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Functionalized Nucleoside 5′-triphosphates for In Vitro Selection of New Catalytic Ribonucleic Acids

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Abstract—A series of novel 2′-modified nucleoside 5′-triphosphates was synthesized. The amino, imidazole, and carboxylate functionalities were attached to the 5-position of pyrimidine base of these molecules through alkynyl and alkyl spacers, respectively. Two different phosphorylation methods were used to optimize the yields of these highly modified triphosphates.

Recently, much attention has been focused on the development of functionalized nucleotides suitable for in vitro selection with the hope of increasing the potential of nucleic acids for binding and catalysis.[1–6] For RNA in vitro selections modifications should be at the nucleotide level so that they can be incorporated simply and efficiently using RNA polymerase without problematic side reactions associated with synthetic posttranscriptional modification.

When designing monomeric nucleoside triphosphates for selection of therapeutic catalytic RNAs one has to take into account nuclease stability of such molecules in biological sera. A common approach to increase RNA stability is to replace the sugar 2′-OH group with other groups like 2′-fluoro, 2′-*O*-methyl or 2′-amino. Fortunately such 2′-modified pyrimidine 5′triphosphates are shown to be substrates for RNA polymerases.[5,7] On the other hand it was shown that variety of substituents at the 5-position of the pyrimidine is well tolerated by T7 RNA polymerase,[1] most likely because the natural hydrogen-bonding pattern of these nucleotides is preserved. We have chosen 2′-fluoro and 2′-*O*-methyl pyrimidine nucleosides as starting materials for attachment of different functionalities to the 5-position of the base. Both rigid (alkynyl) and flexible (alkyl) spacers were used. The choice of imidazole, amino and carboxylate pendant groups is based on their ability to act as general acids, general bases, nucleophiles and metal ligands, all of which can improve the catalytic effectiveness of selected nucleic acids.

2′-*O*-Methyluridine was 3′,5′-bis-acetylated using acetic anhydride in pyridine and then converted to its 5-iodo derivative **1a** using I_2/ceric ammonium nitrate reagent[8] (Scheme 1). Both reactions proceeded in a quantitative yield and no chromatographic purifications were needed. Coupling between **1a** and *N*-trifluoroacetyl propargylamine using copper(I) iodide and tetrakis (triphenylphosphine)-palladium(0) catalyst as described by Hobbs[9] yielded **2a** in 89% yield. Selective *O*-deacylation with aqueous NaOH afforded **3a**, which was phosphorylated with $POCl_3$/triethylphosphate (TEP) in the presence of 1,8-bis(dimethylamino)naphthalene (Proton-Sponge) (Method A).[10] The intermediate nucleoside phosphorodichloridate was condensed in situ with tri-*n*-butylammonium pyrophosphate. At the end the *N*-TFA group was removed with concentrated ammonia. 5′-Triphosphate was purified on Sephadex DEAE A-25 ion exchange column using a linear gradient of 0.1–0.8 M triethylammonium bicarbonate (TEAB) for elution. Traces of contaminating inorganic pyrophosphate are removed using C-18 RP HPLC to afford analytically pure material. Conversion into Na-salt was achieved by passing the aqueous solution of triphosphate through Dowex 50WX8 ion exchange resin in Na^+ form to afford **4a** in 45% yield (see Table 1). When Proton-Sponge was omitted in the first phosphorylation step, yields were reduced to 10–20%. Catalytic hydrogenation of **3a** yielded 5-aminopropyl derivative **5a**, which was phosphorylated under conditions identical to those described for propynyl derivative **3a** to afford triphosphate **6a** in 50% yield.

For the preparation of imidazole derivatized triphosphates **9a**,**b** and **11a**,**b** we developed an efficient synthesis of

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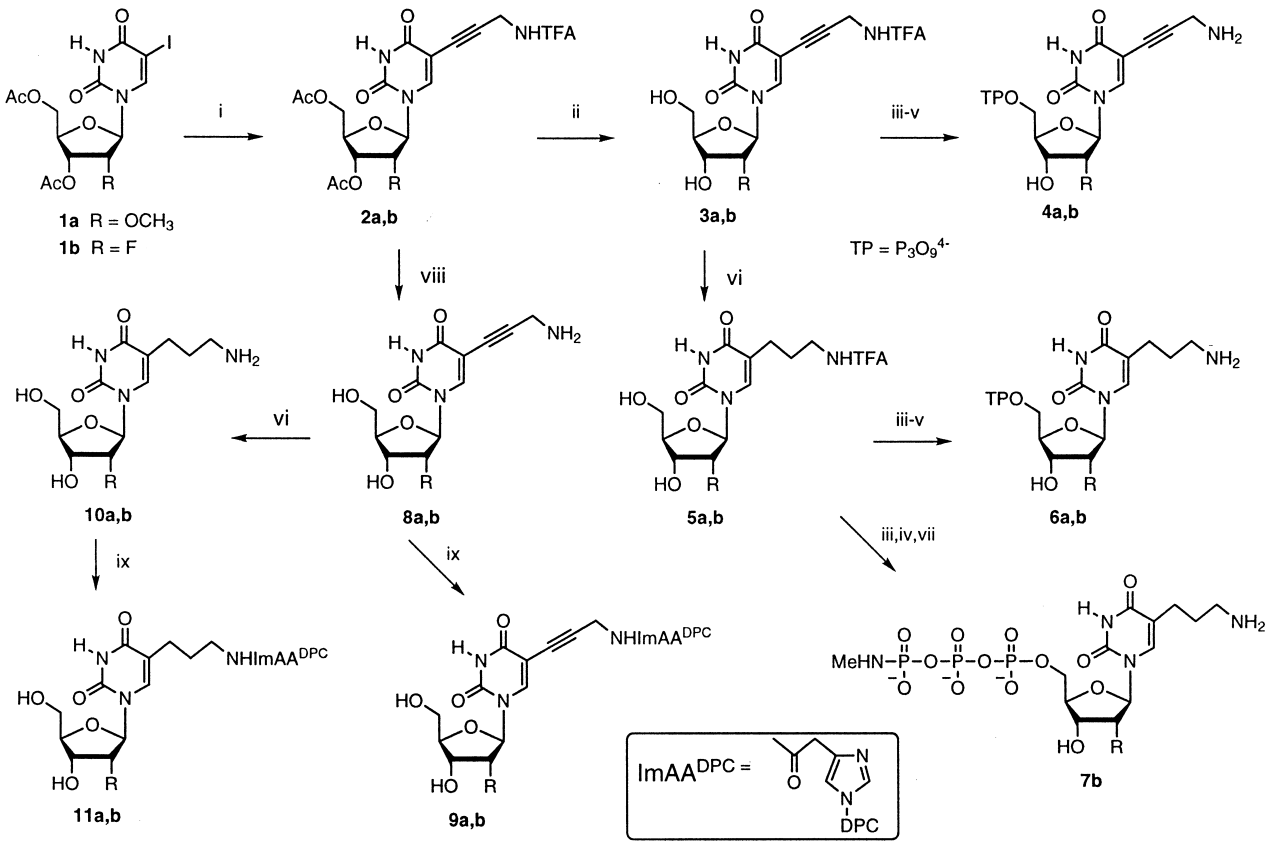
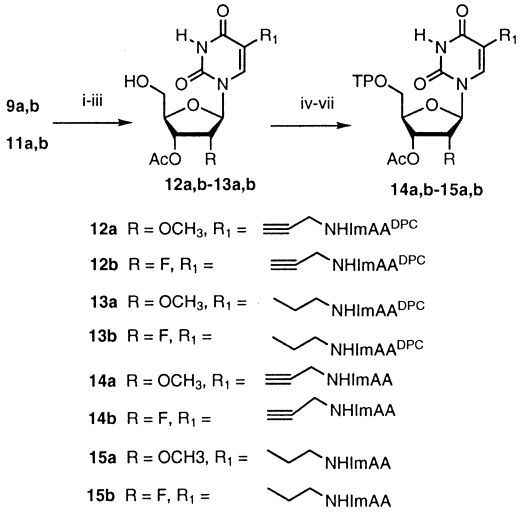


Scheme 1. Synthesis of 5-[3-aminopropynyl(propyl)]uridine 5′-triphosphates and 4-imidazoleacetic acid conjugates: Reagents and conditions: (i) *N*-TFA propargylamine, CuI, tetrakis(Ph_3P)Pd(0), Et_3N, DMF, 16 h; (ii) aq NaOH, pyr, MeOH, 0 °C, 1 h; (iii) $POCl_3$, Proton-Sponge, $(EtO)_3PO$, 2 h; (iv) *n*-Bu_3N PPi, MeCN, 15 min; (v) 1M $Et_3NH^+HCO_3^-$, then NH_4OH, 16 h; (vi) H_2, 5% Pd-C, 24 h, 40 psi; (vii) 40% $MeNH_2$, 3 h; (viii) NH_4OH, 4 °C, 16 h; (ix) $ImAA^{DPC}$, EDC·HCl, DMF,16 h.

N-diphenylcarbamoyl 4-imidazole-acetic acid ($ImAA^{DPC}$). Transient protection of the carboxyl group as a TMS-ester using TMS-Cl/pyridine followed by DPC-Cl allowed for a clean and quantitative conversion of 4-imidazoleacetic acid (ImAA) to its *N*-DPC protected derivative.

Complete deacylation of **2a** afforded 5-(3-aminopropynyl) derivative **8a**, which was condensed with 4-imidazoleacetic acid in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) to afford **9a** in 68% yield. Catalytic hydrogenation of **8a** yielded 5-(3-aminopropyl) derivative **10a** which was condensed with $ImAA^{DPC}$ to yield conjugate **11a** in 32% yield. Yields in these couplings were greatly improved when 5′-OH was protected with DMT group (not shown) thus efficiently preventing undesired 5′-*O*-esterification. Both **9a** and **11a** failed to yield triphosphate products in reaction with $POCl_3$/TEP/Proton-Sponge.

On the contrary, phosphorylation of 3′-*O*-acetylated derivatives **12a** and **13a** using 2-chloro-4*H*-1,3,2- benzodioxaphosphorin-4-one followed by pyrophosphate addition and oxidation (Method B,[11] Scheme 2) afforded the desired triphosphate **14a** and **15a** in 57% yield, respectively. 2′-Deoxy-2′-fluoro nucleoside 5′-triphosphates containing amino- (**4b** and **6b**) and imidazole- (**14b** and **15b**) linked groups were synthesized in a manner analogous to that described for the preparation of 2′-*O*-methyl nucleoside 5′-triphosphates (Schemes 1 and 2).



Scheme 2. Synthesis of 5-[3-(*N*-4-imidazoleacetyl)-aminopropynyl (propyl)]uridine 5′-triphosphates: Reagents and conditions: (i) DMT-Cl, pyr, 16 h; (ii) Ac_2O, pyr, 2 h; (iii) 3%TCA, CH_2Cl_2, 2 h; (iv) 2-Cl-4*H*-1,3,2-benzodioxaphosphorin-4-one, pyr, dioxane, 30 min; (v) *n*-Bu_3N PPi, DMF, 30 min; (vi) I_2, pyr-H_2O, 20 min; (vii) NH_4OH, 2 h.

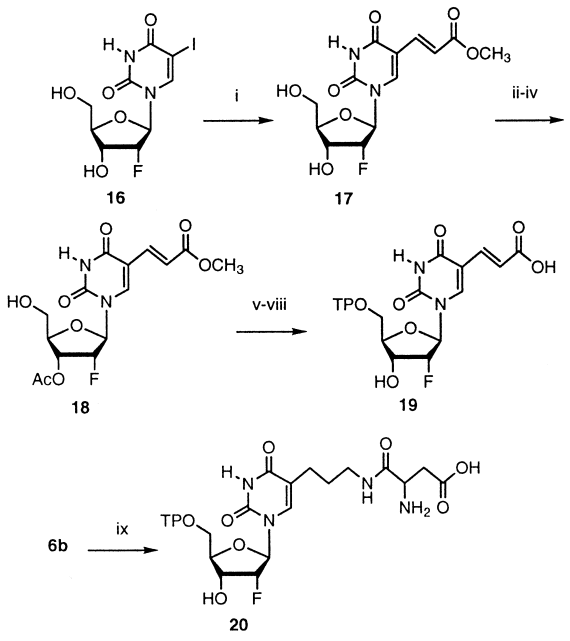
Again, only Ludwig–Eckstein's phosphorylation[11] worked for the preparation of 4-imidazoleacetyl derivatized triphosphates.

It is worth noting that when 'one-pot, two-steps' phosphorylation reaction (Method A)[10] of **5b** was quenched

with 40% aqueous methylamine instead of TEAB or H_2O, the γ-amidate **7b** was generated as the only detectable product. Similar reaction was reported recently for the preparation or γ-amidate of pppA2′p5′A2′p5′A.[12]

A carboxylate group was introduced at the 5-position of uridine both on the nucleoside level and post-synthetically (Method C, Scheme 3).

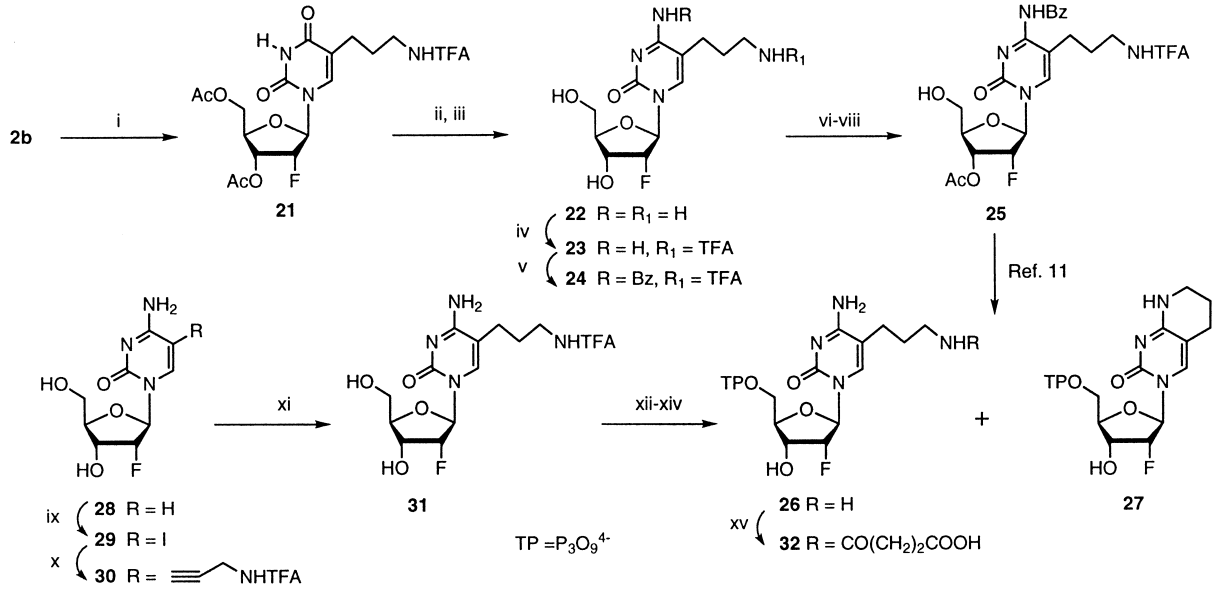
5-Iodo-2′-deoxy-2′-fluorouridine (**16**) was coupled with methyl acrylate using modified Heck reaction[13] to yield **17** in 85% yield. 5′-*O*-Dimethoxytritylation, followed by in situ 3′-*O*-acetylation and subsequent detritylation afforded 3′-protected derivative **18**. Phosphorylation using 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one followed by pyrophosphate addition and oxidation[11] afforded the desired triphosphate **19** in 54% yield.[14] On the other hand, 5-(3-aminopropyl)uridine 5′-triphosphate **6b** was coupled with *N*-hydroxysuccinimide ester of Fmoc-Asp-OFm to afford, after removal of Fmoc and Fm groups with diethylamine, the desired aminoacyl conjugate **20** in 50% yield.



Scheme 3. Synthesis of carboxylate tethered uridine. Reagents and conditions: (i) methyl acrylate, Ph_3P, Pd(II)acetate, Et_3N, dioxane, 30 min, reflux; (ii) DMT-Cl, pyr, 16 h; (iii) Ac_2O, pyr, 3 h; (iv) 3% TCA, CH_2Cl_2, 1 h; (v) 2-Cl-4*H*-1,3,2-benzodioxaphosphorin-4-one, pyr, dioxane, 30 min; (vi) *n*-Bu_3N PPi, DMF, 30 min; (vii) I_2, pyr-H_2O, 20 min; (viii) 1N NaOH, 5 h; (ix) Fmoc-Asp-OFm NHS-ester, DMF-0.1M $Na_2B_4O_7$, 16 h, then Et_2NH, 3 h.

Cytidine derivatives comprising 3-aminopropyl and 3(*N*-succinyl)aminopropyl groups were synthesized according to Scheme 4. Peracylated 5-(3-aminopropynyl)uracil derivative **2b** is reduced using catalytic hydrogenation and then converted in seven steps and 5% overall yield into 3′-acetylated cytidine derivative **25**. This synthesis was plagued by poor solubility of intermediates and formation of the N4-cyclized byproduct during ammonia treatment of the 4-triazolyl intermediate. Phosphorylation of **25** as described in ref. 11 yielded triphosphate **26** and N4-cyclized product **27** in 1:1 ratio. They were easily separated on Sephadex DEAE A-25 ion exchange colum using 0.1–0.8 M TEAB gradient. It appears that under basic conditions the free primary amine can displace any remaining intact 4-NHBz group leading to the cyclized product. This is similar to displacement of 4-triazolyl group by primary amine as mentioned above.

We reasoned that utilization of N^4-unprotected cytidine would solve this problem. This lead to an improved synthesis of **26**: Iodination of 2′-deoxy-2′-fluorocytidine (**28**) provided the 5-iodo derivative **29** in 58% yield.



Scheme 4. Synthesis of 5-(3-aminoalkyl) and 5-[3(*N*-succinyl)aminopropyl] functionalized cytidine. Reagents and conditions: (i) H_2, 5% Pd-C, MeOH, 24 h, 40 psi; (ii) $POCl_3$, 1,2,4-triazole, Et_3N, MeCN, 16 h; (iii) NH_4OH, dioxane, 16 h; (iv) CF_3COOEt, Et_3N, MeOH, reflux, 3 h; (v) Bz_2O, EtOH, reflux, 5 h; (vi) DMT-Cl, pyr, 16 h; (vii) Ac_2O, pyr, 3 h; (viii) 3% TCA, CH_2Cl_2, 3 h; (ix) HIO_3, I_2, AcOH, CCl_4, H_2O, 45 °C, 4 h; (x) *N*-TFA propargylamine, CuI, tetrakis(Ph_3P)Pd(0), Et_3N, DMF, 16 h; (xi) H_2, 5% Pd-C, MeOH, 72 h, 40 psi; (xii) $POCl_3$, Proton-Sponge, $(MeO)_3PO$, 2 h; (xiii) *n*-Bu_3N PPi, MeCN, 15 min; (xiv) NH_4OH, 4 °C, 16 h; (xv) succinic anhydride, DMF-0.1 M $Na_2B_4O_7$ 1:1, 16 h.

Table 1. 5-Functionalized nucleoside 5′-triphosphates

Compound	Method	Yield (%)	^{31}P NMR (δ, *j* [Hz])		
			Pα	Pβ	Pγ
4a	A	45	−10.50 (d, *J* = 20)	−11.48 (d, *J* = 20)	−22.75 (t, *J* = 20)[a]
4b	A	36	−10.50 (d, *J* = 20)	−12.97 (d, *J* = 20)	−22.38 (t, *J* = 20)[b]
6a	A	50	−10.35 (d, *J* = 20)	−11.66 (d, *J* = 20)	−22.83 (t, *J* = 20)[a]
6b	B	54	−7.02 (d, *J* = 20)	−11.24 (d, *J* = 20)	−21.84 (t, *J* = 20)[a]
7b	A	28	−0.48 (d, *J* = 20)	−11.55 (d, *J* = 20)	−22.56 (t, *J* = 20)[a]
14a	B	57	−10.26 (d, *J* = 20)	−11.59 (d, *J* = 20)	−22.82 (t, *J* = 20)[a]
14b	B	17	−7.12 (d, *J* = 20)	−11.47 (d, *J* = 22)	−22.13 (t, *J* = 20)[a]
15a	B	57	−10.35 (d, *J* = 20)	−11.66 (d, *J* = 20)	−22.83 (t, *J* = 20)[a]
15b	B	14	−6.51 (d, *J* = 21)	−11.40 (d, *J* = 21)	−21.90 (t, *J* = 20)[a]
19	B	54	−12.34 (d, *J* = 21)	−13.53 (d, *J* = 21)	−25.61 (t, *J* = 21)[b]
20	C[c]	25	−8.60 (d, *J* = 219)	−11.41 (d, *J* = 19)	−22.38 (t, *J* = 19)[a]
26	A, (B)	37, (22)	−11.56 (d, *J* = 20)	−13.59 (d, *J* = 20)	−24.12 (t, *J* = 20)[b]
27	B	22	−12.28 (d, *J* = 21)	−13.47 (d, *J* = 21)	−25.42 (t, *J* = 20)[b]
32	C[c]	36	−12.20 (d, *J* = 21)	−13.46 (d, *J* = 21)	−25.37 (t, *J* = 21)[b]

[a]Na^+ salt, recorded in D_2O.
[b]TEA^+, salt recorded in CD_3OD.
[c]Post-synthetic coupling.

This compound was then smoothly converted into 5-(3-aminopropynyl) derivative **30**. Hydrogenation afforded 5-(3-aminopropyl) derivative **31**, which was phosphorylated directly with $POCl_3$/PPi to afford **26** in 37% yield. Reaction of the 5′-triphosphate **26** with succinic anhydride yielded succinylated derivative **32** in 36% yield.

All new nucleoside triphosphates gave correct molecular peaks when analyzed by MALDI TOF mass spectrometry.[15]

In conclusion, 2′-modified uridine and cytidine triphosphates containing reactive functionalities tethered to the 5-position were synthesized. Their ability to serve as substrates for RNA polymerases and subsequent utilization in in vitro selection of new nuclease-resistant phosphodiesterases will be reported in due course.

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