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Synthesis of morpholine nucleoside triphosphates

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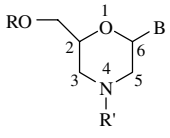
Abstract—Triphosphates of all four ribonucleoside derived morpholine subunits were synthesized and characterized by ^{1}H and ^{31}P NMR, UV and mass spectroscopy.

A wide variety of nucleoside and oligonucleotide analogues is currently used in molecular biology, biochemistry and other areas.[1] Morpholine derivatives (Fig. 1, **1**–**4**) obtained from natural ribonucleosides and oligomers based on these subunits attract special attention[2,3] due to the parent compounds being inexpensive, the sequence specificity of the oligomers, and little or no activity not bound with the antisense effect.[2,3]

Some morpholine nucleosides derived from natural ribonucleosides were obtained many years ago.[4] A number of oligonucleotide analogues based on morpholine subunits were patented.[5,6] Surprisingly, only a few papers have been devoted to the obvious application of the above-mentioned nucleoside derivatives as chain terminators in DNA sequencing.[7] One of the reasons may be the necessity to transform the monomers into the triphosphate form (Fig. 1, **5**–**8**). In spite of the extensive potential nucleoside analogue triphosphates offer,[8] morpholine nucleoside triphosphates are not commercially available. We now report the successful synthesis of all four nucleoside triphosphate monomers.

According to a recent review[9] there is no general and high-yielding route to nucleoside triphosphates. The most widely used 'one-pot, three step' protocol,[10] which involves the reaction of nucleosides with $POCl_3$ and then with pyrophosphate, is not successful for all nucleoside derivatives due to highly variable reaction times and imperfect selectivity in the phosphorylation step of the primary hydroxyl group.[9]

Following the published procedure[6] we synthesized morpholine nucleoside derivatives as base protected subunits with only the hydroxyl function free (Fig. 1, **9**–**12**). Thus we could use methods that do not assume a selectivity in the phosphorilation of the primary hydroxyl group in the triphosphate synthesis. First, we tried the elegant method[11] using 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one. This reagent is active



1	R = H	R' = H	B = Ura
3	R = H	R' = H	B = Cyt
5	R = ppp	R' = H	B = Ura
7	R = ppp	R' = H	B = Cyt
9	R = H	R' = Tr	B = Ura
11	R = H	R' = Tr	B = N^4-*Bz*-Cyt

2	R = H	R' = H	B = Ade
4	R = H	R' = H	B = Gua
6	R = ppp	R' = H	B =Ade
8	R = ppp	R' = H	B = Gua
10	R = H	R' = Tr	B = N^6-*Bz*-Ade
12	R = H	R' = Tr	B = N^2-*iBu*-Gua

ppp = Triphosphate
Tr = Triphenylmethyl

Figure 1. Ribonucleoside derived morpholine monomers.

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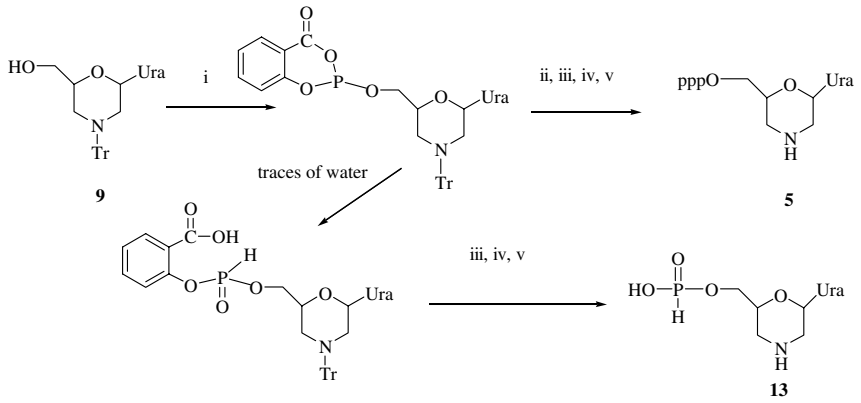


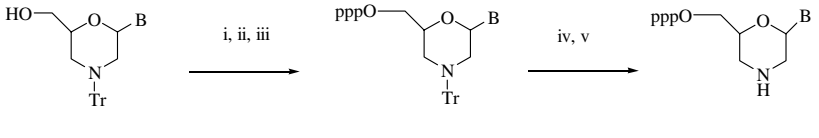
Figure 2. Synthesis of the morpholine uridine triphosphate under the Ludwig–Eckstein conditions: (i) 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one, (ii) $(n\text{-}Bu_4N^+)_2H_2P_2O_7$, (iii) $I_2/Py/H_2O$, (iv) H_2O, (v) $HOAc/H_2O$.

enough to phosphorylate the hydroxyl group in a short time and does not react with the carbonyl functions of uracil and guanine. We succeeded in obtaining the uracil derivative **5** starting from **9** under the Ludwig–Eckstein conditions[11] in 15% yield after deblocking and purification (Fig. 2). The main by-product **13** was identified as the 2-(*H*-phosphono)oxymethyl derivative of the morpholine nucleoside by ^{31}P NMR[12] and anion exchange HPLC (Milichrom-4, Russia). 2-(Phospho)oxymethyl-6-(uracil-1-yl)morpholine used as a reference substance for the HPLC was a generous gift of Dr. A. S. Levina.

The above by-product was a result of a fast hydrolysis of the phosphitalated intermediate prior to the addition of pyrophosphate and was not oxidized to the monophosphate.

As the Ludwig–Eckstein procedure seemed to be too sensitive to moisture, we tried to use $POCl_3$ in pyridine as the phosphorylating agent (Fig. 3). A possible problem with this method could be the formation of pyridinium derivatives of the uracil and guanine rings and their subsequent transformation into cytosine and diaminopurine derivatives, respectively, during ammonia treatment.[13] Fortunately, deblocking of the uracil derivative did not require an ammonia treatment.

Typically, one of the protected morpholine nucleosides **9**–**12** (0.1 mmol) was dissolved in anhydrous pyridine (1 mL) and the solution was chilled in an ice-bath. Phosphorus oxychloride (0.018 mL, 0.2 mmol) was then added to the solution. The reaction mixture was stirred for 15 min, then a 0.5 M solution of bis(tetra-*n*-butylammonium) pyrophosphate in acetonitrile (0.6 mL), prepared from aqueous tetra-*n*-butylammonium hydroxide and tetrasodium pyrophosphate, as in the synthesis of the bis(tri-*n*-butylammonium) salt,[11] was added and the cooling was removed. After 20 min, 1 mL of water was added, the reaction mixture was stirred 30 min and evaporated. The residue was dissolved in water (20 mL) and extracted with dichloromethane (10 mL). The aqueous layer was evaporated to dryness and the residue was dissolved in concentrated ammonia (10 mL) (in the case of **14** ammonia treatment was omitted), the solution was stirred at room temperature for 24 h in the cases of **15** and **16**, and for 48 h in the case of **17**. After the base deprotection was complete the solution was evaporated and the residue was dissolved in 10 mL of 80% aqueous acetic acid. After 30 min, the reaction mixture was evaporated several times with water, the residue was dissolved in 50 mL of 0.1% aqueous acetic acid and the solution was applied to a column (2 × 9 cm) packed with Polisil SA (Vector, Russia). Chromatography was performed with a linear gradient of 300 mL each of 0.1% acetic acid and 1.2 M NaCl in 0.1% acetic acid. Fractions containing the product were combined and diluted tenfold with water, then applied to a column (2.8 × 6 cm) packed with DEAE-Sephadex A-25 (Pharmacia, Sweden). Elution was performed with a linear gradient of 150 mL each of water and 1 M NH_4HCO_3. Appropriate fractions were pooled and evaporated. The residue was coevaporated several times with aqueous ethanol to remove traces of buffer. Compounds **5**–**8** were precipitated as disodium salts by the addition of a ten-



14 B = Ura **15** B = N^6-*Bz*-Ade
16 B = N^4-*Bz*-Cyt **17** B = N^2-*iBu*-Gua

Figure 3. Synthesis of the morpholine nucleoside triphosphates: (i) $POCl_3$, Py, (ii) $(n\text{-}Bu_4N^+)_2H_2P_2O_7$, (iii) H_2O, (iv) NH_2/H_2O (except when B = Ura), (v) $HOAc/H_2O$.

Table 1. NMR and UV spectral data

	^{31}P, δ (ppm) J (Hz)[a]	^{1}H, δ (ppm)[b] J (Hz)	$[M-H]^{-}$[c]	A_{250}/A_{260}[d]	A_{280}/A_{260}[d]
5	−7.6 (d, 1P, P_γ $J_{\beta\gamma}$ 18.2); −10.75 (dt, 1P, P_α, J_{PH} 9.4, $J_{\alpha\beta}$ 19.6); −22.2 (t, 1P, P_β)	7.96 (d, 1H, H6-Ura, J_{56} 8.0); 6.00 (m, 2H, H5-Ura, H6-Mor); 4.40 (m, 1H, H2-Mor); 4.19 (m, 2H, 2-CH_2OR-Mor); 3.37 (m, 2H, H3-Mor); 3.11 (m, 1H, H5-Mor); 3.00 (m, 1H, H5-Mor)	466.25	0.77	0.39
6	−5.7 (d, 1P, P_γ $J_{\beta\gamma}$ 19.7); −10.3 (dt, 1P, P_α, J_{PH} 8.1, $J_{\alpha\beta}$ 18.6); −21.0 (t, 1P, P_β)	8.31 (s, 1H, H8-Ade); 8.20 (s, 1H, H2-Ade); 5.98 (d, 1H, H6-Mor, J 8.1); 4.31 (m, 1H, H2-Mor); 4.04 (m, 2H, 2-CH_2OR-Mor); 3.44 (m, 1H, H5-Mor); 3.32 (m, 1H, H3-Mor); 3.21 (m, 1H, H3-Mor); 2.90 (m, 1H, H5-Mor)	489.22	0.78	0.16
7	−6.6 (d, 1P, P_γ, $J_{\beta\gamma}$ 21.1); −10.9 (dt, 1P, P_α, J_{PH} 7.5, $J_{\alpha\beta}$ 19.1); −22.2 (t, 1P, P_β)	7.86 (d, 1H, H6-Cyt, J_{56} 8.0); 6.08 (d, 1H, H6-Mor, J 7.8); 5.82 (d, 1H, H5-Cyt, J_{56} 8.0); 4.20–4.00 (m, 3H, H2-Mor, 2-CH_2OR-Mor); 3.21 (m, 2H, H3-Mor); 3.06 (m, 2H, H5-Mor)	465.33	0.84	0.85
8	−8.2 (d, 1P, P_γ, $J_{\beta\gamma}$ 19.4); −11.1 (dt, 1P, P_α, J_{PH} 9.7, $J_{\alpha\beta}$ 19.1); −22.6 (t, 1P, P_β)	8.10 (s, 1H, H8-Gua); 6.07 (d, 1H, H6-Mor, J 10.4); 4.55 (m, 1H, H2-Mor); 4.19 (m, 2H, 2-CH_2OR-Mor); 3.69 (m, 1H, H5-Mor); 3.55 (m, 2H, H3-Mor); 3.13 (m, 1H, H5-Mor)	505.13	1.10	0.71
pppUp-(dT)	−0.49 (s, 1P, $P_{diester}$); −9.9 (d, 1P, P_γ, $J_{\beta\gamma}$ 21.1); −11.1 (d, 1P, P_α, $J_{\alpha\beta}$ 19.1); −22.5 (t, 1P, P_β)	7.98(d, 1H, H6-U, J 8.4); 7.74 (s, 1H, H6-dT); 6.34 (t, 1H, H1′-dT, J 7.0); 6.03 (m, 2H, H1′-U, H5-U); 5.41 (m, 1H, H3′-U); 4.53 (m, 2H, H3′-dT, H2′-U); 4.30–4.10 (m, 6H, H5′-dT, H5′-U, H4′-dT, H4′-U); 2.39 (m, 2H, H2′-dT); 1.93 (s, 3H, CH_3-dT)	—	0.66	0.54
ppp(dU)-p(5BrdU)	−0.78 (s, 1P, $P_{diester}$); −10.0 (d, 1P, P_γ, $J_{\beta\gamma}$ 21.0); −11.1 (d, 1P, P_α, $J_{\alpha\beta}$ 19.4); −22.5 (t, 1P, P_β)	8.31(s, 1H, H6-(5BrdU)); 8.05 (d, 1H, H6-dU, J 8.0); 6.39 (m, 2H, H1′-(5BrdU), H1′-T); 6.06 (d, 1H, H5-dU, J_{56} 8.0); 5.04 (m, 1H, H3′-dU); 4.37–4.12 (m, 6H, H5′-dU, H5′-(5BrdU), H4′-dU, H4′-(5BrdU)); 2.72–2.32 (m, 4H, H2′-(5BrdU), H2′-dU)	—	0.75	0.76

[a] Spectra were recorded in D_2O, in P–H coupled and P–H decoupled mode in the cases of **5**–**8**, and in P–H decoupled mode in the cases of dinucleotides.

[b] Spectra were recorded in D_2O. Numbering see in Figure 1.

[c] Spectra were recorded on a Reflex III instrument (Brucker Daltonics, Germany) in negative detector mode with dihydroxybenzoic acid as matrix. The spectrometer was calibrated using a dNTPs calibration standard.

[d] Obtained in analytical HPLC. Column: Polisil SA (Vector, Russia), 2×60 mm, 0.01 mm particles. Solvent: 0–0.3 M KH_2PO_4 in 30% acetonitrile, pH 7.5.

fold volume of 2% $NaClO_4$ in acetone to the aqueous solutions of products. Yields were between 25% and 40%. NMR, UV and mass-spectral characteristics are summarized in Table 1. The main by-products were identified as 2-(phospho)oxymethyl derivatives according to ^{31}P and ^{1}H NMR, UV spectra and HPLC data (not shown). No heterocyclic base modification was detected.

Monophosphorylated deblocked morpholine nucleosides could be exploited as the starting materials to obtain the desired triphosphates by another approach, for example, as described by Bogachev.[14]

The approach presented in this paper allows the preparation of triphosphate morpholine nucleoside derivatives in acceptable yields starting from properly protected compounds and deblocking of intermediates with aqueous ammonia and acidic treatment if necessary.

This scheme could be applied, in principle to a wide range of protected derivatives of nucleosides and oligonucleotides in order to transform them into triphosphates. To illustrate this, we synthesized 5′-triphosphates pppUp(dT) and ppp(dU)p(5BrdU) (Table 1) starting from the properly protected dinucleosides with a free 5′-hydroxyl group using the above protocol. Yields were 10–15%. It should be mentioned that in these cases the formation of side products with altered UV spectra were detected and this was the main reason for diminished yields. Obviously, the procedure described has to be studied further for application to nucleosides.

Substrate properties of morpholine nucleoside triphosphates in DNA synthesis catalyzed with eukaryotic DNA polymerases β and λ were investigated. Morpholine nucleosides analogues demonstrated inhibition of DNA synthesis to different extents depending on the enzyme used. The results obtained will be published elsewhere.

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