 H, 1′-H-Ad, 1′-H-Thym), 7.56 (s, 1 H, 6-H-Thym), 8.15 (s, 1 H, 2-H-Ad), 8.33 (s, 1 H, 8-H-Ad) ppm. ^{13}C NMR (100.6 MHz, D_2O): δ = 11.5, 36.4, 38.7, 39.1, 60.6, 65.0, 69.1, 70.0, 72.9, 81.0, 84.4, 84.8, 84.9, 85.1, 86.8, 111.2, 119.0, 136.7, 148.1, 151.3, 152.5, 155.4, 166.0, 172.4 ppm. (Es$^-$/TOF): *m*/*z* calculated for $C_{24}H_{33}N_9O_{12}P$ [M], 670.1986, found 670.1963.

Procedure for Investigating the Stability of AECM-AT Dimer 10 to Different Basic Deprotection Solutions: Compound **10** (0.5 mg/flask) was transferred into different 10 mL conical flasks from an aqueous stock solution of known concentration. The solutions were lyophilised, and the dry compound **10** was dissolved in the different solutions (2 mL of each), which were then left for different times and at different temperatures (see below). The tests were run in duplicate. 1) NH_3 (aq.) 30%, 24 h, 55 °C; 2) NH_3 (aq.) 30%, 48 h, 55 °C; 3) ethylenediamine (20% in methanol), 24 h, 20 °C; 4) ethylenediamine (20% in methanol), 48 h, 20 °C); 5) NH_3 (saturated in methanol), 24 h, 20 °C; 6) NH_3 (saturated in MeOH), 48 h, 20 °C; 7) NH_4OH (30% aq.)/EtOH (3:1), 24 h, 20 °C. After incubation, each sample was concentrated to dryness under reduced pressure, then water (2 mL) was added, and the sample was freeze-dried. The material in each flask was then dissolved in buffer A and subjected to RP-HPLC analysis as described above.

Procedures for Investigating the Resistance of AECM-AT 10 To Enzymatic Degradation (by PDE I and II): For the enzyme Phosphodiesterase I from *Crotalus adamanteus* venom, which cleaves oligonucleotides at the phosphate to give a 3′-OH and a 5′-phosphate, the following procedure was used. An assay buffer was prepared containing Tris (tris(hydroxymethyl)aminomethane; 100 mM), NaCl (100 mM), and $MgCl_2$ (14 mM) at pH 8.9. The dApT and the 2′-*O*-AECM-ApT **10** dinucleotides were each dissolved in the assay buffer to give assay solutions (AS-I) with concentrations of 0.07 mM. A stock solution of the enzyme was prepared containing enzyme (100 units), Tris (110 mM), magnesium chloride (15 mM) and NaCl (110 mM) at pH 8.9 in sterile water/glycerol (1:1) in a total volume of 1.47 mL, to give a total concentration of 68 U enzyme/mL. Enzyme stock solution (0.022 mL) was then added to assay buffer (0.078 mL) to give a new enzyme solution (ES-I) with a concentration of 0.15 U/mL. For enzymatic degradation studies with the PDE I, Eppendorf tubes were loaded with ApT or CMAT AS-I solutions (0.99 mL) to which enzyme solution ES-I (0.01 mL) was added. The reactions were incubated at 37 °C, and aliquots of 0.1 mL were taken and quenched at different reaction times. For the quenching, EDTA solution (75 mM; 0.02 mL) was added to each aliquot, and the solution was then filtered through a Micron YM-3-filter. The resulting solutions were then analysed by RP-HPLC as described above.

For Phosphodiesterase II from Bovine spleen, which cleaves oligonucleotides at the phosphate to give a 3′-phosphate and a 5′-OH, the following procedure was used. An assay buffer (pH 6.5) containing succinate buffer (s-2378; 250 mM) was prepared. The 2′-*O*-CMAT dimer and dApT were each dissolved in the assay buffer to give assay solutions (AS-II) with concentrations of 0.07 mM. A stock solution of the enzyme (ES-II) was prepared containing enzyme (3 units/mL) in sterile water. For the enzyme assay with PDE II, Eppendorf tubes were loaded with the respective ApT and CMAT assay solutions (AS-II; 0.495 mL), and then enzyme solution ES-II (0.005 mL) was added. The reactions were incubated at 37 °C, and 0.1 mL aliquots were taken and quenched at different times. To quench the reaction, the enzyme was removed by filtration through a Micron YM-3-filter. The quenched reaction mixtures were analysed by RP-HPLC as described above.

Supporting Information (see footnote on the first page of this article): Additional NMR spectroscopic data, including 2D ^{1}H–^{13}C correlation spectra (HMQC and HMBC) of compounds **2** and **4**.

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