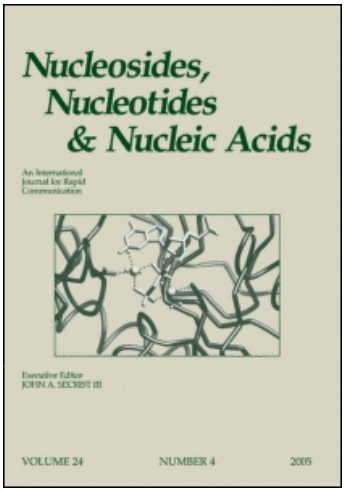


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# Synthesis of Pyrophosphates for In Vitro Selection of Catalytic RNA Molecules

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# SYNTHESIS OF PYROPHOSPHATES FOR IN VITRO SELECTION OF CATALYTIC RNA MOLECULES

**Hyo-Joong Kim, Myong Jung Kim, Nilesh Karalkar, Daniel Hutter, and Steven A. Benner**

*Foundation for Applied Molecular Evolution, Gainesville, Florida, USA*

□ *Reported here are synthetic routes to pyrophosphates linking riboflavin with various nucleosides. The focus is on a flavin-uracil dinucleotide having a biotin tag on the uracil, a molecule that has potential value in the selection of RNA enzymes that catalyze the template-directed polymerization of RNA in the 3′-to-5′ direction, which is the direction opposite that catalyzed by standard protein polymerases. Two detailed procedures are presented to prepare this new compound, as well as one procedure to prepare the new flavin-2,6-diaminopurine dinucleotide.*

**Keywords** Dinucleotides; flavin; origin of life; ribozymes



Following the discovery of RNA molecules that have catalytic activity as phosphotransesterases,[1,2] and the proposal that an episode of early life on Earth may have used RNA as its sole genetically encoded component,[3,4] many workers have considered the possibility of selecting for RNA molecules that catalyze the template-directed synthesis of RNA. Obtaining such catalysts would be a step toward realizing a "synthetic biology,"[5] and would also add support to an RNA world hypothesis for the origin of life.

RNA polymerases known in natural biology synthesize phosphodiester bonds via a process where the nucleophilic reactant (the 3′-OH group of the 3′-terminal ribonucleotide) is delivered by a primer, while the electrophilic reactant, a phosphorus atom having a pyrophosphate unit as the leaving group, is delivered by an incoming monomer. This means that the nucleophilic component in the reaction is tightly held to the template

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by Watson-Crick pairing that extends the length of the primer–template duplex, while the electrophilic component (the monomer) is held in the reacting complex by only (two or three) hydrogen bonds. Thus, in protein polymerases from the modern world, much of the binding energy holding the incoming monomer in the proper orientation must come from binding interactions between the triphosphate and the protein catalyst.

This places multiple demands on the structure of any RNA molecule that might catalyze the polymerization of RNA, demands that may be found only infrequently in a library of random RNA sequences. For example, it may be difficult to uncover an RNA molecule (a polyanion) that binds to a pyrophosphate leaving group (also a polyanion). Interactions between the incoming triphosphate and the RNA catalyst may be difficult to find that do not discriminate between the four standard nucleobases. While we cannot today calculate the size of a library that is needed to contain (with, for example, 50% probability) a single RNA molecule that solves all of these problems, estimates can be made based on the types of RNA catalysts that *have* emerged from random libraries,[6] and the size appears to be quite large, perhaps as large as $10^{30}$ random RNA sequences.



With these thoughts in mind, we considered an architecture that reverses the direction of RNA synthesis, from 5′-to-3′ to 3′-to-5′, and changes the leaving group (Figure 1). In this architecture, the leaving group is not pyrophosphate, but rather the phosphate ester of an alcohol. The electrophilicity of phosphorus is similar for leaving groups of the type O-$PO_2$-O-R and O-$PO_2$-O-$PO_3^=$ (the standard triphosphate); the free energy of hydrolysis of a pyrophosphate bond is about the same as the free energy of hydrolysis of a triphosphate. In this architecture, however, R can be chosen to be a better "handle" for the desired RNA catalyst to bind.

In choosing the R group "handle," we began by considering small molecules for which RNA molecules had already been developed to bind. Flavin was a particularly interesting ligand in this respect, as RNA molecules that bind flavin have been discovered by selection among random RNA libraries,[7] and have been optimized by deliberate structural change.[8]

This allows the starting point for catalyst selection to contain two flavin-binding RNA aptamers and a reversed direction of oligonucleotide synthesis. This has several hypothetical advantages. First, the architecture should allow *both* the nucleophilic and electrophilic components to be held by strong, multipoint noncovalent attachments (Figure 1). Thus, the incoming nucleophile on the monomer is held in the reaction complex by hydrogen bonding to the template (as in the standard architecture), and to a flavin. The electrophilic center, now provided by the primer, is held in the complex by template–primer interactions, as well as to the catalyst through the second flavin aptamer.

To implement this idea, a set of four flavin-pyrophosphate substrates are needed. These fall into the general structure of flavin adenine dinucleotide,

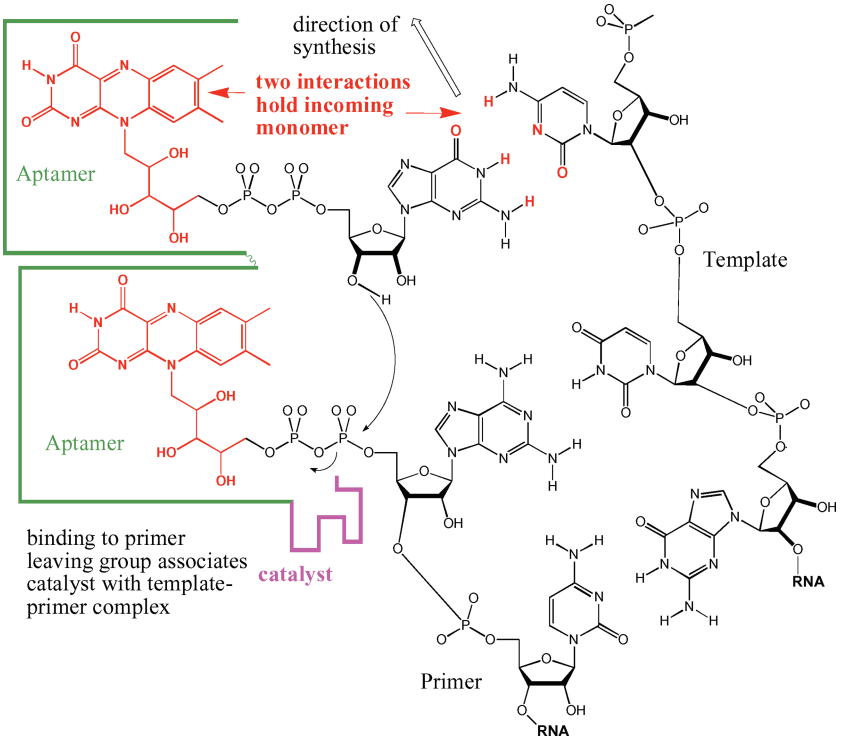
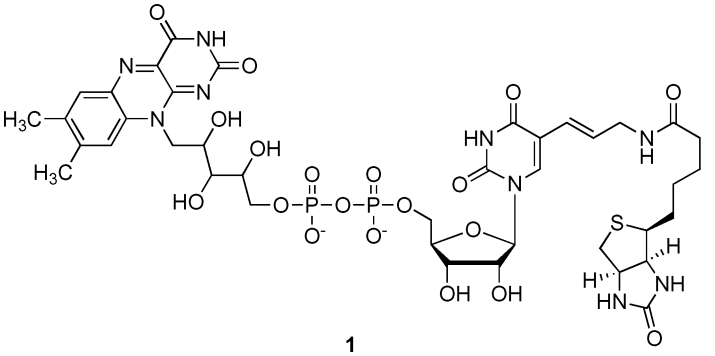



**FIGURE 1** Schematic diagram of a conceptual 3′-to-5′ reverse polymerization reaction that is the application of the pyrophosphates prepared here.

which is one of the substrates that would be used in this architecture. Pyrophosphates of this type have long been known, back to early work of Khorana,[9] who prepared flavin adenine dinucleotide by the coupling of flavin mononucleotide with adenosine-5′-monophosphate that had been activated as its corresponding morpholidate, with a low level of protection. In the Moffatt-Khorana procedure, the 2′- and 3′-hydroxyl groups of the



**1**

**FIGURE 2** The chemical structure of the pyrophosphate with a biotin tag.

adenosine, the ribitol hydroxyl groups of the riboflavin, and the exocyclic amine functionalities were all not protected. The coupling was performed in pyridine over 2 days.

For several of the pyrophosphates needed for this architecture, this approach was successful in our hands. In particular, the 2,6-diaminopurine-flavin, guanine-flavin,[10] and the cytosine-flavin[10] dinucleotides were all readily prepared from their morpholidates. The procedure for 2,6-diaminopurine-flavin dinucleotide, a new compound, is included in the experimental section.

Attempts to prepare biotinylated uracil-flavin dinucleotide (Figure 2) unexpectedly encountered problems, however. These focused on the difficulty of obtaining the precursor biotinylated 5-aminoallyluridine 5′-monophosphate, in part due to an irreproducible selectivity in the phosphorylation of biotinylated 5-aminoallyluridine using standard conditions (triethylphosphate as a solvent, $POCl_3$, and a trace amount of water).[11] These conditions are used for the preparation of nucleoside 5′-monophosphates. Further optimization of the coupling reaction, which remains slow, using the manganese dication is also reported.

Given the potential value of these and related compounds, we present detailed experimental procedures for the two new species. We focus on the most difficult pyrophosphate **1**, the species where the nucleobase carries a biotin tag.

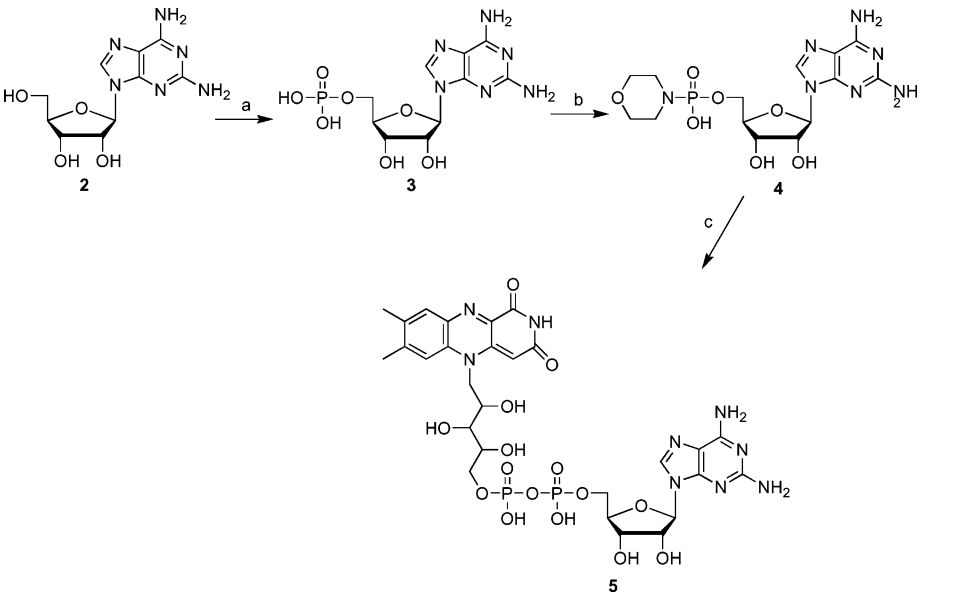


## RESULTS AND DISCUSSION

First, 2,6-diaminopurine-flavin dinucleotide was synthesized as shown in Scheme 1. 2-Aminoadenosine **2** was phosphorylated with phosphorus oxychloride in triethyl phosphate to give the monophosphate **3** in 40% yield. Compound **3** was refluxed with morpholine and dicyclohexylcarbodiimide in a mixture of $H_2O$ and *t*-butyl alcohol to afford the morpholidate **4** in 79% yield. The coupling of compound **4** with pyridinium riboflavin-5′-phosphate was performed via a slightly modified Moffatt-Khorana procedure to give flavin 2,6-diaminopurine dinucleotide **5**. The reaction was monitored by $^{31}P$ NMR. Compound **5** was purified by ion-exchange HPLC, followed by RP-HPLC, and identified by $^{1}H$ and $^{31}P$ NMR ($\delta$ −10.66 and −11.37).

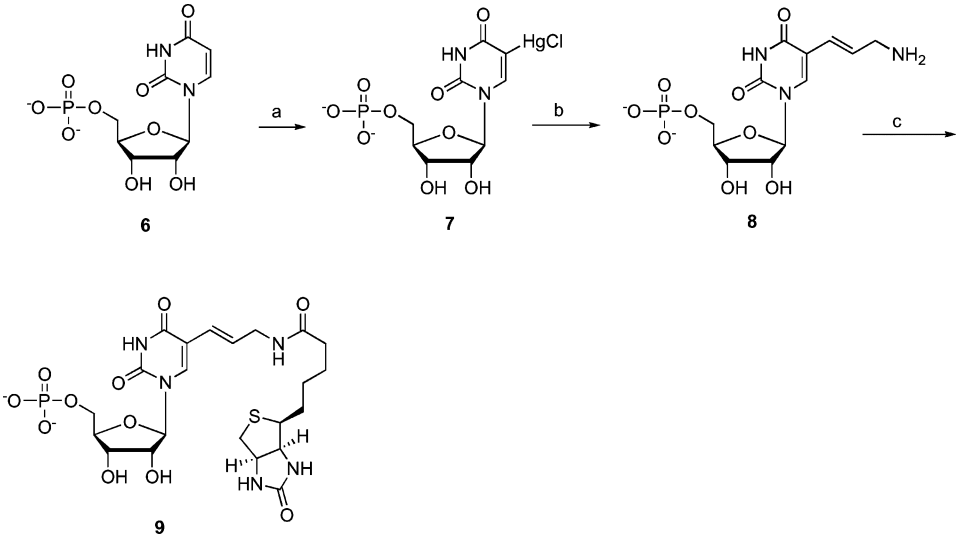
The first route to the biotinylated intermediate **9** was attempted from the commercially available uridine-5′-monophosphate (Scheme 2). This appeared to be a strategically wise choice, as the route was known in the literature,[12] and avoided the need to later phosphorylate an advanced synthetic intermediate having multiple hydroxyl groups.

However, in our hands, the reaction of the mercuric compound **7** with trifluoroacetyl allylamine (Scheme 2) gave only low yields of **8**, as

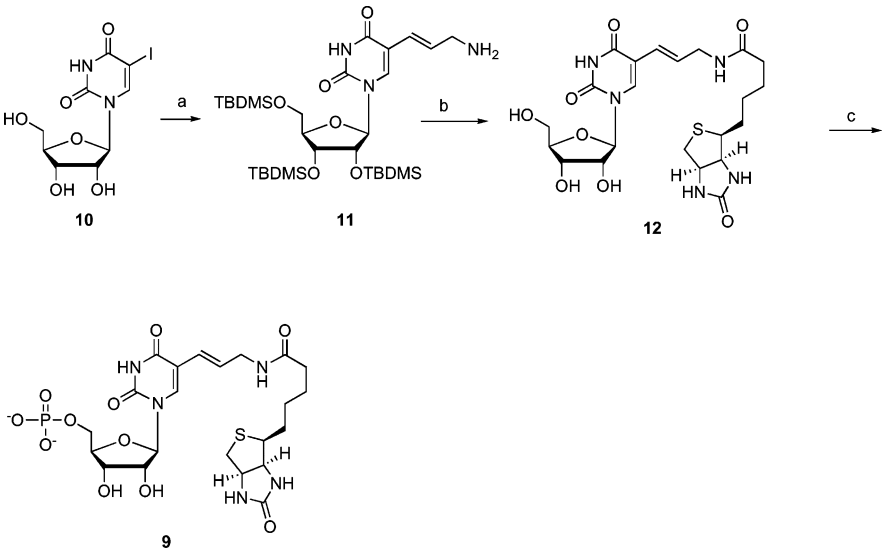


**SCHEME 1** Synthesis of 2,6-diaminopurine-flavin dinucleotide. Conditions: (a) $(EtO)_3PO$, $H_2O$, $POCl_3$, 0°C, 18 hours (40%); (b) morpholine, DCC, *t*-BuOH, $H_2O$, reflux, 6 hours (79%); (c) FMN-pyridinium salt, pyridine, $Bu_3N$, 1-tetrazole, RT, 2 days (11%).





**SCHEME 2** Attempted synthesis of the biotinylated monophosphate **9**. Conditions: (a) $Hg(OAc)_2$, LiCl, NaOAc, pH 6 buffer; (b) (1) trifluoroacetyl allylamine, $K_2PdCl_4$, NaOAc buffer, DMF, 80°C, 2 hours; (2) $NH_4OH$, RT; (c) biotin *N*-hydroxysuccinimide, pyridine, $Et_3N$.




**SCHEME 3** Synthesis of the monophosphate **9** with partial protection. Conditions: (a) (1) trifluoroacetyl allylamine, $Na_2PdCl_4$, NaOAc buffer, DMF, 80°C, 2 hours; (2) TBDMSCl, imidazole, DMF; (3) $NH_4OH$, MeOH, RT, 17 hours (40% for 3 steps); (b) (1) biotin *N*-hydroxysuccinimide, pyridine, $Et_3N$, RT, 2 hours; (2) $Et_3N$ 3HF, THF, RT (75% for two steps); (c) triethylphosphate, $POCl_3$, $H_2O$, 0°C, 18 hours, (45%).



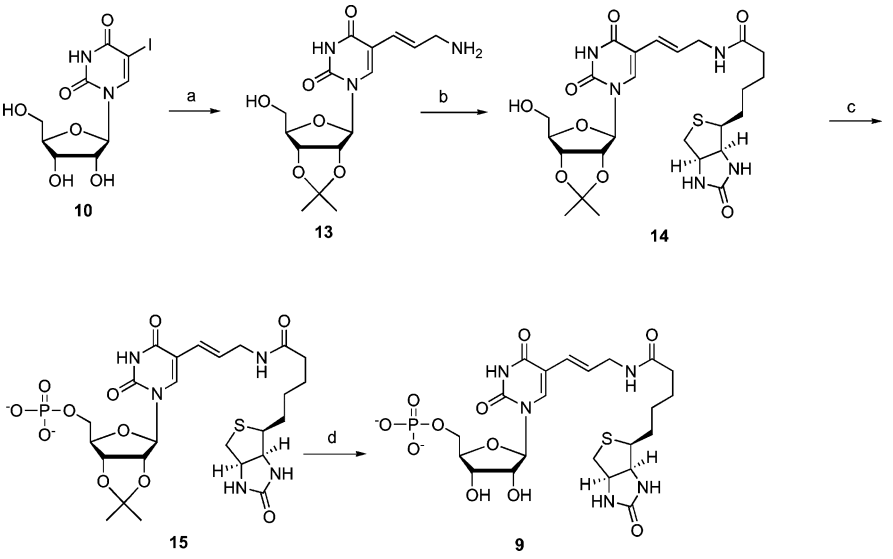
well as several impurities that were not separable by preparative column chromatography.

In contrast, the Heck reaction of iodouridine **10** with trifluoroacetyl allylamine in the presence of $Na_2PdCl_4$ gave the coupled product in good yield (Scheme 3).[13] The initial product, a trialcohol, was difficult to purify. Therefore, the catalyst was removed by purification using column chromatography, the crude product was converted to the *tris*-TBDMS ether, and the trifluoroacetyl group was removed to give the allylamine compound **11** in 40% overall yield.

The amino group of the allylamine compound **11** was then reacted with the activated biotin derivative biotin *N*-hydroxysuccinimide. The silyl groups were then removed with $Et_3N{\cdot}3HF$ to give the biotinylated uridine **12** in 75% overall yield. Treating with three equivalents of $POCl_3$ in triethylphosphate in the presence of a small amount of $H_2O$ gave regioselectively the desired monophosphate **9**, but only in 45% yield, and only in the best experiment.

The inconsistent regioselective phosphorylation required us to develop a more reliable method for the phosphorylations. First, the 2′- and 3′-hydroxyl groups were protected following the procedure in Scheme 4. It was expected that protection of the 2′, 3′-diol would give a substrate that was more consistently phosphorylated by increasing the solubility of the reactant and removing the possibility of unwanted 2′- or 3′-phosphorylation.

After Heck reaction of iodouridine **10** with trifluoroacetyl allylamine, the product was purified by column chromatography and treated with



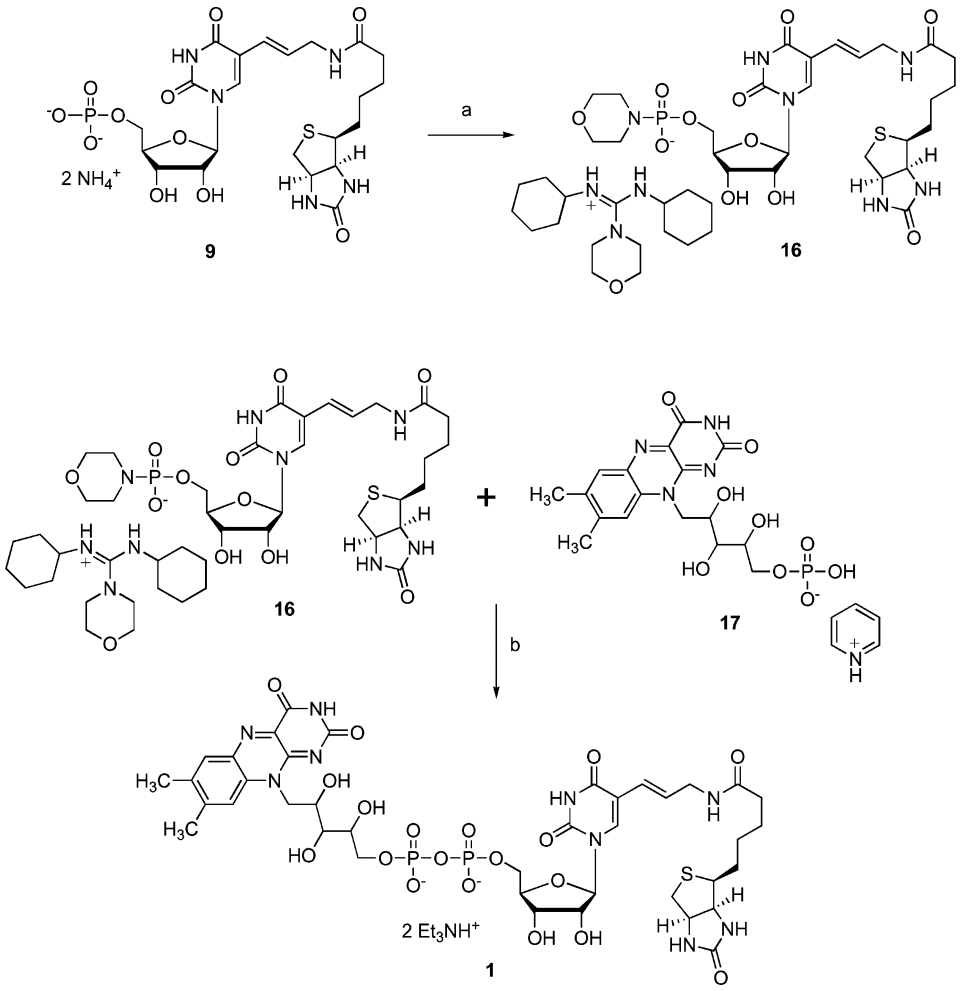

**SCHEME 4** Synthesis of the monophosphate **9** with full protection. Conditions: (a) (1) trifluoroacetyl allylamine, $Na_2PdCl_4$, NaOAc buffer, DMF, 80°C, 2 hours; (2) 2,2-dimethoxypropane, *p*-TsOH, acetone, RT, 1 hour; (3) $MeNH_2$, RT, 3 hours, (67% for 3 steps); (b) biotin *N*-hydroxysuccinimide, pyridine, $Et_3N$, RT, 2 hours (72%); (c) triethylphosphate, $POCl_3$, 0°C, 18 hours, (56%); (d) HCl, $H_2O$, RT, 18 hours (64%).



2,2-dimethoxypropane in acetone in the presence of *p*-TsOH, followed by treatment with 40% methylamine solution. This gave the 2′,3′-protected allylamine derivative **13** in 67% yield (Scheme 4). Then, **13** was biotinylated to give **14** and converted to the monophosphate **15** by treatment of $POCl_3$ in triethylphosphate in 56% yield. Finally, deprotection of isopropylidene of **15** by HCl in water gave the key intermediate **9**.

With the monophosphate **9** in hand, pyrophosphate **1** was accessible using an adaptation of a procedure of Lee *et al.* (Scheme 5).[14] First, the monophosphate group in **9** was converted to its morpholidate with dicyclohexylcarbodiimide to give **16** in 82% yield. The morpholidate **16** was then treated with flavin mononucleotide **17** as its trioctylamine salt in pyridine and acetonitrile in the presence of tetrazole in the dark. The progress of the reaction was followed by analytical ion-exchange HPLC; a photodiode array detector allowed for easy identification of the different compounds according to their UV spectra; the desired pyrophosphate shows, in addition to a flavin absorbance, a shoulder at 300 nm, arising from the uridine heterocycle conjugated with the double bond of the linker carrying biotin). After 6 days at room temperature, all of the morpholidate had been consumed. The desired pyrophosphate was recovered from the reaction mixture by ion-exchange and reverse-phase HPLC to give a yellow solid **1** in 5% yield.

By changing the acid catalyst from tetrazole to $MnCl_2$[15] we were able to shorten the reaction time to less than one day and double the yield,




**SCHEME 5** Synthesis of the pyrophosphate **1**. Conditions: (a) DCC, morpholine, *t*-BuOH, $H_2O$, reflux, 4 hours (52%); (b) trioctylamine, pyridine, tetrazole, RT, 6 days (5%) or $MnCl_2$, $Na_2SO_4$, formamide, RT, 18 hours (10%).

which also made the HPLC purification easier, as more of the nearby FMN peak had been transformed into product. We are now conducting in vitro selection of catalytic RNA; these results will be reported in due course.

## EXPERIMENTAL

### 2-Aminoadenosine-5-monophosphate (3)

To a cooled mixture of 2-aminoadenosine (500 mg, 1.77 mmol) in $(EtO)_3PO$ (4.5 mL) was added a mixture of $(EtO)_3PO$ (4.5 mL), water (6 $\mu$L), and $POCl_3$ (0.5 mL, 5.36 mmol) at 0°C; reaction mixture was kept at 0°C for 18 hours. The reaction mixture was poured into 1 M TEAB (2

mL) in water (20 mL) and extracted with EtOAc (2 × 10 mL). The aqueous layer was rotary evaporated under reduced pressure and the residue was purified by flash chromatography (silica, *i*-PrOH:$NH_4OH$:$H_2O$ = 6:3:2) to give **3** (ammonium salt, 270 mg, 0.71 mmol, 40%) as a solid.

### 2-Aminoadenosine 5′-Phosphomorpholidate (4)

To a refluxed solution of **3** (270 mg, 0.71 mmol) and morpholine (0.24 mL, 2.75 mmol) in *t*-BuOH (7 mL) and $H_2O$ (7 mL) was added a solution of DCC (585 mg, 2.83 mmol) in *t*-BuOH (10 mL). The reaction mixture was refluxed for 6 hours, cooled to RT and filtered (washed with *t*-BuOH). The filtrate was rotary evaporated under reduced pressure, dissolved in $H_2O$ (20 mL) and washed with ether (10 mL × 3). The aqueous layer was rotary evaporated under reduced pressure. The residue was dissolved in MeOH (10 mL) and ether was added. The precipitate was filtered and washed with ether to give **4** (410 mg, 0.57 mmol, 79%) as a solid. $^1$H NMR (300 MHz, $D_2O$) $\delta$ 7.95 (s, 1H), 5.78 (d, 1H, $J$ = 4.8 Hz), 4.61 (t, 1H, $J$ = 5.3 Hz), 4.36 (t, 1H, $J$ = 4.7 Hz), 4.16 (m, 1H), 3.85–3.88 (m, 2H), 2.77–3.66 (m, 18H), 0.95–1.76 (m, 20H); $^{31}$P NMR ($D_2O$) $\delta$ 7.45.



### Flavin-2-aminoadenosine dinucleotide (5)

Flavin mononucleotide (FMN) (220 mg, 0.41 mmol) and compound **4** (300 mg, 0.41 mmol) were dried at 100°C under high vacuum for 2 h. A mixture of FMN and $Bu_3N$ (0.1 mL, 0.42 mmol) in pyridine (5 mL) was concentrated. The mixture was coevaporated with pyridine (3 mL × 2). Compound **4** was added to this mixture and coevaporated with pyridine (3 mL × 2). To this mixture were added pyridine (6 mL) and 1-tetrazole (0.45 M in $CH_3CN$, 2.73 mL) at RT. After being stirred at RT for 2 days, the reaction mixture was rotary evaporated under reduced pressure and quenched with sat. $NH_4HCO_3$ solution. The mixture was washed with ether (10 mL), concentrated and purified by ion-exchange HPLC with a linear gradient of ammonium bicarbonate (10–250 mM, followed by reverse-phase HPLC with a linear gradient of acetonitrile, 0–60% in 25 mM TEAA. After lyophilization, compound **5** (45 mg, 11%) was obtained as its triethylammonium salt. $^1$H NMR (300 MHz, $D_2O$) $\delta$ 7.86 (s, 1H), 7.58 (s, 1H), 7.46 (s, 1H), 5.52 (d, 1H, $J$ = 5.1 Hz), 4.82 (m, 1H), 3.76–4.61 (m, 11H), 3.02 (q, $Et_3N$), 2.30 (s, 3H), 2.25 (s, 3H), 1.11 (t, $Et_3N$); $^{31}$P NMR ($D_2O$) $\delta$ −10.66 (d), −11.37 (d). The phosphorus–phosphorus coupling constant is 22.0 Hz. Mass spectrometry (HPLC/ESI-MS) observed a single charged $[M\text{-}H]^-$ at m/e 799.2.

### 5-Aminoallyl-2′,3′,5′-tri-*O*-*tert*-butyldimethylsilyl-uridine (11)

A mixture of 5-iodouridine **10** (2 g, 5.4 mmol), $Na_2PdCl_4$ (1.43 g, 4.9 mmol) and trifluoroacetyl allylamine (6 mL) in DMF (20 mL) and NaOAc buffer (10 mL, 0.1 N, pH 5.2) was heated to 80°C for 2 hours. The mixture was cooled to RT and rotary evaporated under reduced pressure. The product was purified from the residue by flash chromatography (silica, gradient $CH_2Cl_2$:MeOH = 7:1 to 4:1). The appropriate fraction (by tlc) was collected, evaporated and dissolved in DMF (40 mL). To this solution were added imidazole (2.94 g, 43.2 mmol) and TBDMSCl (2.93 g, 19.4 mmol) at RT. After being stirred overnight at RT, the reaction mixture was poured into water (200 mL) and extracted with ether (200 mL × 2). The combined organic layers were dried over $MgSO_4$, filtered and concentrated by rotary evaporation. The residue was purified by flash chromatography (silica, hexane:EtOAc = 4:1). The appropriate fraction was collected and evaporated and treated with 28% aq. $NH_4OH$ (20 mL) in MeOH (40 mL). The mixture was stirred at RT for 17 hours and evaporated. The residue was purified by flash chromatography (silica, $CH_2Cl_2$:MeOH = 6:1) to give **11** as a light yellow solid (1.38 g, 40% for 3 steps). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 7.69 (s, 1H), 6.65 (m, 1H), 6.25 (d, 1H, $J$ = 15.9 Hz), 5.84 (d, 1H, $J$ = 6.9 Hz), 4.31 (dd, 1H, $J$ = 4.5, 6.6 Hz), 4.06 (dd, 1H, $J$ = 1.5, 4.5 Hz), 3.92 (m, 1H), 3.83 (dd, 1H, $J$ = 4.8, 11.4 Hz), 3.70 (1H, $J$ = 3.6, 11.4 Hz), 3.35–3.48 (m, 4H), 0.91, 0.89, 0.81 (3s, 27H), 0.11, 0.10, 0.09, 0.00, −0.09 (5s, 18H).



### 5-Biotinylaminoallyl-uridine (12)

A mixture of compound **11** (467 mg, 0.73 mmol), biotin-*N*-hydroxysuccinimide ester (298 mg, 0.87 mmol) and $Et_3N$ (0.2 mL, 1.44 mmol) in pyridine (15 mL) was stirred at RT for 2 hours and concentrated. The residue was diluted with $CH_2Cl_2$ (200 mL) and washed with 0.1 N HCl (20 mL). The organic layer was washed with sat. $NaHCO_3$ solution, dried over $Na_2SO_4$, filtered, and concentrated. The residue was purified by flash chromatography (silica, $CH_2Cl_2$:MeOH = 8:1) to give the protected intermediate as a white solid (545 mg, 86%). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 11.56 (s, 1H), 7.97 (t, 1H, $J$ =5.7 Hz), 7.57 (s, 1H), 6.40 (m, 3H), 6.07 (d, 1H, $J$ = 16.2 Hz), 5.86 (d, 1H, $J$ = 7.2 Hz), 4.26 (m, 2H), 4.09 (m, 1H), 4.04 (m, 1H), 3.93 (m, 1H), 3.70-3.87 (m, 4H), 3.05 (m, 1H), 2.71 (m, 1H), 2.54 (d, 1H, $J$ = 12.3 Hz), 0.05 (t, 1H, $J$ = 7.5 Hz), 1.29–1.69 (m, 6H), 0.90, 0.88, 0.80 (3s, 27H), 0.1, 0.09, 0.07, −0.01, −0.11 (5s, 18H).

A mixture of biotinylated compound (545 mg, 0.63 mmol) and triethylamine trihydrofluoride (Aldrich, 1.02 mL, 6.26 mmol) in THF (10 mL) was stirred at 50°C for 16 hours and concentrated. The residue was recrystallized from MeOH to give **12** as a white solid (286 mg, 87%). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 11.44 (s, 1H), 8.08 (s, 1H), 7.96 (t, 1H, J = 5.6 Hz), 6.36-6.45

(m, 3H), 6.10 (d, 1H, $J$ = 15.9 Hz), 5.77 (d, 1H, $J$ = 4.8 Hz), 5.40 (br s, 1H), 5.21 (br s, 1H), 5.08 (br s, 1H), 4.28 (m, 1H), 4.10 (m, 1H), 4.05 (m, 1H), 3.97 (m, 1H), 3.84 (m, 1H), 3.55–3.76 (m, 4H), 3.07 (m, 1H), 2.79 (dd, 1H, $J$ = 5.1, 12.6 Hz), 2.55 (d, 1H, $J$ = 11.7 Hz), 2.07 (t, 2H, $J$ = 15.0 Hz), 1.30–1.63 (m, 6H).

### 5-Biotinylaminoallyl-uridine 5′-monophosphate (9)

To a cooled mixture of compound **12** (280 mg, 0.53 mmol) in $(EtO)_3PO$ (2.5 mL) was added a mixture of $(EtO)_3PO$ (2 mL), water (5 $\mu$L), and $POCl_3$ (0.15 mL, 1.61 mmol) at 0°C and reaction mixture was kept at 0°C for 18 hours. The reaction mixture was neutralized with NaOH (1 N) and extracted with EtOAc (2 × 10 mL). The aqueous layer was concentrated in vacuo and the residue was purified by flash chromatography (silica, *i*-PrOH:$NH_4OH$;$H_2O$ = 6:2:2) to give **9** as a white solid (ammonium salt, 150 mg, 45 %). $^1$H NMR (300 MHz, $D_2O$) $\delta$ 7.83 (s, 1H), 6.29 (m, 1H), 6.17 (d, 1H, $J$ = 16.2 Hz), 5.84 (d, 1H, $J$ = 5.1 Hz), 4.42 (m, 1H), 4.14–4.28 (m, 4H), 3.98 (m, 2H), 3.76 (m, 2H), 3.14 (m, 1H), 2.80 (dd, 1H, $J$ = 5.1, 13.2 Hz), 2.59 (d, 1H, $J$ = 13.2 Hz), 2.16 (t, 1H, $J$ = 7.1 Hz), 1.24–1.58 (m, 6H); $^{31}$P NMR ($D_2O$) $\delta$ 0.12. Mass spectrometry (HPLC/ESI-MS) observed a single charged $[M\text{-}H]^-$ at m/e 604.3.

### 5-Aminoallyl-2′,3′-*O*-isopropylidene-uridine (13)

A mixture of 5-iodouridine **10** (2.10 g, 5.7 mmol), $Na_2PdCl_4$ (1.43 g, 4.9 mmol) and trifluoroacetyl allylamine (6 mL) in DMF (20 mL) and NaOAc buffer (10 mL, 0.1 N, pH 5.2) was heated to 80°C for 2 hours. The mixture was cooled to RT and evaporated and purified by flash chromatography (silica, gradient $CH_2Cl_2$:MeOH = 7:1 to 4:1). The appropriate fraction was collected and evaporated and treated with acetone (100 mL), 2,2-dimethoxypropane (5 mL, 40 mmol) and *p*-TsOH·$H_2O$ (0.80 g, 4.2 mmol). The mixture was stirred at rt for 1 hour and basified by the addition of $NH_4OH$ (2 mL), evaporated, and resolved by flash chromatography (silica, gradient ethyl acetate:hexane = 1:1 to 2:1 to 3:1). The appropriate fraction was collected and evaporated and treated with methylamine (10 mL, 40% in water). The mixture was stirred at RT for 3 hours and evaporated. The residue was purified by flash chromatography (silica, gradient $CH_2Cl_2$:MeOH = 10:1 to 6:1 to 3:1) to give **13** as a light yellow solid (1.3 g, 67% for 3 steps). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 11.5 (br s, 1H), 8.05 (s, 1H), 8.0 (br s, 2H), 6.57 (m, 1H), 6.33 (d, 1H, $J$ = 16 Hz), 5.85 (d, 1H, $J$ = 3 Hz), 5.25 (t, 1H, $J$ = 5 Hz), 4.91 (dd, 1H, $J$ = 6, 3 Hz), 4.76 (dd, 1H, $J$ = 6, 3 Hz), 4.11 (dd, 1H, $J$ = 7.5, 3.6 Hz), 3.58 (m, 2H), 3.50 (d, 1.5H, $J$ = 6 Hz), 3.17 (d, 0.5H, $J$ = 2.7 Hz), 1.47 (s, 3H), 1.27 (s, 3H).

### 5-Biotinylaminoallyl-2′,3′-*O*-isopropylidene-uridine (14)

To a mixture of the amine **13** (555 mg, 1.64 mmol) and biotin *N*-hydroxysuccinimide (586 mg, 1.71 mmol) in pyridine (10 mL) was added triethylamine (0.46 mL, 3.28 mmol) and the mixture was stirred at RT for 2 hours. The volatiles were removed by evaporation, and the residue was resolved by flash chromatography (silica, gradient $CH_2Cl_2$:MeOH = 8:1 to 6:1 to 4:1 to 3:1) to give **14** as a white solid (670 mg, 72%). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 11.48 (s, 1H), 7.99 (t, 1H, $J$ = 5.4 Hz), 7.94 (s, 1H), 6.3∼6.4 (m, 3H), 6.10 (d, 1H, $J$ = 15.9 Hz), 5.85 (d, 1H, $J$ = 2.7 Hz), 5.18 (t, 1H, $J$ = 5.1 Hz), 4.92 (dd, 1H, $J$ = 6.3, 2.7 Hz), 4.76 (dd, 1H, $J$ = 6.3, 3.3 Hz), 4.28 (m, 1H), 4.09 (m, 1H), 3.73 (m, 1H), 3.59 (m, 1H), 3.0∼3.1 (m, 3H), 2.5∼2.9 (m, 3H), 2.0∼2.1 (m, 2H), 1.4∼1.6 (m, 5H), 1.2∼1.4 (m, 5H), 1.17 (t, 2H, $J$ = 7.5 Hz).

### 5-Biotinylaminoallyl-2′,3′-*O*-isopropylidene-uridine 5′-monophosphate (15)



To a suspension of compound **14** (565 mg, 1.00 mmol) in triethylphosphate (10 mL) was added $POCl_3$ (270 $\mu$L, 3.00 mmol) at 0°C. The mixture was stirred at 0°C for 18 hours, diluted with water (20 mL), neutralized by 1 N NaOH solution, and extracted with ethyl acetate (2 × 20 mL). The aqueous phase was evaporated and the residue was purified by flash chromatography (silica, gradient *i*PrOH:$NH_4OH$:$H_2O$ = 6:1:1 to 5:1:1 to 4:1:1) to give **15** as a light tan solid (380 mg, 56%). $^1$H NMR (300 MHz, $D_2O$) $\delta$ 7.73 (s, 1H), 6.3 (m, 1H), 6.17 (d, 1H, $J$ = 16.2 Hz), 5.79 (br s, 1H), 4.8∼5.0 (m, 2H), 4.46 (m, 2H), 4.27 (m, 1H), 3.99 (m, 3H), 3.79 (m, 1H), 3.19 (br s, 1H), 2.6∼2.9 (m, 2H), 2.19 (m, 2H), 1.2∼1.7 (12H); $^{31}$P NMR ($D_2O$) $\delta$ –0.049 (s).

### 5-Biotinylaminoallyl-uridine 5′-monophosphate (9)

A solution of compound **15** (380 mg, 0.56 mmol) in $H_2O$ (60 mL) and conc. HCl (0.3 mL) was stirred at RT overnight. The mixture was neutralized aqueous NaOH (1 N) and evaporated. The residue was purified by flash chromatography (silica, gradient *i*PrOH:$NH_4OH$:$H_2O$ = 6:1:1 to 5:2:2) to give **9** as a light tan solid (230 mg, 64%). The analytical data are shown for compound **9** above.

### 5-Biotinylaminoallyl-uridine 5′-phosphomorpholidate (16)

A mixture of compound **9** (230 mg, 0.36 mmol) and morpholine (0.13 mL, 1.4 mmol) in *t*-BuOH (5 mL) and $H_2O$ (5 mL) was heated to reflux. To this mixture was added a solution of DCC (309 mg, 1.50 mmol) in *t*-BuOH (6 mL). The resulting mixture was refluxed for 4 hours. After

cooling to RT, the mixture was filtered through Celite and washed with *t*-BuOH. The combined filtrate was evaporated and dissolved in $H_2O$ (20 mL) and extracted with ethyl ether (2 × 20 mL). The aqueous phase was evaporated and the residue was dispersed in methanol (3 mL) and treated with ethyl ether (30 mL). The ether layer was decanted away and the resulting solid was dried under high vacuum to give **16** as a light tan solid (180 mg, 52%). $^1$H NMR (300 MHz, $D_2O$) $\delta$ 7.72 (s, 1H), 6.23 (m, 2H), 5.83 (d, 1H, $J$ = 5.1 Hz), 4.42 (m, 1H), 4.12–4.46 (m, 4H), 3.93 (m, 2H), 3.76 (m, 2H), 2.79–3.67 (m, 18H), 2.59 (d, 1H, $J$ = 12.6 Hz), 2.15 (m, 1H), 1.03–1.78 (m, 26H); $^{31}$P NMR ($D_2O$) $\delta$ 7.31.

### Flavin-5-biotinylaminoallyl-uridine dinucleotide (1)

***Method 1***

FMN as its pyridinium salt (45 mg, 0.084 mmol) in pyridine (2 mL) was treated with trioctylamine (0.037 mL, 0.084 mmol) and stirred for 10 minutes and evaporated. The residue was dissolved in pyridine (2 mL) and evaporated to dryness. To this solid in flask (10 mL) was added compound **16** (90 mg, 0.093 mmol) and treated with pyridine (2 mL) and 0.45 M tetrazole in MeCN (0.67 mL). The resulting mixture was stirred at RT and periodically analyzed by analytical ion-exchange HPLC (Dionex DNAPac PA-100 4 × 250-mm column, eluent A = 25 mM TEAA, pH 7, eluent B = 100 mM NaCl in 25 mM TEAA, pH 7, gradient from 0 to 100% B in 30 minutes; morpholidate **16**: $R_t$ = 3 minutes; FMN: $R_t$ = 12 minutes, plus small peaks at 13 minutes; pyrophosphate **1**: $R_t$ = 14 minutes). After 6 days, all of the morpholidate **16** had been comsumed. The volatiles were removed by evaporation and the residue was dissolved in water (10 mL) and filtered through a 0.2-$\mu$m filter. The filtrate was purified by ion-exchange HPLC (GE Healthcare HiPrep 16/10 DEAE FF column, eluent A = 10 mM $NH_4HCO_3$, eluent B = 500 mM $NH_4HCO_3$, gradient from 0 to 100% B in 50 minutes, flow rate 3 mL/minutes). The fraction between 28 and 36 minutes was collected and lyophilized and further purified by reverse-phase HPLC (Waters Nova Pak HR C18 6-$\mu$m 19 × 300-mm column, eluent A = 10% $CH_3CN$ in 25 mM TEAA pH 7, eluent B = 60% $CH_3CN$ in 25 mM TEAA pH 7, gradient from 0 to 50% B in 25 minutes, flow rate 5 mL/minutes). The peak at 21 minutes was collected and lyophilized to give an orange solid (5 mg, 5%). Mass spectrometry (HPLC/ESI-MS) observed a single charged $[M\text{-}H]^-$ at m/e 1042.2.

***Method 2***

A mixture of FMN pyridinium salt (45 mg, 0.084 mmol), morpholidate **16** (90 mg, 0.093) and sodium sulfate (26 mg, 0.183) in 0.2 M $MnCl_2$ in formamide (0.7 mL, 0.14 mmol) was stirred at RT for 18 h. Analytical ion-exchange HPLC (conditions as in method 1) showed complete consump-

tion of the morpholidate **16**. The mixture was diluted with $CH_3CN$ (10 mL). The resulting yellow solid was filtered and dissolved in water (10 mL) and filtered through a 0.2-$\mu$m filter. The filtrate was purified by ion-exchange HPLC and reverse-phase HPLC (conditions as in method 1) to give an orange solid (10 mg, 10%) still containing some excess triethylammonium salt. $^1$H NMR (300 MHz, $D_2O$) $\delta$ 7.74 (s, 2H), 7.63 (s, 1H), 6.11 (m, 1H), 5.96 (d, 1H, $J = 15.9$ Hz), 5.66 (d, 1H, $J = 4.8$ Hz), 3.70-4.43 (m, 14H), 3.63 (m, 2H), 3.13 (m, 1H), 3.04 (q, $Et_3N$), 2.76 (dd, 1H, $J = 5.1, 13.2$ Hz), 2.54 (d, 1H, $J = 12.9$ Hz), 2.08 (t, 2H, $J = 7.1$ Hz), 1.32-1.52 (m, 6H) (s, 1H), 1.13 (t, $Et_3N$); $^{31}$P NMR ($D_2O$) $\delta$ −10.72 (m), −11.46 (m).

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