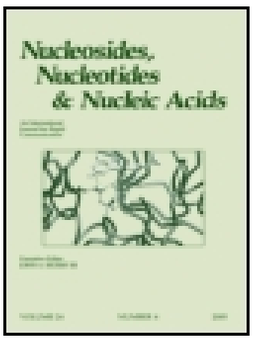

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# Effective Synthesis of Fluorescently Labeled Morpholino Nucleoside Triphosphate Derivatives

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**ABSTRACT**

Morpholino nucleoside triphosphates (A, U, G, C, T) bearing the active functional amino group tethered to morpholine residue and their fluorescently labeled derivatives were synthesized. All compounds were characterized by $^{1}H$, $^{13}C$, and $^{31}P$ NMR, and mass spectrometry. A possibility of using fluorescently labeled morpholino nucleoside triphosphates as chain terminators in DNA sequencing is discussed.

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Morpholino nucleosides; triphosphates; fluorescent labeling

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## Introduction

DNA sequencing has become an indispensable method for basic biological research and in numerous applications such as diagnostics, biotechnology, and others.[1,2] The latest achievements in technology were used for the development of the automated sequencing of DNA. There are two variants of automated DNA sequencing using the fluorescence detection, i.e., dye-labeled primer sequencing[3–5] and dye-labeled terminator sequencing, when the fluorescent dyes are attached to the terminating dideoxynucleoside triphosphates (ddNTP).[6–8] However, the ddNTP synthesis is complicated because of the multistage process and the instability of the glycosidic bond. In addition, the fluorescent dyes are usually attached to position 5 of pyrimidines or to position 7 of 7-deazapurines.[9–11] For these reasons, numerous by-products are formed during the synthesis, and the yield of ddNTP may be low. That is why either the search for new methods of the synthesis of fluorescent derivatives of 2′,3′-dideoxynucleosides triphosphates or the development of a new type of chain terminators is an urgent task.

The derivatives of morpholino nucleosides and nucleotides modified at the sugar-phosphate backbone are widely used as nucleic acid mimetics in the molecular biological studies.[12] The synthesis of nucleobase-functionalized morpholino-modified nucleoside monomers was reported recently.[13,14] Morpholino nucleoside

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triphosphates and some their derivatives functionalized at morpholine residue were shown to be substrates for DNA polymerase $\beta$ and HIV-1 reverse transcriptase.[15] It has been also found that the *N*-methylene carboxyl morpholino derivative of thymidine triphosphate is a substrate for Taq DNA polymerase.[16] This morpholino triphosphate acts as a chain terminator in DNA sequencing.

The promising properties of the morpholino nucleoside triphosphates as potential chain terminators for DNA sequencing or inhibitors of some viral DNA polymerases motivated us to develop the efficient method for the synthesis of these triphosphates containing functionally active alkylamino group suitable for fluorescent or reporter labeling.

## Results and discussion

The convenient synthesis of morpholino nucleoside triphosphates without additional functional groups tethered to morpholine residue or to nucleobase was reported.[17] Since many of widely used fluorescent dyes have the functional carboxyl group, it would be useful to introduce the active amino group in the nucleoside triphosphate structure for the attachment of the fluorescent label. It was shown that extending the linker arm between nucleotide triphosphate and a dye facilitates the incorporation of terminating nucleotide in DNA chain.[18] We report here the synthesis of morpholino nucleoside triphosphates (A, U, G, C, T) containing the extended hydrophilic linker bearing the alkyl amino group and the efficient procedure for fluorescent labeling of these compounds with carboxyl dye. The linker is tethered to morpholine residue of the molecule. The presence of glycol fragments in the linker should provide hydrophilicity of the alkyl chain for better solubility and an avoidance of formation of triphosphate aggregates at high NTP concentrations.

*N*-Trityl protected morpholino nucleosides were prepared from base-protected ribonucleosides by Summerton et al.[19] Taking in mind their standard protocol, we have synthesized morpholino nucleosides containing the extended hydrophilic linker (Scheme 1) starting from ribonucleosides with protected heterocyclic bases (**1a-e**) and using an aqueous solution of an extended alkyl diamine buffered with boric acid instead of an ammonium salt. The key feature of our procedure was the careful maintaining of pH in the reaction mixture close to 9.0 during the reaction of dialdehyde derivatives **2a-e** with diamine. The products obtained after the reduction of the intermediate Schiff base **3a-e** were separated by reverse phase chromatography (RPC) to afford morpholino derivatives of nucleosides **4a-e**. Compounds **4a-e** were trifluoroacetylated to give protected derivatives of morpholino nucleosides **5a-e** in overall yields of 55–65%.

The method of the synthesis of morpholino nucleosides starting from 5′-*O*-protected ribonucleosides is described in the literature.[20] The authors reported that the presence of the protective group at the 5′-position of ribonucleosides considerably increased the yields of the morpholino monomers, for example, from 12 to 52% for morpholinothymidine nucleoside. In an earlier study of the same

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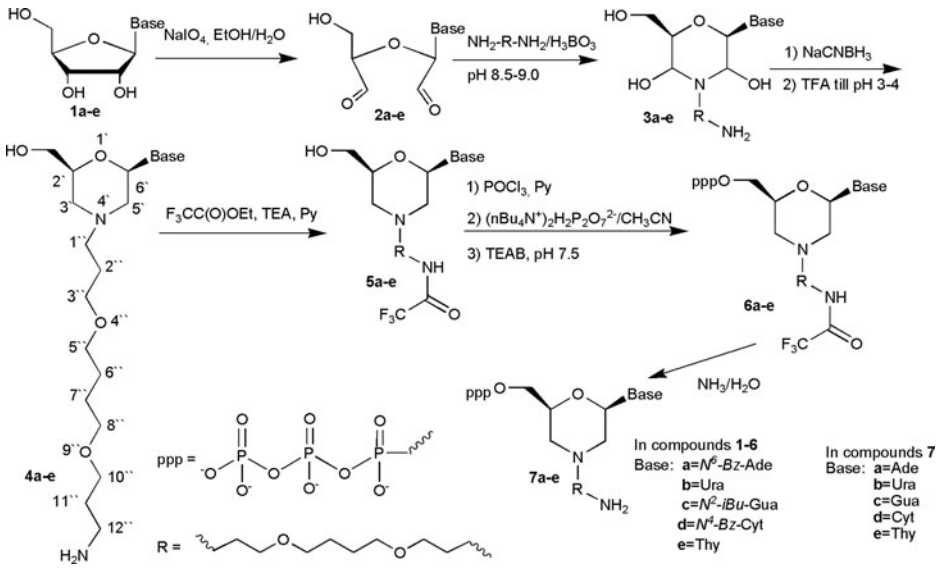


**Scheme 1.** Synthesis of morpholino nucleoside triphosphates containing the extended hydrophilic linker.

authors[21] morpholino nucleosides were obtained using the standard protocol without the additional protection of 5′-OH group. This result may give an impression that the Summerton's protocol for the morpholino nucleoside synthesis[19] is not sufficiently effective and does not provide reasonable yields of the final products. Using our improved procedure we synthesized various types of morpholino nucleosides including Summerton's in high yields without the 5′-*O*-protection.[17,22–25] Most likely, we obtained the reasonable yields of the target morpholino nucleosides due to the careful monitoring of the pH in the reaction mixture during the Schiff base formation. Nevertheless, the advisability of the 5′-*O*-protection of ribonucleosides before the formation of the morpholino ring remains to be discussed.

At present, the "one-pot, three step" protocol[26] is the most widely used for obtaining nucleoside triphosphates from nucleosides. This method involves the reaction of unprotected nucleosides with $POCl_3$ in trimethyphosphate/trialkylphosphate and then with pyrophosphate. Due to the presence of the highly reactive alkyl amino group in compounds **4a-e**, this method cannot be used in our case. Therefore, we applied the previously developed strategy for triphosphorylation of suitably protected dinucleoside derivatives.[27] The primary alkyl amino group of the linker was trifluoroacetylated (Scheme 1, compounds **5a-e**) before the introduction of the triphosphate residue. The products after triphosphorylation were purified by RPC giving compounds **6a-e**. The protective groups were removed from crude protected triphosphates **6a-e** by the treatment with concentrated aqueous ammonia. After the deprotection of bases and linker the target compounds were purified by ion exchange chromatography to afford triphosphates **7a-e**. Typical yields of the triphosphates **7a-e** were 50–60%.

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The conjugation of fluorescent dyes to nucleotides containing the aliphatic amino group is mainly carried out by using their activated esters in aqueous organic media. *N*-Hydroxysuccinimidyl esters are used most frequently. We synthesized *N*-hydroxysuccinimidyl esters of cyanine (Cy5) and rhodamine (TAMRA) dyes by conjugation of the fluorescent dye with *N*-hydroxysuccinimide in the presence of *N,N′*-dicyclohexylcarbodiimide in DMSO. Our attempts to attach these activated dyes to the aliphatic amino group of morpholino nucleoside triphosphate (A, U) in aqueous organic media failed; the yield of target product did not exceed 5%. Recently, we performed the successful fluorescent labeling of dideoxyuridine triphosphate derivative using pentafluorophenyl ester of Cy5.[28] We prepared pentafluorophenyl ester of TAMRA in a quantitative yield by conjugation with pentafluorophenol under the action of *N,N′*-diisopropylcarbodiimide in anhydrous DMF similarly to previously published protocol for Cy5[28] and used it for acylation of the aliphatic amino group of morpholino nucleoside triphosphates **7a-e** (Scheme 2). Fluorescently labeled morpholino nucleoside triphosphates **8a-e** were obtained in high yields (80–85% after purification by RPC). Thus, the use of pentafluorophenyl ester of the dye provides the highly efficient synthesis of labeled triphosphate derivatives **8a-e**. Taking into account the similar results of the fluorescent labeling of dideoxyuridine-5′-triphosphate,[28] we conclude that the use of pentafluorophenyl esters of fluorescent dyes would be preferable for obtaining fluorescently labeled triphosphates.

In preliminary experiments on studying the substrate properties of triphosphates **7a-e** and **8a-e** in DNA synthesis, morpholino nucleoside analogues **7a-e** demonstrated some inhibition of the DNA synthesis catalyzed by DNA polymerase $\beta$ and reverse transcriptase of human immunodeficiency virus (HIV-1 RT) in primer extension essay. It was found out that Taq DNA polymerase utilizes thymine derivative **8e** more correctly than uracil containing compound **8b** in sequencing reaction. Thus, it is necessary to use thymine morpholino nucleoside triphosphate instead of more available uracil-containing triphosphate.

In conclusion, an effective synthetic protocol for preparing fluorescently labeled morpholino nucleoside triphosphates bearing an extended hydrophilic linker tethered to morpholine residue is developed. The synthesis of morpholino nucleoside triphosphates and their fluorescent derivatives is easier and occurs in higher yields as compared to 2′,3′-dideoxyribonucleoside-5′-triphosphates and their fluorescent analogues. A new family of DNA terminators may be proposed based on these triphosphates.

## Experimental

We used sodium periodate, pentafluorophenol (Acros Organics, USA), *N,N′*-dicyclohexylcarbodiimide (Merck, Germany). All other reagents and solvents were from Sigma–Aldrich (USA) and Reachem (Russia). NMR spectra were recorded on a Bruker AV-300, AV400 and DRX-500 instruments (Bruker, Germany) in appropriate deuterated solvents at 30°C. Chemical shifts ($\delta$) are reported in ppm relative

to TMS signals. In the case of $^{31}P$ and $^{19}F$, external standards of 85% $H_3PO_4$ and $C_6F_6$, respectively, were used. Coupling constants *J* are reported in Hertz. MALDI–TOF mass spectra were registered on an Autoflex III mass spectrometer (Bruker Daltonics, Germany) using 2,5-dihydroxybenzoic acid as a matrix (MALDI–TOF) in positive or negative mode or on an Agilent ESI MSD XCT Ion Trap (Agilent Technologies, USA) (ESI) in The Center of Cooperative Use ("Proteomics," Russian Academy of Sciences). Thin layer chromatography (TLC) was carried out on Kieselgel 60 F254 plates (Merck, Germany) in the proper solvent systems (see later) and visualized by UV irradiation or ninhydrin (amine groups). The preparative RPC was performed using Porasil C 18 (55–105 $\mu$m, 125 A) (Waters, USA); anion exchange chromatography, DEAE Sephadex A-25 (Pharmacia, Sweden). Eluent composition is given in v/v per cent. Bis(tetra-*n*-butylammonium) pyrophosphate was prepared as in Ref. [29].

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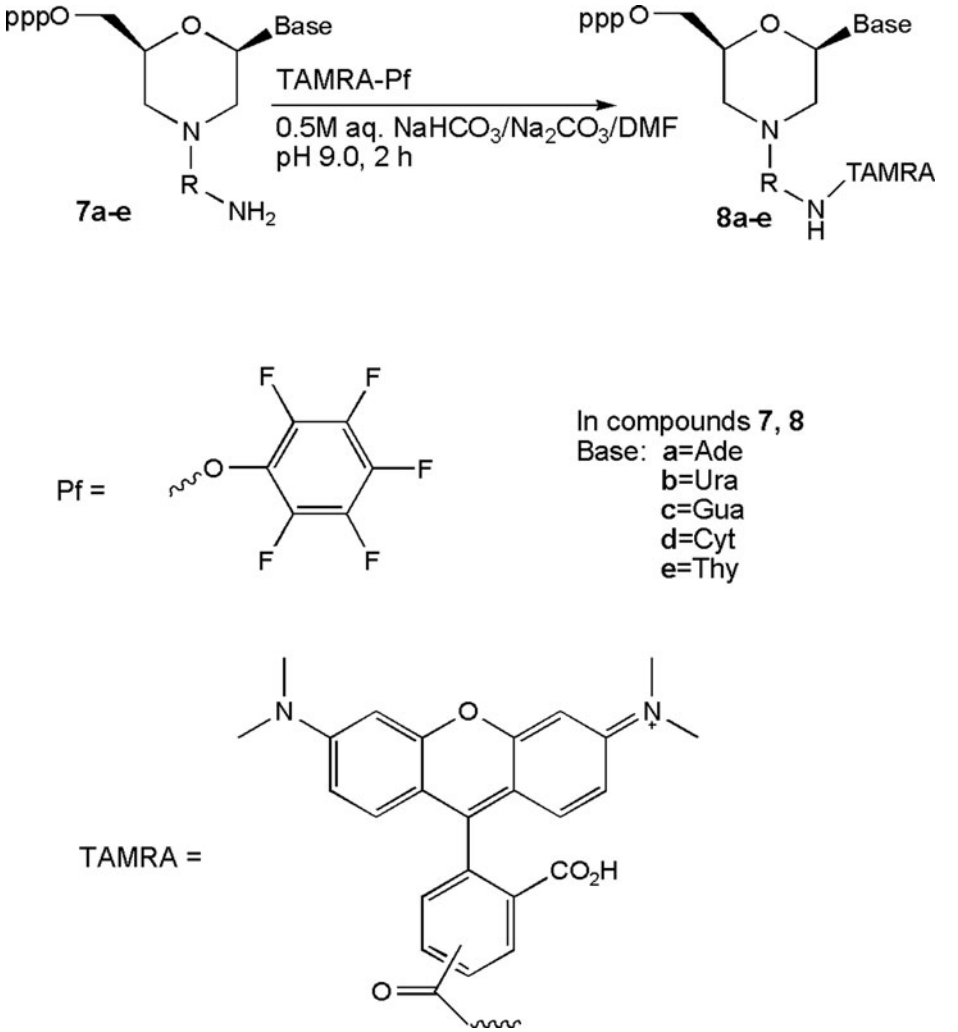


**Scheme 2.** Fluorescent labeling of morpholino nucleoside triphosphates.

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**General procedure for the synthesis of compounds 5a-e**. The protected ribonucleosides **1a-e** (1 mmol) were suspended in ethanol (16 mL). A sodium periodate (0.224 g, 1.05 mmol) was dissolved in a warm water (1 mL) and was added to the nucleoside suspension. After 15 min of stirring, a 4,9-dioxadodecane-1,12-diamine (0.256 mL, 1.2 mmol) in a mixture of water (1.28 mL) and boric acid (0.235 g, 3.8 mmol) was added to the reaction mixture. If the pH of the resulting mixture was below 8.5, triethylamine (TEA) was added till pH 9.0. The pH of the reaction mixture was checked up 2–3 times when stirring and TEA was added till pH 9.0. After 1.5–2 h, the reaction mixture was filtered. The precipitate was washed with ethanol (3 × 1 mL). Sodium cyanoborohydride (0.082 g, 1.3 mmol) was added to the filtrate and the mixture was stirred for 40 min. The reaction mixture was adjusted to pH 3–4 with trifluoroacetic acid (TFA). After 2 h, the solution was evaporated under reduced pressure. The products **4a-e** were purified by RPC in a linear gradient of EtOH in water (0–50%) containing 0.1% TFA. The appropriate fractions were evaporated. The crude products were dissolved in methanol (1.5 mL). TEA (0.417 mL, 3 mmol) and ethyl trifluoroacetate (0.357 mL, 3 mmol) were added to the solution of morpholino nucleoside. The progress of reaction was monitored by TLC (EtOH/DCM, 1/9). After the reaction was complete, the solvent was evaporated under reduced. The oil residue was purified by RPC in a linear gradient of MeCN in water (0–60%) to give the products **5a-e**.

***2-Hydroxymethyl-4-[-4,9-dioxa-12-(N-trifluoroacetylamino)dodecyl]-6-($N^6$-benzoyladenin-9-yl)morpholine* (*5a*)**: yield 65%; $R_f = 0.17$ (EtOH/DCM, 1/9); $^1$H NMR (300 MHz, $CD_3CN$) $\delta$ 9.56 (br s, 1H, NH), 8.66 (s, 1H, H-8), 8.31 (s, 1H, H-2), 8.01 (dt, 2H, $J = 7.2$, 1.6 Hz, Bz), 7.71 (br s, 1H, NH), 7.64 (tt, 1H, $J = 7.3$, 1.3 Hz, Bz), 7.54 (tt, 2H, $J = 7.4$, 1.5 Hz, Bz), 5.93 (dd, 1H, $J = 10.1$, 2.6 Hz, H6′), 3.96–3.86 (m, 1H, H2′), 3.58–3.54 (m, 2H, 2′-$\underline{CH_2}$OH), 3.47–3.28 (m, 10H, H3″, H5″, H8″, H10″, H12″), 3.15 (dt, 1H, $J = 10.7$, 2.0 Hz, H5′), 2.88 (dt, 1H, $J = 11.3$, 2.0 Hz, H3′), 2.58–2.45 (m, 3H, H1″, H3′), 2.04 (app t, 1H, $J = 11.1$ Hz, H5′), 1.81–1.66 (m, 4H, H2″, H11″), 1.56 (m, 4H, H6″, H7″); $^{13}$C NMR (400 MHz, DMSO-$d_6$) $\delta$ 166.13, 156.65 (q, $J_{CF} = 35.8$ Hz), 152.32, 150.89, 143.28, 133.75, 132.96, 128.96, 125.78, 116.04 (q, $J_{CF} = 288.0$ Hz), 79.68, 77.48, 70.32, 70.24, 68.50, 67.76, 62.48, 55.65, 55.05, 54.27, 37.17, 28.97, 26.86, 26.47); $^{19}$F NMR (300 MHz, $CD_3CN$) $\delta$ 87.75; MS (ESI) m/z: $[M+H]^+$ calcd. for $C_{29}H_{39}F_3N_7O_6$ 638.2914, found 638.192.

***2-Hydroxymethyl-4-[4,9-dioxa-12-(N-trifluoroacetylamino)dodecyl]-6-(uracil-1-yl)morpholine(5b)***: yield 60%; $R_f$ 0.28 (EtOH/DCM, 1/9); $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 11.29 (s, 1H, NH), 9.30 (br t, 1H, $J = 6.7$ Hz, NH), 7.62 (d, 1H, $J = 8.0$ Hz, H6), 5.58 (d, 1H, $J = 8.0$ Hz, H5), 5.53 (dd, 1H, $J = 10.1$, 2.0 Hz, H6′), 4.70 (br t, 1H, $J = 5.5$ Hz, 2′-$CH_2\underline{OH}$), 3.69–3.61 (m, 1H, H2′), 3.45–3.37 (m, 2H, 2′-$\underline{CH_2}$OH), 3.36–3.28 (m, 8H, H3″, H5″, H8″, H10″), 3.20 (app q, 2H, $J = 6.5$ Hz, H12″), 2.83 (br d, 1H, $J = 11.0$ Hz, H5′), 2.77 (br d, 1H, $J = 11.0$ Hz, H3′), 2.36 (t, 2H, $J = 7.3$ Hz, H1″), 1.97 (app t, 1H, $J = 10.5$ Hz, H3′), 1.81 (app t, 1H, $J = 10.9$ Hz, H5′), 1.70–1.58 (m, 4H, H2″, H11″), 1.50–1.44 (m, 4H, H6″, H7″); $^{13}$C NMR (500 MHz, DMSO-$d_6$) $\delta$ 162.89, 156.13 (q, $J_{CF} = 35.9$ Hz), 149.99,

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140.90, 115.88 (q, $J_{CF}$ = 293 Hz), 101.66, 79.03, 76.95, 69.84, 69.72, 67.98, 67.29, 62.02, 55.41, 54.45, 53.52, 36.71, 28.49, 26.37, 25.98; $^{19}$F NMR (300 MHz, $CD_3CN$) $\delta$ 87.72; MS (ESI) *m/z*: $[M+H]^+$ calcd. for $C_{21}H_{34}F_3N_4O_7$ 511.2380, found 511.094.

***2-Hydroxymethyl-4-[4,9-dioxa-12-(N-trifluoroacetylamino)dodecyl]-6-($N^2$-isobutyrylguanin-9-yl)morpholine(5c)***: 65% yield, $R_f$ = 0.18 (EtOH/DCM, 1/9); $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 12.04 (br.s, 1H, NH), 11.67 (s, 1H, NH), 9.35 (brt, 1H, *J* = 5.4 Hz, NH), 8.12 (s, 1H, H8), 5.60 (dd, 1H, *J* = 10.5, 2.2 Hz, H6′), 4.80 (br t, 1H, *J* = 5.6 Hz, 2′-$CH_2$OH), 3.96–3.87 (m, 1H, H2′), 3.45–3.23 (m, 10H, 2′-$CH_2$OH, H3″, H5″, H8″, H10″), 3.18 (app q, 2H, *J* = 6.6 Hz, H12″), 3.03 (br d, 1H, *J* = 10.7 Hz, H5′), 2.82 (br d, 1H, *J* = 10.4 Hz, H3′), 2.73 (sep, 1H, *J* = 6.8 Hz, CH($CH_3$)$_2$), 2.58–2.37 (m, 4H, H3′, H1″), 1.97 (app t, 1H, *J* = 11.1 Hz, H5′), 1.70–1.58 (m, 4H, H2″, H11″), 1.52–1.40 (m, 4H, H6″, H7″), 1.08 (br.d, 6H, *J* = 6.7 Hz, CH($CH_3$)$_2$); $^{13}$C NMR (400 MHz, DMSO-$d_6$) $\delta$ 180.65, 156.62 (q, $J_{CF}$ = 36.0 Hz), 155.27, 148.75, 148.60, 137.85, 120.28, 116.37 (q, $J_{CF}$ = 36.0 Hz), 79.21, 74.26, 70.32, 70.25, 68.43, 67.76, 62.54, 55.65, 54.84, 53.48, 37.16, 35.20, 28.97, 26.81, 26.48, 19.32, 19.28; $^{19}$F NMR (300 MHz, $CD_3CN$) $\delta$ 87.97; MS (ESI) *m/z*: $[M+H]^+$ calcd. for $C_{26}H_{41}F_3N_7O_7$ 620.3020, found 620.369.

***2-Hydroxymethyl-4-[4,9-dioxa-12-(N-trifluoroacetylamino)dodecyl]-6-($N^4$-benzoylcytozin-1-yl)morpholine (5d)***: yield 55%; $R_f$ = 0.25 (EtOH/DCM, 1/9); $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 11.31 (s, 1H, NH), 9.39 (br t, 1H, *J* = 5.6 Hz, NH), 817 (d, 1H, *J* = 7.6 Hz, H6), 8.01 (dt, 2H, *J* = 7.1, 1.7 Hz, Bz), 7.63 (tt, 1H, *J* = 7.4, 1.4 Hz, Bz), 7.51 (tt, 2H, J = 7.5, 1.4 Hz, Bz), 7.37, (d, 1H, *J* = 7.6 Hz, H5), 5.71 (dd, 1H, *J* = 9.4, 2.4 Hz, H6′), 4.82 (t, 1H, *J* = 5.8 Hz, 2′-$CH_2$OH), 3.49 (app t, 1H, *J* = 5.6 Hz, 2′-$CH_2$OH), 3.45–3.26 (m, 8H, H3″, H5″, H8″, H10″), 3.23 (app q, 2H, *J* = 6.2 Hz, H12″), 3.01 (br.d, 1H, *J* = 10.8, H5′), 2.86 (d, 1H, J = 10.0 Hz, H3′), 2.41 (t, 2H, *J* = 7.2 Hz, H1″), 1.96–1.83 (m, 2H, H3′, H5′), 1.74–1.61 (m, 4H, H2″, H11″), 1.55–1.44 (m, 4H, H6″, H7″); $^{13}$C NMR (400 MHz, DMSO-$d_6$) $\delta$ 167.76, 163.49, 156.52 (q, $J_{CF}$ = 34.8 Hz), 154.09, 145.95, 133.44, 133.14, 128.80, 127.87, 116.37 (q, $J_{CF}$ = 286.0 Hz), 96.83, 80.89, 77.39, 70.21, 70.10, 68.35, 67.65, 62.40, 56.60, 54.80, 53.95, 37.06, 28.85, 26.74, 26.35; $^{19}$F NMR (400 MHz, DMSO-$d_6$) $\delta$ 91.79; MS (ESI) *m/z*: $[M+H]^+$ calcd. for $C_{28}H_{39}F_3N_5O_7$ 614.2802, found 614.184.

***2-Hydroxymethyl-4-[4,9-dioxa-12-(N-trifluoroacetylamino)dodecyl]-6-(thymin-1-yl)morpholine(5e)***: yield 60%; $R_f$ = 0.30 (EtOH/DCM, 1/9); $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 11.39 (s, 1H, NH), 9.39 (br t, 1H, *J* = 5.36 Hz, NH), 7.55 (q, 1H, *J* = 1.1 Hz, H6), 5.58 (br d, 1H, *J* = 9.7 Hz, H6′), 4.80 (t, 1H, *J* = 5.6 Hz, 2′-$CH_2$OH), 3.74–3.63 (m, 1H, H2′), 3.51–3.28 (m, 10H, 2c-CH2OH, H3″, H5″, H8″, H10″), 3.23 (app q, 2H, *J* = 6.7 Hz, H12″), 2.92–2.77 (m, 2H, H3′, H5′), 2.42 (br t, 2H, *J* = 8.1 Hz, H1″), 2.10 (br.t, 1H, *J* = 10.7 Hz, H3′), 1.88 (br t, 1H, *J* = 10.74 Hz, H5′), 1.78 (d, 3H, *J* = 1.1 Hz, $CH_3$), 1.75–1.61 (m, 4H, H2″, H11″), 1.54–1.47 (m, 4H, H6″, H7″); $^{13}$C NMR (300 MHz, DMSO-$d_6$) $\delta$ 163.68, 156.19 (q, $J_{CF}$ = 35.7 Hz), 150.11, 136.47, 116.11 (q, $J_{CF}$ = 287.7 Hz), 109.44, 78.79, 76.90, 69.91, 69.79, 68.02, 67.35, 60.06, 55.19, 54.59, 53.53, 36.77, 28.56, 26.32, 26.05,

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12.02; $^{19}$F NMR (300 MHz, DMSO-$d_6$) $\delta$ 88.23; MS (ESI) $m/z$: $[M+H]^+$ calcd. for $C_{22}H_{35}F_3N_4O_7$ 525.2536, found 525.093.

**General procedure for the synthesis of compounds 7a-e**. Nucleosides **5a-e** (0.1 mmol) were dissolved in anhydrous pyridine (1 mL) and the solution was chilled in an ice bath. Phosphorous oxychloride (0.027 mL, 0.3 mmol) was added to the solution of nucleoside and reaction mixture was stirred for 15 min (TLC control EtOH/DCM = 1/9). After the reaction was complete, the 0.5 M solution of bis(tetra-*n*-butylammonium) pyrophosphate in acetonitrile (1.5 mL, 0.75 mmol) was added. The reaction mixture was stirred at room temperature for 40 min (TLC iPrOH/con.aq. $NH_3/H_2O$ = 7/1/2) and then was quenched with 1M TEAB (pH 7.5, 20 mL). After 1 h, the reaction mixture was evaporated under reduced pressure. The residue was purified by RPC in a linear gradient of EtOH in water (0–50%) to give the compounds **6a-e**. The crude products **6a-e** were dissolved in con. aq. ammonia (10 mL) and stirred for 24 h. Then the reaction mixture was evaporated under reduced pressure. The residue after evaporation was dissolved in 20% aq. ethanol (50 mL) and applied to a column packed with DEAE-Sephadex A25 in 20% aqueous ethanol. The target products were purified by an anion exchange chromatography. Elution was performed with a linear gradient of $NH_4HCO_3$ (0–1 M) in 20% aqueous EtOH. The appropriate fractions were pooled and evaporated. The residue was dissolved in water and the target triphosphates were precipitated by tenfold volume of 4% $NaClO_4$/acetone.

***2-(O-Triphosphohydroxymethyl)-4-(4,9-dioxa-12-aminododecyl)-6-(adenine-9-yl)morpholine(7a)***: yield 60%; $R_f$ = 0.36 (iPrOH/con.aq. $NH_3/H_2O$ = 6/1/3); $^1$H NMR (300 MHz, $D_2O$) $\delta$ 8.36 (s, 1H, H8), 8.24 (s, 1H, H2), 5.99 (dd, 1H, $J$ = 10.7, 2.1 Hz, H6′), 4.34–4.24 (m, 1H, H2′), 4.13 (dd, 2H, $J$ = 6.8, 4.8 Hz, 2′-$\underline{CH_2}$Oppp), 3.66–3.46 (m, 8H, H3″, H5″, H8″, H10″), 3.32 (br d, 1H, $J$ = 11.0 Hz, H5′), 3.19 (br d, 1H, $J$ = 11.8 Hz, H3′), 3.09 (t, 2H, $J$ = 7.3 Hz, H1″), 2.91 (app t, 1H, $J$ = 11.1 Hz, H3′), 2.85–2.63 (m, 2H, H12″), 2.47 (app t, 1H, $J$ = 11.5 Hz, H5′), 1.99–1.79 (m, 4H, H2″, H11″), 1.69–1.53 (m, 4H, H6″, H7″); $^{31}$P NMR (300 MHz, $D_2O$) $\delta$ −7.09 (d, 1P, $J$ = 19.3 Hz, $P_\gamma$), −10.60 (d, 1P, $J$ = 18.9 Hz, $P_\alpha$), −21.43 (t, 1P, $J$ = 19.2, $P_\beta$).

***2-(O-Ttriphosphohydroxymethyl)-4-(4,9-dioxa-12-aminododecyl)-6-(uracil-1-yl)morpholine(7b)***: yield 50%; $R_f$ = 0.24 (iPrOH/con.aq. $NH_3/H_2O$ = 6/1/3); $^1$H NMR (300 MHz, $D_2O$) $\delta$ 7.87 (d, 1H, $J$ = 8.1 Hz, H6), 5.89 (d, 1H, $J$ = 8.1 Hz, H5), 5.56 (dd, 1H, $J$ = 10.3, 2.7 Hz, H6′), 4.26–4.15 (m, 1H, H2′), 4.11 (dd, 2H, $J$ = 7.2, 4.3 Hz, 2′-$\underline{CH_2}$Oppp), 3.66–3.48 (m, 8H, H3″, H5″, H8″, H10″), 3.23–3.13 (m, 2H, H3′, H5′), 3.09 (t, 2H, $J$ = 7.1 Hz, H1″), 2.82–2.62 (m, 2H, H12″), 2.50–2.35 (m, 2H, H3′, H5′), 2.01–1.79 (m, 4H, H2″, H11″), 1.68–1.54 (m, 4H, H6″, H7″); $^{31}$P NMR (300 MHz, $D_2O$) $\delta$: −8.27 (d, 1P, $J$ = 19.9 Hz, $P_\gamma$), −10.72 (d, 1P, $J$ = 19.2 Hz, $P_\alpha$), −21.89 (t, 1P, $J$ = 19.5, $P_\beta$).

***2-(O-Triphosphohydroxymethyl)-4-(4,9-dioxa-12-aminododecyl)-6-(guanine-9-yl)morpholine(7c)***: yield 48%; $R_f$ = 0.12 (iPrOH/con.aq. $NH_3/H_2O$ = 6/1/3); $^1$H NMR (300 MHz, $D_2O$) $\delta$: 8.04 (s, 1H, H8), 5.87 (dd, 1H, $J$ = 10.5, 2.0 Hz,

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H6′), 4.38–4.27 (m, 1H, H2′), 4.10–4.07 (m, 2H, 2′-$\underline{CH_2}$Oppp), 3.69–3.47 (m, 8H, H3″, H5″, H8″, H10″), 3.37 (br d, 1H, $J$ = 10.8 Hz, H5′), 3.27 (br d, 1H, $J$ = 12.4 Hz, H3′), 3.12 (t, 2H, $J$ = 7.3 Hz, H1“), 2.95 (t, 1H, $J$ = 11.1 Hz, H3′), 2.90–2.70 (m, 2H, H12″), 2.52 (t, 1H, $J$ = 11.5 Hz, H5′), 2.04–1.84 (m, 4H, H2”, H11″), 1.71–1.55 (m, 4H, H6″, H7″); $^{31}$P NMR (300 MHz, $D_2O$) $\delta$: −8.17 (d, 1P, $J$ = 19.2 Hz, $P_\gamma$), −10.68 (d, 1P, $J$ = 19.4 Hz, $P_\alpha$), −21.35–22.38 (t, 1P, $J$ = 18.7 Hz, $P_\beta$).

***2-(O-Triphosphohydroxymethyl)-4-(4,9-dioxa-12-aminododecyl)-6-(cytozine-1-yl)morpholine(7d)***: yield 59%; $R_f$ = 0.28 (iPrOH/con.aq. $NH_3/H_2O$ = 6/1/3); $^1$H NMR (300 MHz, $D_2O$) $\delta$ 7.85 (d, 1H, $J$ = 7.6 Hz, H6), 6.09 (d, 1H, $J$ = 7.6 Hz, H5), 5.93 (dd, 1, $J$ = 10.4, 2.1 Hz, H6′), 4.29–4.19 (m, 1H, H2′), 4.15 (dd, 2H, $J$ = 7.7, 5.1 Hz, 2′-$\underline{CH_2}$Oppp), 3.68–3.47 (m, 8H, H3″, H5″, H8″, H10″), 3.33–3.22 (m, 2H, H3′, H5′), 3.12 (t, 2H, $J$ = 7.4 Hz, H1″), 2.93–2.68 (m, 2H, H12″), 2.61–2.42 (m, 2H, H3′, H5′), 2.04–1.81 (m, 4H, H2″, H11″), 1.70–1.54 (m, 4H, H6″, H7″); $^{31}$P NMR (300 MHz, $D_2O$) $\delta$ −8.50 (d, 1P, $J$ = 19.5 Hz, $P_\gamma$), −10.74 (d, 1P, $J$ = 19.5 Hz, $P_\alpha$), −21.99 (t, 1P, $J$ = 19.5, $P_\beta$).

***2-(O-Triphosphohydroxymethyl)-4-(4,9-dioxa-12-aminododecyl)-6-(thymin-1-yl)morpholine(7e)***: yield 60%; $R_f$ = 0.25 (iPrOH/con.aq. $NH_3/H_2O$ = 6/1/3); $^1$H NMR (300 MHz, $D_2O$) $\delta$: 7.71 (q, 1H, $J$ = 1.1, H6), 5.88 (dd, 1H, $J$ = 10.6, 2.4 Hz, H6′), 4.26–4.16 (m, 1H, H2′), 4.13 (dd, 2H, $J$ = 7.2, 4.6 Hz, 2′-$\underline{CH_2}$Oppp), 3.68–3.49 (m, 8H, H3″, H5″, H8″, H10″), 3.21–3.06 (m, 4H, H3′, H5′, H1″), 2.83–2.62 (m, 2H, H12″), 2.52–2.33 (m, 2H, H3′, H5′), 2.04–1.81 (m, 7H, H2″, H11″, $CH_3$), 1.71–1.56 (m, 4H, H6″, H7″); $^{31}$P NMR (300 MHz, $D_2O$) $\delta$: −7.65 (d, 1P, $J$ = 20.1 Hz, $P_\gamma$), −10.6 (d, 1P, $J$ = 19.3 Hz, $P_\alpha$), −21.56 (t, 1P, $J$ = 19.3, $P_\beta$).

**General procedure for synthesis of compounds 8a–e**. Morpholino nucleoside triphosphates **7a-e** (7 μmol) were dissolved in 0.5 M carbonate-bicarbonate buffer pH 9.0 (0.30 mL). A pentafluorophenyl ester of TAMRA (0.012 g, 19 mmol) in DMF (0.60 mL) was added to the solution. The reaction mixture was stirred for 2 h, and then was pooled in 4% $LiClO_4$ in acetone (15 mL) and centrifuged. The precipitate was washed with acetone (2 × 15 mL) and dried in air. RPC was used for the purification of the target products (linear gradient of MeCN 0–30% in 0.03 M $LiClO_4$). The appropriate fractions were pooled and evaporated. The residue was dissolved in water. The target triphosphates were precipitated by tenfold volume of acetone, dissolved in water and precipitated with 4% $NaClO_4$/acetone.

***Conjugate 8a***: MS (MALDI-TOF) $m/z$: $[M\text{-}2H]^-$ calcd. for $C_{45}H_{57}N_9O_{17}P_3$ 1088.3091, found 1088.66.

***Conjugate 8b***: MS (MALDI-TOF) $m/z$: $[M]^+$ calcd. for $C_{44}H_{58}N_6O_{19}P_3$ 1067.2964, found 1067.54

***Conjugate 8c***: MS (MALDI-TOF) $m/z$: $[M\text{-}2H]^-$ calcd. for $C_{45}H_{57}N_9O_{18}P_3$ 1104.3040, found 1104.70.

**Conjugate 8d**. MS (MALDI-TOF) $m/z$: $[M\text{-}2H]^-$ calcd. for $C_{44}H_{57}N_7O_{18}P_3$ 1064.2978, found 1064.63.

**Conjugate 8e**. MS (MALDI-TOF) $m/z$: $[M\text{-}2H]^-$ calcd. for $C_{45}H_{58}N_6O_{19}P_3$ 1079.2975, found 1079.61.

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