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Synthesis of Fluorophosphate Nucleotide Analogs and Their Characterization as Tools for ^{19}F NMR Studies

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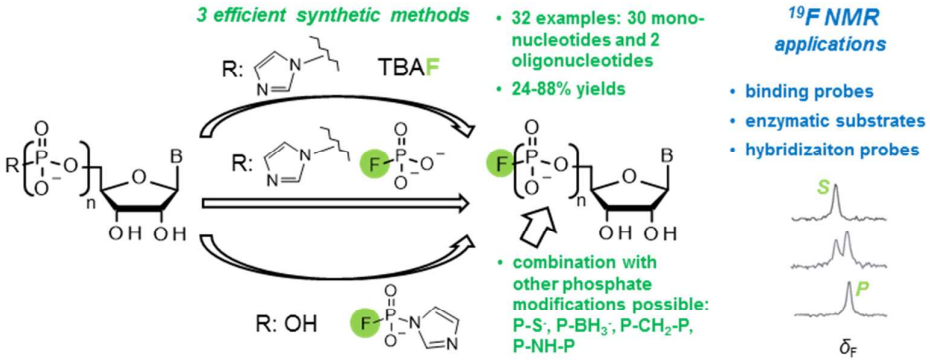


ABSTRACT To broaden the scope of existing methods based on ^{19}F nucleotide labeling, we developed a new method for the synthesis of fluorophosphate (oligo)nucleotide analogs containing an O to F substitution at the terminal position of the (oligo)phosphate moiety and evaluated them as tools for ^{19}F NMR studies. Using three efficient and comprehensive synthetic approaches based on phosphorimidazolide chemistry and tetra-*n*-butylammonium

fluoride, fluoromonophosphate or fluorophosphate imidazolide as fluorine sources, we prepared over 30 fluorophosphate-containing nucleotides, varying in nucleobase type (A, G, C, U, m^7G), phosphate chain length (from mono- to tetra-) and presence of additional phosphate modifications (thio-, borano-, imido-, methylene-). Using fluorophosphate imidazolide as fluorophosphorylating reagent for 5′-phosphorylated oligos we also synthesized oligonucleotide 5′-(2-fluorodiphosphates), which are potentially useful as ^{19}F NMR hybridization probes. The compounds were characterized by ^{19}F NMR, and evaluated as ^{19}F NMR molecular probes. We found that fluorophosphate nucleotide analogs can be used to monitor activity of enzymes with various specificities and metal-ion requirements, including human DcpS enzyme – a therapeutic target for spinal muscular atrophy. The compounds can also serve as reporter ligands for protein binding studies, as exemplified by studying interaction of fluorophosphate mRNA cap analogs with eukaryotic translation initiation factor (eIF4E).

INTRODUCTION

^{19}F nuclei have several beneficial properties for NMR applications, including a nuclear spin of $^1/_2$, high magnetogyric coefficient, and 100% abundance, resulting in a ^{19}F NMR sensitivity equal to 83% of ^1H NMR. However, unlike protons, ^{19}F is almost never present in natural biomolecules, has a higher chemical shift dispersion, and is more sensitive to changes in the local environment.[1] As such, fluorine-labelled compounds and their transformations can be selectively observed by ^{19}F NMR even in complex mixtures.[2] Consequently, ^{19}F-labeled nucleotides and nucleic acids have broad utility in biophysical and biological experiments that utilize ^{19}F NMR, including binding experiments, enzymatic activity assays, and structural and functional studies.[3] For example, 2-fluoro ATP has been employed as a reporter substrate for ^{19}F NMR-based screening of enzyme therapeutic targets.[3b] A macromolecular trifluorothymidine prodrug has been recently designed as a potential anticancer theranostic

agent with utility in ^{19}F magnetic resonance imaging.[4] For those various applications ^{19}F-labels are usually introduced within either the nucleobase or the ribose moiety. However, such modifications sometimes disturb the physicochemical and biological properties of (oligo)nucleotides or require laborious synthetic procedures. Therefore, there is a need for ^{19}F-labeled (oligo)nucleotides with increased functionalities and/or synthetic availabilities. We envisaged that the introduction of fluorine atom at the terminal position of an (oligo)phosphate to form a fluorophosphate moiety by an efficient and straightforward synthetic method would provide a viable alternative to existing ^{19}F-labeling methods, which usually require multistep syntheses of modified nucleosides and subsequent incorporation into (oligo)nucleotides by chemical or chemo-enzymatic approaches.

The O to F substitution at the terminal phosphate produces a chemically stable analog that is isostructural and isoelectronic to unmodified nucleotides, but has a smaller negative net charge at physiological pH, a lower affinity to divalent metal cations, and properties similar to those of corresponding phosphate methyl esters under hydrolytic conditions.[5] As a result, fluorophosphate analogs may be considered close mimics to either terminally-protonated nucleoside 5′-(oligo)phosphates or nucleoside 5′-phosphosulfates (Fig. 1) and may replace nucleotides as substrates in some enzymatic reactions (Table S1).[6] Several enzymes that recognize fluorophosphate analogs are therapeutic targets and thus ^{19}F NMR-based assays that facilitate the discovery of inhibitors for such targets are of general interest. To our knowledge, fluorophosphate nucleotide analogs have not been used in ^{19}F NMR assays to date, and one of the reasons for that may be their limited synthetic availability.

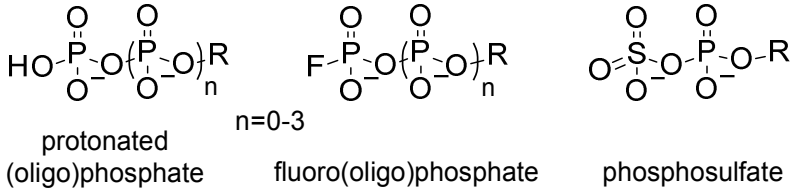


Figure 1. The general structures of fluorophosphate nucleotide analogs and structurally related compounds found in nature. R is 5′-nucleoside moiety.

Nucleoside 5′-fluoromonophosphate derivatives (phosphorofluoridates - NMPF, phosphorofluoridothioates and phosphorofluoridodithioates) are accessible by several, usually efficient methods based either on phosphorus (V) or phosphorus (III) chemistry and substitution by F^- or oxidative fluorination, respectively.[7] Conversely, only few methods for the synthesis of nucleoside fluoro di- and triphosphates (NDPF and NTPF) have been reported. The first synthesis of ATPF has been carried out by coupling of ADP and fluorophosphate tributylammonium salts using Michelson method (diphenylchlorophosphate in pyridine) and isolated in about 35% yield after quite complex chemoenzymatic work-up. [5a] GTPF has been synthesized directly from GTP by treatment with dinitrofluorobenzene, but isolated only in 5–10% yield, [5d] whereas ADPF has been synthesized in 25% yield by reacting adenosine 5′-phosphomorpholidate and fluoromonophosphate in pyridine.[6a,8] Notably, all the reported procedures gave moderate to low yields and have been demonstrated only on a single target molecule. To our knowledge, nucleoside 5′-fluorotetraphosphates (NP_4F) or nucleoside oligophosphate analogs combining the fluorophosphate with other phosphate-modifications have not been reported so far.

In this work, our goal was to provide a comprehensive chemical method for the synthesis of various fluorophosphate-containing nucleotide analogs – from mono- to tetraphosphates – and to evaluate them as tools for ^{19}F NMR experiments in biological or medicinal context. We assumed that our synthetic method should not only provide access to nucleotides differing in nucleobase type and oligophosphate chain length, but also enable combining the presence of

fluorophosphate group with other phosphate modifications. Due to resistance to enzymatic degradation and differences in electronic structure, nucleotides containing phosphate modifications such as imidodiphosphate, methylenebisphosphonate, phosphorothioate or boranophosphate are valuable tools for structural and mechanistic studies on nucleotide-binding proteins and enzymes and often exhibit beneficial biological properties or inhibitory effects.[9] Combination of those modifications with fluorophosphate could be advantageous in some ^{19}F NMR-based binding experiments and also provide new types of nucleotide mimics for mechanistic studies.

To achieve our goal, we first developed three efficient synthetic methods for nucleotide analogs containing the fluorophosphate moiety. The methods were based on phosphorimidazolide chemistry and three different, easily available fluorine sources: tetra-*n*-butylammonium fluoride (TBAF), triethylammonium fluoromonophosphate (FP) or a new reagent, fluoromonophosphate imidazolide lithium salt (FPIm). To demonstrate the utility of our methods we synthesized a set of over 30 fluorophosphate nucleotide analogs of various structural complexity, improving the efficiencies of the previously reported compounds and preparing numerous previously unknown ones. We also, for the first time, obtained oligonucleotides carrying a (2-fluorodiphosphate) moiety at the 5′-end. The synthesized nucleotides were characterized by ^{19}F NMR and their potential utility as reporter substrates in ^{19}F NMR-monitored enzymatic experiments, reporter ligands for binding studies and hybridization probes was also demonstrated.

RESULTS AND DISCUSSION

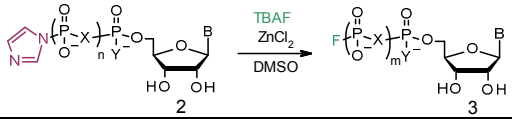
As a starting point for the syntheses we chose to explore the reactivity of imidazole activated nucleotides (NMPIm and NDPIm) in aprotic polar solvents in the presence of divalent metal chlorides (MCl_2; usually $MgCl_2$ or $ZnCl_2$) as Lewis acid reaction mediators. Such approach

has been previously used with success for pyrophosphate bond formation reactions leading to various mono- and dinucleoside oligophosphates and their phosphate-modified derivatives.[9c,10] After initial optimization studies we identified three complementary synthetic approaches that could lead to fluorophosphate nucleotide analogs (Fig. S1). The first approach employed a nucleophilic substitution of imidazole-activated nucleotides with a fluoride anion (from TBAF) in the presence of excess divalent metal chloride ($MgCl_2$ or $ZnCl_2$) yielding a fluorophosphate derivative of a nucleotide with the same phosphate chain length (Table 1). The second approach, was similar, but utilized triethylammonium fluoromonophosphate as a nucleophile yielding a fluorophosphate-containing product with the phosphate chain elongated by one subunit (Table 2). Finally, in the third approach a reversed activation scheme was applied, i.e. an imidazole activated fluorophosphate was coupled with non-activated nucleotide to afford, similarly as in the second approach, a product with the phosphate chain elongated by one subunit (Table 3).

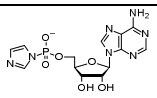
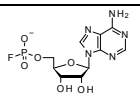
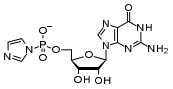
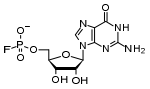
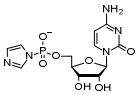
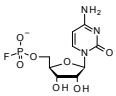
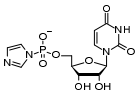
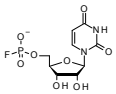
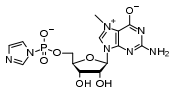
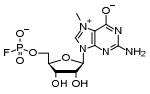
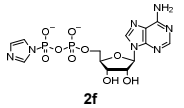
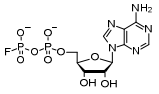
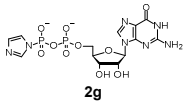
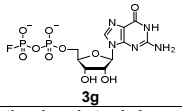
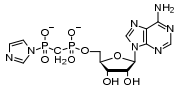
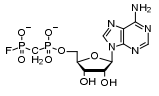
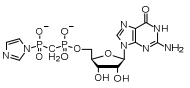
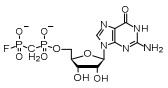
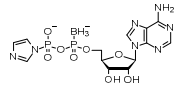
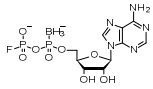
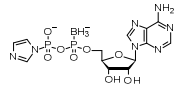
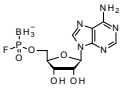
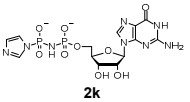
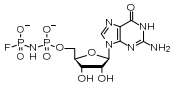
The synthesis employing fluoride as a nucleophile was tested in the presence of 4–10-fold excess of TBAF, in either DMSO or DMF, and in the presence of excess $ZnCl_2$, or of an excess $MgCl_2$ or in the absence of divalent metal chloride. The reaction was efficient only in DMSO and with $ZnCl_2$ as a mediator. As monitored by RP HPLC combined with MS(-)ES analysis of the UV-absorbing peaks (Fig. 2), adenosine phosphorimidazolide (**2a**; t_R = 12 min) treated with 6 equiv. of TBAF in DMSO at r.t. and in the presence of 8 equiv. of $ZnCl_2$ was nearly quantitatively converted into AMPF (**3a**; t_R = 10.8 min, *m/z* = 348) within 2 h (the reaction could be accelerated by addition of higher excess of TBAF). In the absence of mediator or in the presence of $MgCl_2$ no reaction was observed. Adenosine, guanosine, uridine, cytidine and 7-methylguanosine 5′-fluoromonophosphates were synthesized under these conditions and isolated after DEAE Sephadex ion-exchange purification in very good

yields (58–88%) and high purity by HPLC, ^{1}H and ^{31}P NMR (Table 1, entries 1–5 and Supporting Information).

Table 1. Synthesis of fluorophosphate nucleotide analogs from nucleotide imidazolide derivatives and TBAF.



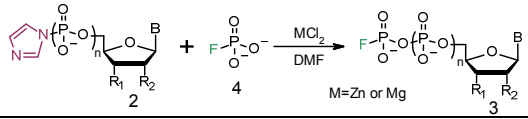
2 3

Entry	Starting material	Product	Yield %[a](%)[b]
Nucleotides unmodified in the phosphate chain			
1	 **2a**	 **3a**	86 (91)
2	 **2b**	 **3b**	88 (95)
3	 **2c**	 **3c**	83 (99)
4	 **2d**	 **3d**	78 (95)
5	 **2e**	 **3e**	58 (90)
6	 **2f**	 **3f**	67 (83)
7	 **2g**	 **3g**	42 (69)
Nucleotides modified in the phosphate chain			
Entry	Starting material	Product	Yield %[a](%)[b]
8	 **2h**	 **3h**	82 (99)
9	 **2i**	 **3i**	51 (64)
10	 **2j**	 **3j**	39 (57)
11	 **2j**	 **3k**	49 (86)
12	 **2k**	 **3l**	25 (65)

[a]Isolated yield; [b] conversion calculated based on the reaction RP HPLC profile

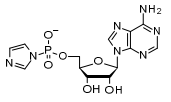
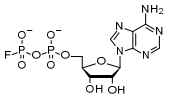
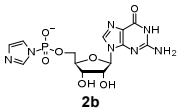
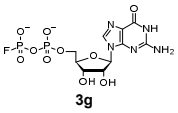
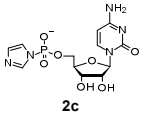
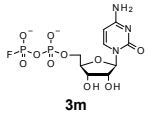
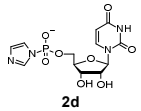
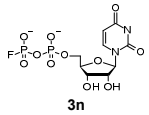
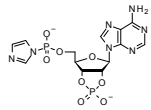
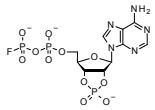
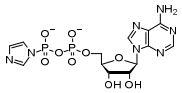
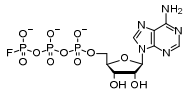
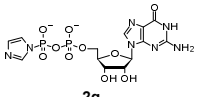
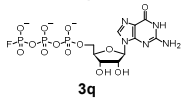
Imidazolide derivatives of nucleoside diphosphates, ADP (**2f**) and GDP (**2g**) were under similar conditions converted into corresponding 5′-fluorodiphosphates, ADPF (**3f**) and GDPF (**3g**), respectively, within 2–4 h (Table 1, entries 6, 7). However, after longer reaction times (>24 h) or in the presence of 10-fold or higher TBAF excess, a fluoride-mediate cleavage of the pyrophosphate was observed as a side reaction. For example, the initially formed ADPF (**3f**) was partially cleaved to a mixture of AMPF (*m/z* 348) by HPLC and AMP (*m/z* 346) as the only UV-absorbing by-products.[11]

Table 2. Synthesis of fluorophosphate nucleotide analogs from nucleotide imidazolide derivatives and FP (4).



2 4 3

Nucleotides modified in the phosphate chain

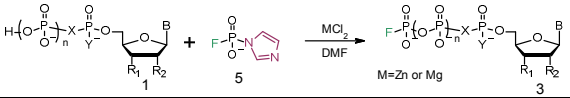
Entry	Starting material	Product	Yield %[a] (%)[b]
13	 **2a**	 **3f**	73 (90)
14	 **2b**	 **3g**	n.d.(68)
15	 **2c**	 **3m**	54 (82)
16	 **2d**	 **3n**	76 (80)
17	 **2l**	 **3o**	65 (81)
18	 **2f**	 **3p**	n.d.(65)
19	 **2g**	 **3q**	n.d.(66)

[a]Isolated yield; [b] conversion calculated based on reaction RP HPLC profile

The reaction with TBAF was also performed on activated phosphate-modified NDP derivatives, including imidazolides of nucleoside α,β-methylenebisphosphonates (**2h, 2i**), α-boranodiphosphate (**2j**) and α,β-imidodiphosphate (**2k**), (Table 1, entries 8–12). For compound **2h** (t_R 6.5 min, Fig. 2B) the conversion into corresponding fluoromethylenebisphosphonate (**3h**, t_R 5.0 min) was nearly quantitative, and due to the high chemical stability P-CH_2-P bond no fluoride-mediated cleavage was observed even in the presence of high TBAF concentrations. Adenosine α-boranodiphosphate imidazolide (**2j**), which was a mixture of two (S_P and R_P) diastereomers, was also efficiently converted into two diastereomers of ADPαBH_3F (**3j**).However, if reaction with TBAF was prolonged or higher excess of TBAF was used, **3j** was efficiently cleaved by fluoride producing two diastereomers of AMBH_3F (**3k**) as major products (Table 1, entries 10 and 11; Fig. S2). Imidodiphosphate derivative **2k** was also converted into corresponding fluoroimidodiphosphate (**3l**), however, the reaction was notably slower, taking over 24 h and reaching only 65% HPLC conversion (Fig. 2C). The major UV-absorbing by-product (t_R = 4.8 min) was identified as $GPNH_2$ (*m/z* = 361). The fluoride-based approach appeared to be less practical for NTPF (and NP_4F) since the synthesis of the required starting materials (corresponding NTP or NP_4 imidazolides) is often problematic as they are more polar and less stable than imidazolides of mono- and diphosphates.

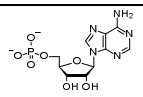
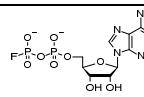
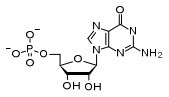
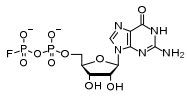
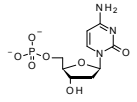
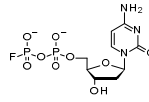
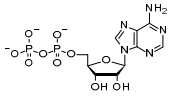
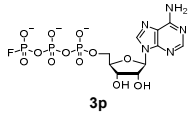
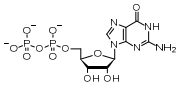
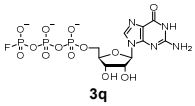
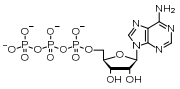
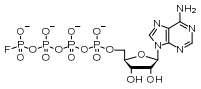
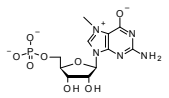
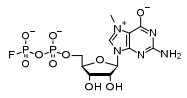
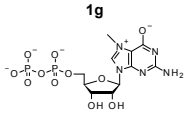
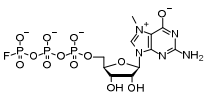
Therefore, we tested the second approach, in which triethylammonium fluoromonophosphate (TEA FP, **4**) was used as a nucleophile instead of fluoride. Reaction of NMPIm and NDPIm with **4** resulted in the formation of a fluorophosphate-containing product with oligophosphate chain one subunit longer compared to the starting imidazolide derivative (NDPF and NTPF, respectively). The reactions were performed in DMF in the presence of excess $MgCl_2$ or $ZnCl_2$.

Table 3. Synthesis of fluorophosphate nucleotide analogs from nucleotides and FPIm (5).

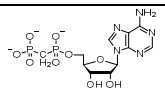
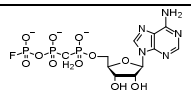
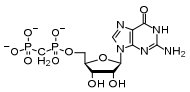
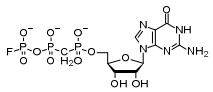
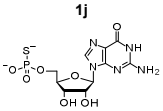
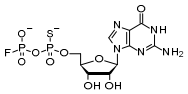
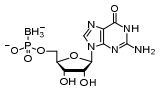
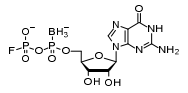
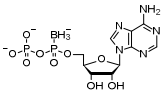
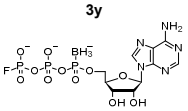
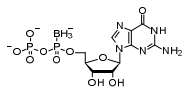
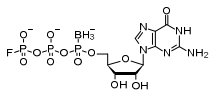
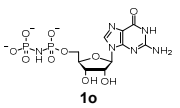
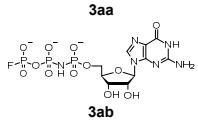
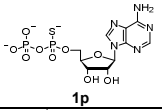
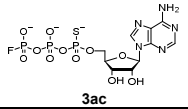


1 5 3

Nucleotides unmodified in the phosphate chain

Entry	Starting material	Product	Yield %[a](%)[b]
20	 **1a**	 **3f**	n.d.(87)
21	 **1b**	 **3g**	69 (75)
22	 **1c**	 **3r**	64 (74)
23	 **1d**	 **3p**	60 (87)
24	 **1e**	 **3q**	56 (78)
25	 **1f**	 **3s**	28 (64)
26	 **1g**	 **3t**	57 (75)
27	 **1h**	 **3u**	58 (70)

Nucleotides modified in the phosphate chain

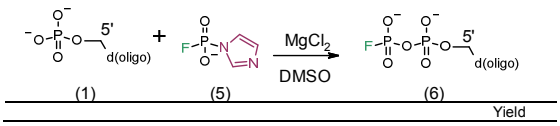
Entry	Substrate	Product	Yield %[a](%)[b]
28	 **1i**	 **3v**	72 (82)
29	 **1j**	 **3w**	63 (72)
30	 **1k**	 **3x**	74 (79)
31	 **1l**	 **3y**	63 (75)
32	 **1m**	 **3z**	65 (74)
33	 **1n**	 **3aa**	68 (90)
34	 **1o**	 **3ab**	70 (80)
35	 **1p**	 **3ac**	60 (74)

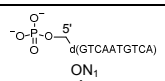
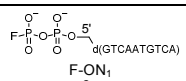
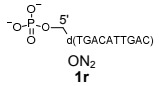
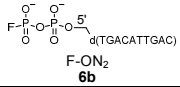
[a]Isolated yield; [b] conversion calculated based on reaction RP HPLC profile

A pilot coupling reaction of **2a** (AMPIm; Fig. 2D) with 1.5 equiv. of **4** in the presence of 8-fold excess of $MgCl_2$ resulted in 90% HPLC conversion into ADPF (**3f**) within 40 min (and isolated yield of 73%), indicating this approach may represent a more straightforward alternative for NDPF. The reaction was similarly efficient in the presence of $ZnCl_2$ (Figure S2), indicating that for compound **4** as a nucleophile both metal chlorides are efficient reaction mediators. The utility of this approach was further confirmed by the synthesis of **3g** (GDPF), **3n** (UDPF), **3m** (CDPF) and **3o** (PADPF) in good yields (68–82) by HPLC, 40–76% isolated, Table 2, entries 14–17; Fig. 2E). The approach was also tested for the synthesis of NTPFs from corresponding NDPIms. In the coupling reactions of ADPIm (**2f**) and GDPIm (**2g**) with **4**, the maximum HPLC conversions reached 65 and 66 %, respectively. Hence, the synthesis was feasible, however, the yield was slightly lower as a result of imidazolide hydrolysis and self-coupling side reactions, which were more abundant in the case of NDPIms because of their lower stability compared to NMPIms. Hence, we developed the third approach avoiding the problems with synthetically challenging or chemically labile imidazolides. This includes not only some of NDP and NTP (e.g. 7-methylguanosine derivatives), but also nucleotides containing the phosphorothioate group, which decompose

with the loss of sulfur under standard activation protocols (i.e. dithiodipyridine/triphenylphosphine system used in this work or activation with CDI). The approach was based on the use of non-activated nucleotides as nucleophiles and, thus, required the use of an electrophilic fluorophosphorylating agent. As such, we synthesized fluorophosphate phosphorimidazolide lithium salt (FPIm, **5**), in a single step from triethylammonium fluorophosphate (**4**), without chromatographic purification and in high yield (75–80%). The compound was stable when stored at 4 °C in the absence of moisture for at least one month. The compound acted as a powerful fluorophosphorylating reagent for various (oligo)phosphates. Several nucleoside fluorodiphosphates (**3f, 3g, 3r, 3t**), fluorotriphosphates (**3p, 3q, 3u**) and even tetraphosphates (**3s**) were synthesized from corresponding non-activated nucleotides (**1**) using reagent **5**. The analogs additionally modified with methylenebisphosphonate (**3v, 3w**) phosphorothiate (**3x, 3ac**), boranophosphate (**3y, 3z, 3aa**) and imidodiphoshate (**3ab**) groups were also synthesized. The reagent gave usually very good HPLC conversion within 0.5 to 2 h with nearly no other UV-absorbing by-products (Fig. 2F,G). However, prolonged reaction time resulted in formation of more polar by-product of m/z value 82 units higher than the expected product, suggesting introduction of an additional fluorophosphate group. MS/MS analysis indicated that the second fluorophosphate group is present at the 2′ or 3′-hydroxyl groups (not shown). Finally, we also found that **5** efficiently reacts with 5′-phosphorylated oligonucleotides in DMSO in the presence of $MgCl_2$ excess to produce oligonucleotide 5′-fluorodiphosphates in 80–90% conversions. For example, 10 nt 5′-phosphorylated oligo ON_1 (t_R = 15.7 min) treated with excess FPIm (**5**) and $MgCl_2$ in DMSO was converted within 24 h and in 83% yield into fluorophosphorylated F-ON_1 (**6a**) (Table 4, Figure 2H), which then was purified by RP HPLC.

Table 4. Synthesis of oligonucleotide 5′-fluorodiphosphates from oligonucleotide 5′-phosphates and FPIm



Entry	Substrate	Product	%[a](%)[ba]
35	 ON_1 **1q**	 $F\text{-}ON_1$ **6a**	20.4 (83)
36	 ON_2 **1r**	 $F\text{-}ON_2$ **6b**	21.2 (86)

[a]Isolated yield; [b] conversion calculated based on reaction RP HPLC profile

Altogether, using the three approaches, we synthesized a set of over 30 fluorophosphate nucleotide analogs (mononucleotides **3** and oligonucleotides **6**; Figure 1, Table S2), varying in oligophosphate chain length (from mono- to tetraphosphate), nucleobase type (A, G, C, U, m^7G), and phosphate modifications which may be useful for conferring enzymatic resistance or tuning their affinity towards proteins. Although neither of the three synthetic approaches was fully universal, they were complementary, enabling us to cover a broad spectrum of nucleotides varying in size, polarity (and thus solubility in organic solvents), chemical reactivity and stability. The compound structures and homogeneities were confirmed by HRMS, ^{1}H NMR, ^{31}P NMR and RP HPLC. In contrast to *O*,*O*-dialkyl phosphorofluoridates, which are rapidly hydrolyzed to phosphate diesters under aqueous conditions,[5b] the fluorophosphate analogs of mononucleotides showed high stability, both in aqueous solutions and upon storage as solid samples. NMPF (except **3k**),[12] NDPF and NTPF were stable in water and in aqueous buffers of pH 5–8 at RT for several weeks or even months. Moreover, during storage NDPF and NTPF appeared to be less susceptible to pyrophosphate bond hydrolysis with the loss of terminal phosphate, which is commonly observed for unmodified NDP and NTP.

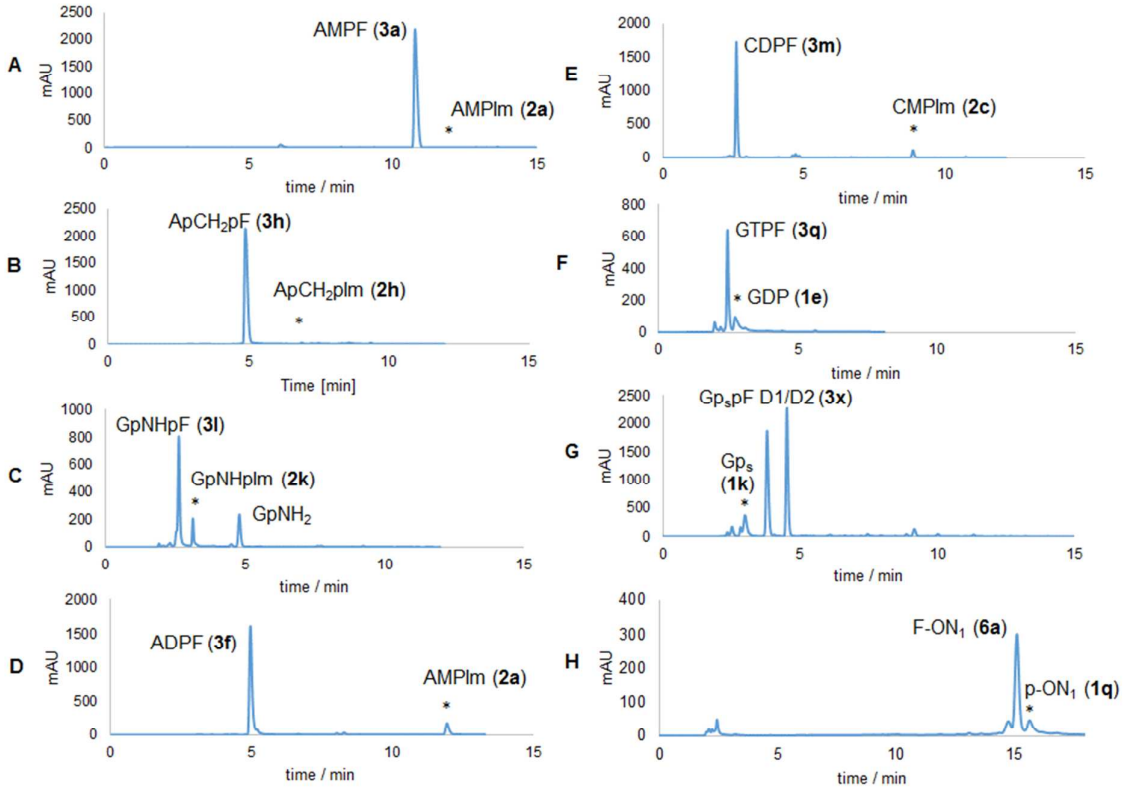


Figure 2. Representative RP HPLC profiles from the syntheses of fluorophosphate nucleotide analogs. Synthesis of A) AMPF (**3a**) from AMPIm (**2a**) and TBAF; B) $ApCH_2pF$ (**3h**) from $ApCH_2pIm$ (**2h**) and TBAF C) GpNHpF (**3l**) from GpNHpIm (**2k**) and TBAF D) ADPF (**3f**) from AMPIm (**2a**) and FP (**4**) E) CDPF from AMPIm (**2a**) and FP (**4**) F) GTPF from GDP (**1e**) and FPIm (**5**) G) Gp_SpF (two P-diastereomers) from GMPS (**1k**) and FPIm (**5**) H) 5′-fluorodiphosphorylated oligo $F\text{-}ON_1$ (**6a**) from 5′-phosphorylated oligo (**1q**) and FPIm (**5**). Asterisks indicate always t_R of the starting material.

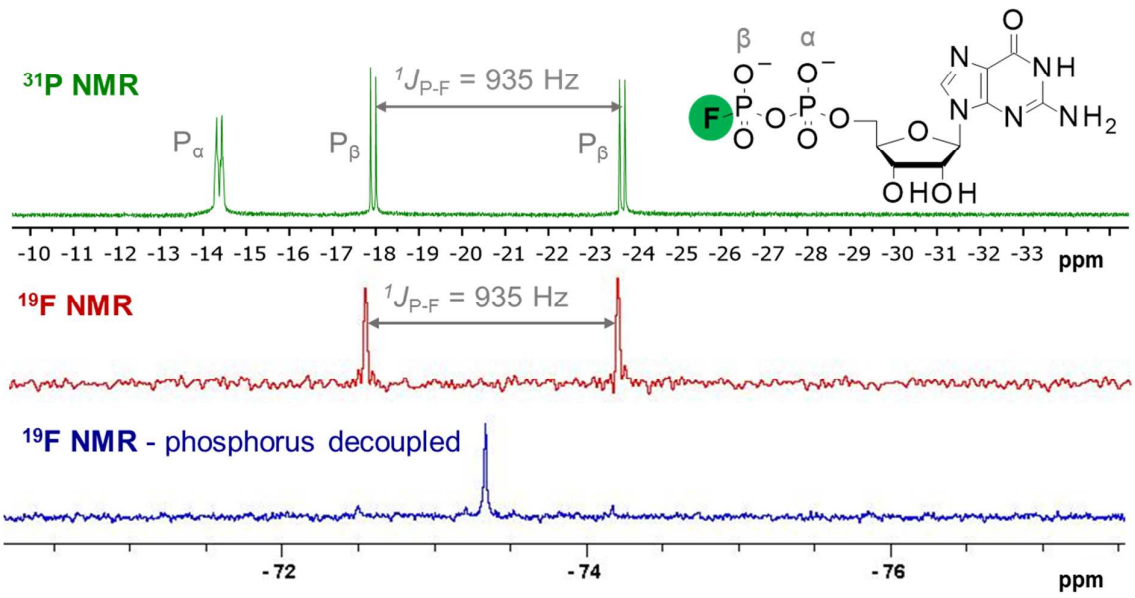


Figure 3. ^{31}P and ^{19}F NMR spectra of compound **3g** (GDPF).

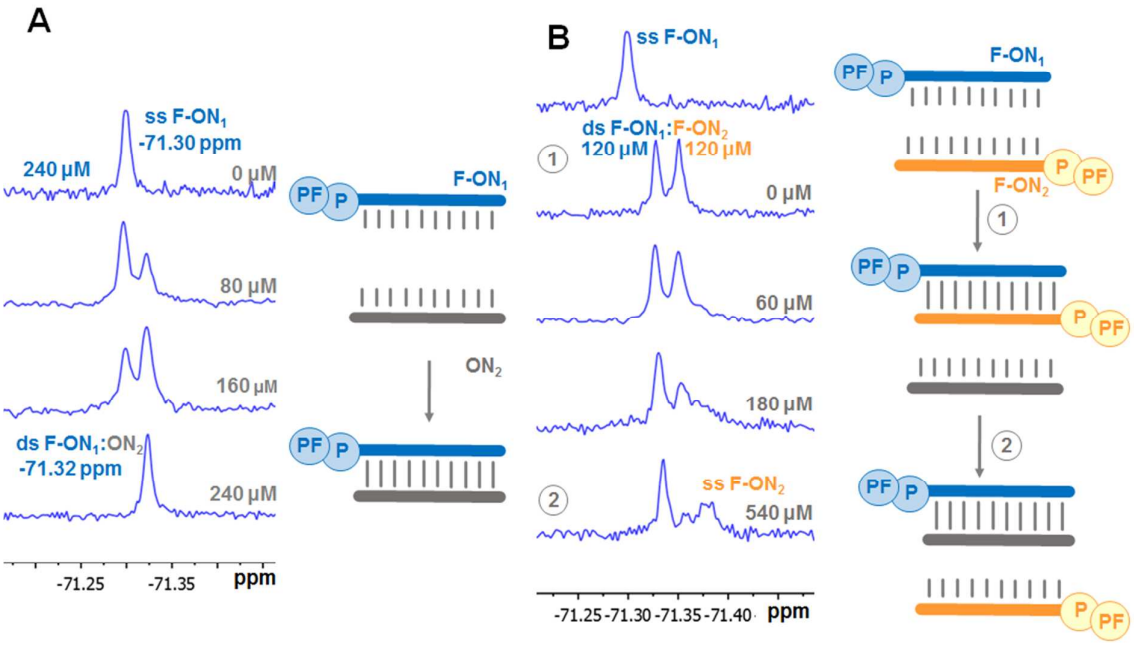


Figure 4. Duplex formation of 5′-fluorodiphosphate oligos can be monitored by ^{19}F NMR. a) Hybridization of 240 µM 5′-fluorodiphosphorylated 10 nt oligo (F-ON$_1$, **6a**) with increasing concentrations of unmodified complementary ON$_2$ (**6b**; 0→240 µM); b) Hybridization of 120 µM F-ON$_1$ with complementary 120 µM F-ON$_2$ and subsequent replacement of F-ON$_2$ by

increasing concentrations of ON_2 (0→540 μM). In each case, only the downfield component of the doublet is shown for clarity.

The next step in our study was characterization of the compounds by ^{19}F NMR (Table 5, Figure S3). The δ_F values were strongly dependent on the length of the phosphate chain and phosphate-modifications, whereas structural alterations in the ribose or nucleobase moiety influenced the δ_F values to a smaller extent (Figures S3 and S4). As for nucleobases, the greatest differences in the δ_F values were observed between purine and pyrimidine nucleosides, whereas in other cases the differences were very small (<0.01 ppm) or undetectable (the differences decreased with increasing oligophosphate chain length). Due to scalar coupling with the adjacent phosphorus nucleus, the ^{19}F NMR signal of each nucleotide appeared as a doublet ranging from -53 to -79 ppm with $^1J_{P\text{-}F}$ ranging from 914 to 1144 Hz (Table 5). The $^1J_{P\text{-}F}$ values for various nucleoside fluoro(oligo)phosphates were virtually the same ($^1J_{P\text{-}F}$ ~935 Hz, **3a-3g**, **3m-3u**). However, if one of the oxygen atoms in the fluorophosphate moiety was replaced by another species (S^-, BH_3^-, NH, or CH_2; **3h-3l**, **3v-3z**, **3ac-3ad**) the value changed up to ±130 Hz. The δ_F values for fluorophosphate nucleotides were almost independent of the buffer, pH, or presence of magnesium ions and slightly dependent from temperature as exemplified on various nucleotide mixtures (Fig. S5), making them good candidates for ^{19}F NMR reporter molecules or binding probes. The δ_F and $^1J_{P\text{-}F}$ values of oligo 5′-(2-fluorodiphosphates) were similar to those of the corresponding mononucleotides; however, the δ_F values varied depending on whether the oligonucleotide was in the single or double stranded form. Notably, the 0.02 ppm difference in δ_F was sufficient to monitor the hybridization of a 5′-fluorodiphosphorylated 10 nt oligonucleotide (F-ON_1, **6a**) with a complementary unlabeled oligo (ON_2, **1q**) (Figure 4). In the case of duplex formation between two complementary 5′-fluorodiphosphorylated oligonucleotides,

namely F-ON_1 (**6a**) and F-ON_2 (**6b**), two separate ^{19}F resonances were observed for each strand. The displacement of F-ON_2 by ON_2 could also be monitored.

Table 5. Representative ^{19}F NMR data for fluorophosphate nucleotides. [a]

Entry	Number	Abbreviation	δ_F (ppm)[a]	$^1J_{P\text{-}F}$ (Hz) [a]
1	**3b**	GMPF	-78.98	933
2	**3k**	AMPBH_3F[b]	-52.74/-52.95[b]	1140[b]
3	**3f**	ADPF	-72.50	934
4	**6a**	Fpp-d(GTCAATGTCA)	-72.59	934
5	**3l**	GpNHpF	-61.54	914
6	**3h**	ApCH_2pF	-53.96	973
7	**3p**	ATPF	-72.26	935
8	**3ac**	Ap_SppF[b]	-72.23[b]	935[b]
9	**3s**	Ap_4F	-72.00	936

[a] ^{19}F NMR spectra recorded at 376 MHz in D_2O at 25 °C; chemical shifts reported relative to aqueous NaF, as the secondary external standard (-121.5 ppm). [b]Mixture of R_P and S_P diastereomers.

The next goal in our study was to preliminarily verify whether the fluorophosphate nucleotide analogs can be of utility in ^{19}F NMR monitored enzymatic-activity assays and binding experiments. For this purpose, we took advantage of commercially available broad specificity enzymes – snake venom phosphodiesterase (SVPDE) or ribonuclease T2 (RNAse T2), as well as of highly specific enzyme that constitutes a real therapeutic target – mRNA decapping scavenger (DcpS), which is an m^7G-nucleotide specific pyrophosphatase that has been identified as a target in spinal muscular atrophy (SMA).[13] Moreover, using eukaryotic translation initiation factor 4E (eIF4E), a non-enzymatic protein recognizing m^7G-capped mRNAs during initiation of translation, we demonstrated possible use of fluorophosphate nucleotide analogs as reporter molecules in binding assays. Finally, we also quantitatively assessed how the terminal O to F substitution influences the protein-nucleotide interactions.

First, we subjected four fluorophosphate nucleotides, (AMPF, ADPF, ATPF and AP_4F; compounds **3a, 3f, 3p**, and **3s**, respectively), to HPLC and ^{19}F NMR-monitored degradation by snake venom phosphodiesterase (SVPDE), which cleaves NTPs, NDPs, and oligos to release nucleoside 5′-monophosphates. All tested nucleotides were viable substrates for SVPDE. Furthermore, there was a sufficient difference in the chemical shift ($\Delta\delta_F > 0.1$ ppm) of the fluorophosphorylated substrate (ADPF, ATPF and AP_4F) and product (i.e. fluoromonophosphate, fluorodiphosphate and fluorotriphosphate, respectively) to adequately monitor the reaction progress (Figure 5A, Figure S6). However, the signal of fluoride released from AMPF was not detected, probably as a result of significant line broadening caused by the presence of Mg^{2+} ions, which are required by SVPDE. The presence of Mg^{2+} caused also slight line broadening and δ_F changes of the fluorophosphate signals, especially in the products, which disappeared upon quenching the reaction with EDTA. Next, we subjected a mixture of all four adenine nucleotides to incomplete hydrolysis by SVPDE, yielding a sample containing four fluorinated substrates and four products. Each fluorophosphate-containing species was characterized by a distinctive δ_F value, meaning that each substrate-product combination could be monitored by ^{19}F NMR (Figure S7, Table S2) and indicating the potential applicability of the analogs in different adenine nucleotide-processing enzymes.

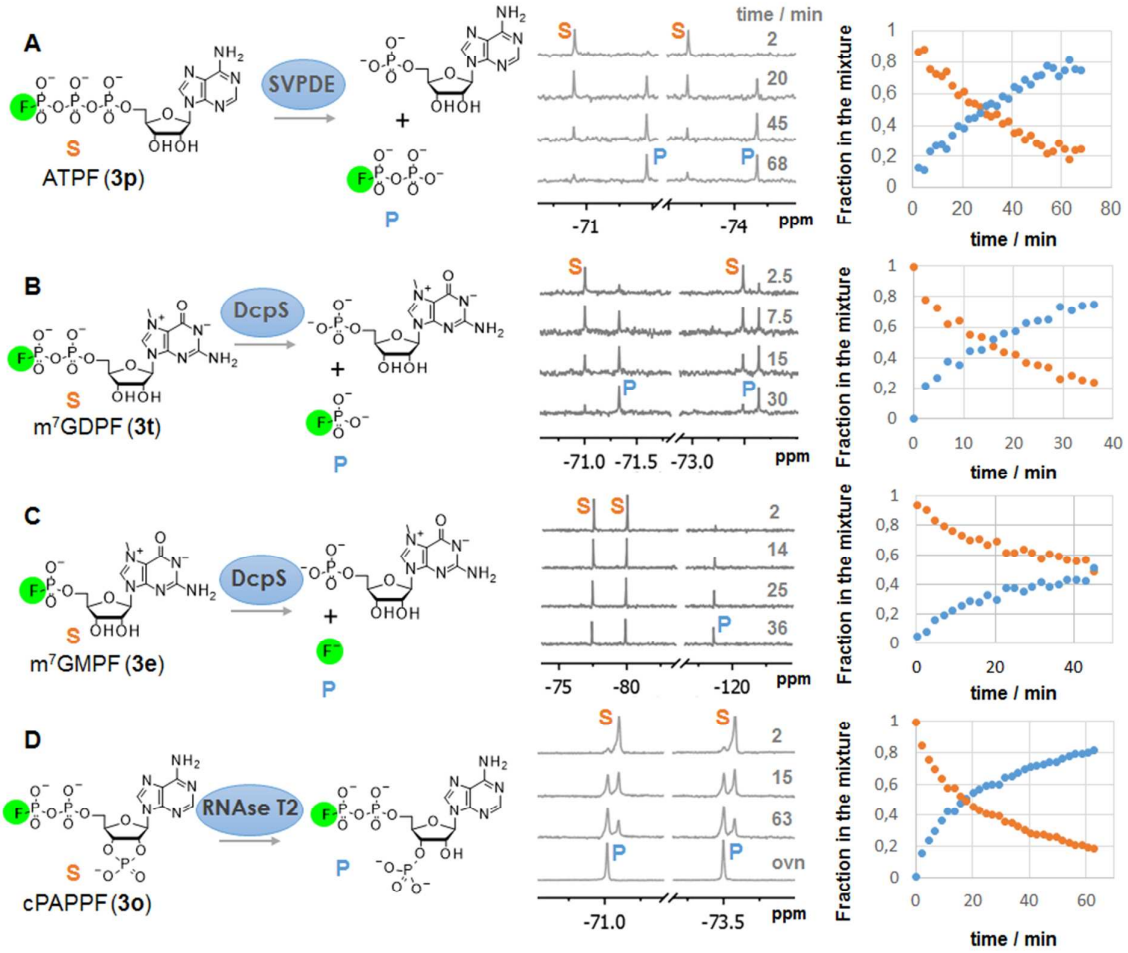


Figure 5. Fluorophosphate nucleotide analogs can be used as reporter substrates in ^{19}F NMR monitored activity-based enzymatic assays. The time course of several reactions was determined by ^{19}F NMR including a) cleavage of 1 mM ATPF **(3p)** by SVPDE, b) cleavage of 0.5 mM m^{7}GDPF **(3t)** by DcpS, c) cleavage of 0.5 mM m^{7}GMPF **(3e)** by DcpS, and d) cleavage of 2 mM cPAPPF **(3o)** by RNAse T2.

7-Methylguanosine (m^{7}G) derivatives **3e**, **3t**, **3u** may be considered analogs of the m^{7}G cap structure present on the 5′ end of mRNA. Therefore, we tested their viability as substrates for human mRNA decapping scavenger enzyme (DcpS). DcpS is a member of the HIT family of pyrophosphatases and hydrolyzes mRNA cap derivatives formed after mRNA degradation by exosomes.[14] It has also been identified as a molecular target in spinal muscular atrophy

(SMA).[13,15] The canonical substrates of DcpS are di- or oligonucleotides with an m^7GpppN_n structure, which are cleaved between the γ and β phosphates to produce m^7GMP and downstream (oligo)nucleotides. Among unmodified mononucleotides, m^7GDP is not a substrate but is a strong inhibitor of DcpS, whereas m^7GTP is cleaved by DcpS, but less efficiently than dinucleotides.[16] Therefore, m^7GMPF (**3e**), m^7GDPF (**3t**), and m^7GTPF (**3u**) are analogs of the reaction product, inhibitor, and substrate, respectively. Surprisingly, as confirmed by HPLC and ^{19}F NMR, all of these compounds were cleaved by DcpS, releasing m^7GMP and fluoride, fluoromonophosphate, or fluorodiphosphate, respectively. In each case, the δ_F values of the reaction substrates and products differed sufficiently to monitor the reaction progress (Figure 5B,C; Figure S8). As such, these novel DcpS substrates offer the possibility to develop novel DcpS assays that are an alternative to previously described radioactive probes.[13]

To test if δ_F changes of the fluorophosphate moiety could also be used to monitor enzymatic processes in which more distant parts of the molecule are modified, we subjected 2′,3′-cyclo-phosphoadenosine 5′-(2-fluorodiphosphate) (**3o,** cPADPF) to cleavage by RNAse T2, producing 3′-phosphoadenosine 5′-(2-fluorodiphosphate) (PADPF). PADPF is an isoelectronic and isostructural analog of phosphoadenosine 5′-phosphosulfate (PAPS),[6a] which serves as a source of ′active sulfate′ in cells, as ATP serves as ′active phosphate′.[17] The enzymatic reaction was easily monitored by ^{19}F NMR due to the appearance of a 0.08 ppm downfield shifted signal upon cleavage of the 2′-phosphoester bond of **3o** by RNAse T2 (Figure 5D).

Apart from activity-based assays, monitoring ligand binding is also of interest, especially in inhibitor discovery. This is particularly important for proteins lacking enzymatic activity or when targeting a particular binding site. As such, we investigated the use of fluorophosphate-containing nucleotides as reporter molecules in ^{19}F NMR-based binding assays. As a model

system, we used eukaryotic translation initiation factor 4E (eIF4E), a protein responsible for the recognition of the mRNA 5′ cap during the recruitment of ribosomes onto mRNA. eIF4E is also a marker protein and therapeutic target in cancer.[18] It has been shown that m^7G-containing mono- and dinucleotides can bind efficiently to eIF4E and that modifications within the phosphate moiety can affect binding affinity.[9c] Therefore, we investigated how the fluorophosphate moiety influences recognition by eIF4E. First, we determined the affinities of fluorophosphate cap analogs **3e**, **3t**, **3u** for eIF4E using fluorescence quenching titration (Figure S10).[19] For comparison, we also determined the K_D value for the complex between DcpS and an unhydrolyzable analog of **3t**, m^7GpNHpF (**3ad**), which was synthesized by treating GpNHpF (**3l**) with CH_3I in DMSO. Replacing the terminal oxygen atom with fluorine resulted in a 10 to 7-fold decrease in affinity towards both proteins as compared to the unmodified parent compound (Table 6), corresponding to a $\Delta G°$ loss of 1.2–1.3 kcal/mol. The affinity of m^7GDPF (**3t**) for eIF4E was 8-fold lower than the affinity of m^7GDP, and only 2–2.5-fold higher than that of m^7GMP and 7-methylguanosine phosphosulfate (m^7GPS), which have the same net charge as m^7GDPF at pH 7.4. Similarly, the affinity of **30** for hDcpS was 8.5-fold lower than that of m^7GpNHp.

Table 6. Binding affinities of fluorophosphate cap analogs and their unmodified or phosphosulfate counterparts for cap-binding proteins, eIF4E and DcpS.

Ligand		**K_D for eIF4E / nM**		
		X=F	**X=O$^-$** [20]	**X=OSO$_3^-$** [21]
m^7GMPX	**(3e)**	$(6.67 \pm 0.98)\cdot 10^3$	667 ± 44	575 ± 145

m^7GDPX	**(3t)**	267.4 ± 5.0	34.6 ± 1.8	n.d.
m^7GTPX	**(3u)**	52.6 ± 3.3	6.9 ± 0.4	n.d.
		K_D for DcpS / nM		
m^7GpNHpX	**(3ad)**	81.8 ± 4.3	9.6 ± 0.4	n.d.

This effect can be attributed to the loss of a single H-bond ($\Delta\Delta G^o$ = -1.3 ± 0.6 kcal/mol[22]) and, thus, well corresponds with the literature suggestions that fluorophosphate closely mimics either a protonated phosphate or phosphosulfate. Next, we used m^7GTPF (**3u**) as a reporter molecule for ^{19}F NMR-monitored binding experiments with eIF4E. Typically, a significant broadening of the ^{19}F signals of the reporter molecule is observed upon protein binding due to increased transverse relaxation rates. Additionally, the Carr-Purcell-Meiboom-Gill (CPMG) sequence is used to attenuate broad (protein bound) resonances of reporter molecules. Subsequently, the protein-bound reporter ligand (in our case **3u**) is displaced with a non-fluorinated ligand of interest, leading to the recovery of the signal belonging to the unbound reporter. In our case, displacement was carried out using m^7GpppG or m^7GppCH_2ppG, two mRNA 5′ cap analogs with differing protein affinities.[23] The apparent dissociation constants (K_{app}, defined as the concentration of free ligand when 50% of the protein is bound to the ligand in the presence of reporter ligand **3u**) obtained by ^{19}F NMR-monitored titration of eIF4E in the presence of m^7GTPF and increasing concentrations of either m^7GpppG or m^7GppCH_2ppG (Figure S11) are shown in Table 7. The values were in qualitative agreement with the fluorescence titration data, as m^7GppCH_2ppG proved to be about a 4-fold tighter binder than m^7GpppG.

Table 7. Binding affinities of non-fluorinated nucleotides for eIF4E determined by ^{19}F NMR ligand displacement with m^7GTPF (**3u**) as a ^{19}F-labeled reporter ligand.

K_D / µM	
m^7GpppG	**m^7GppCH_2ppG**

^{19}F NMR	**Apparent**	7 ± 1	30 ± 4
	Calculated	0.012 ± 0.002	0.054 ± 0.008
Fluorescence[23]		0.0206 ± 0.0009	0.0800 ± 0.0019

Since the affinity of **3u** for eIF4E was known from the fluorescence titration, the actual K_D values (defined as the concentration of free ligand when 50% of the protein is bound to the ligand in the absence of reporter) for m^7GpppG and m^7GppCH$_2$ppG ligands could also be calculated and showed good agreement with the literature data (Table 7).

The presented examples support our initial assumption that fluorophosphate nucleotide analogs are potentially useful as tools in various biologically-oriented ^{19}F NMR studies. The sensitivity of the fluorophosphate moiety to the alterations of local environment is sufficient to observe various enzymatic and non-enzymatic transformations due to chemical shift or relaxation rate changes. We expect fluorophosphate nucleotide analogs to be particularly useful in studying enzymes processing and proteins binding the oligophosphate chain as well as for receptors that are particularly sensitive to the changes within the nucleobase or ribose moieties. Although the introduction of only a single F atom implicates a rather moderate sensitivity, we were able to collect the ^{19}F NMR spectra at concentrations as low as 80 μM with standard NMR probe and only few μM using cryoprobe (the eIF4E binding experiment). Even though such labeling method would not always be competitive to more sensitive labels such as CF_3 or $C(CF_3)_3$, at least in some cases, the lower sensitivity can be compensated for by higher synthetic availability.

CONCLUSION

In summary, a novel, more general and high yielding synthetic methodologies for the preparation of fluorophosphate-containing nucleotides were developed. The first and second

approach were based on divalent-metal cation-mediated coupling of imidazole activated nucleotides with either tetra-*n*-butylammonium fluoride or triethylammonium fluorophosphate, respectively. The first approach gave access to nucleoside 5′-fluoromono-, 5′-fluorodiphosphates and some of their derivatives containing additional phosphate-chain modifications (O to CH_2, O to NH and O to BH_3 substitutions). The second approach was exemplified by the synthesis phosphate unmodified nucleoside 5′-fluorodiphosphates and 5′-fluorotriphosphates. The third approach encompassed reactions of non-activated nucleotides with a novel electrophilic reagent, fluorophosphate imidazolide, and was particularly useful for the syntheses starting from nucleotides that were difficult to activate with imidazole, either because of high polarity or chemical lability (e.g., triphosphates, 5′-phosphorylated oligos, and phosphate-modified). The three approaches gave altogether access to a collection of fluorophosphate nucleotide analogs including nucleotides varying in oligophosphate chain length (from mono- to tetraphosphate), nucleobase type (A, G, C, U, m^7G), and presence of additional phosphate modifications. The majority of compounds were reported for the first time (including 5′-fluorodiphosphorylated oligonucleotides), and some may be considered representatives of novel classes of nucleotide mimics, such as nucleoside 5′-fluoroimidodiphosphates (**3l**, **3ad**), 5′-fluoromethylenebisphosphonates (**3h**, **3i**) or 5′-fluoroboranophosphates (**3j**) and thus, are interesting objects for further studies. Notably, the fluorophosphate nucleotide analogs served as versatile reporter molecules in ^{19}F NMR enzymatic activity and binding assays, revealing their potential utility in inhibitor discovery and ligand-binding studies. The introduction of the ^{19}F label into the oligophosphate is advantageous because virtually the same synthetic protocols can be used for structurally varying nucleotides, whereas for ribose or base fluorinated nucleotides, a change of structure implicates often development of completely new synthetic route. The possibility of using 5′-fluorophosphorylated oligonucleotides for hybridization/dehybridization events is also

potentially interesting, however, more detailed studies into scope and limitations of such application are required. We hope that the improved, efficient synthetic methods and application highlights presented here may encourage broader use of such compounds in ^{19}F NMR studies on nucleotide-processing enzymes and proteins, which may contribute to the development of efficient screening platforms. Further studies on the scope and limitations of applications of nucleotides and oligonucleotides labelled with fluorophosphate moieties are in progress.

EXPERIMENTAL SECTION

I. Chemical synthesis

1. General information

1.1. Starting materials and chemical reagents

Solvents, chemical reagents and starting materials, including nucleotides: 5′-AMP (free acid form), 3′-AMP (free acid form), 5′-GMP (disodium salt), 5′-CMP (disodium salt), 5′-dCMP (disodium salt), 5′-UMP (disodium salt) and ATP (trisodium salt), were from commercial sources. Other nucleotide starting materials (**1** and **2**) were synthesized as described previously: guanosine 5′-monoboranophosphate ($GMPBH_3$,[10c] adenosine 5′-monoboranophosphate, $AMPBH_3$,[10c] adenosine 5′-(1-thiodiphosphate), ADPαS,[10c] adenosine 5′-(1-boranodiphosphate), $ADP\alpha BH_3$,[10c] guanosine 5′-(1-boranodiphosphate), $GDP\alpha BH_3$,[10c] guanosine 5′-imidodihosphate, GpNHp,[24] adenosine 5′-(methylenebisphosphonate), $ApCH_2p$,[25] guanosine 5′-(methylenebisphosphonate), $GpCH_2p$[25] and 2′,3′-cyclic-phosphoadenosine imidazolide, cPAPIm.[21] 5′-Phosphorylated nucleotides p-ON_1 and p-ON_2 (5′-P-d(GTCAATGTCA) and 5′-P-d(TGACATTGAC), respectively, in a desalted form were from commercial source. Non-phosphorylated nucleotide ON_1 (d(TGACATTGAC)) was synthesized using standard solid phase protocol and purified by RP HPLC.

1.2. Nucleotide purification by ion-exchange chromatography using DEAE Sephadex A-25 and conversion into sodium salts

The synthesized nucleotides (**3**) were purified by ion-exchange chromatography on DEAE Sephadex A-25 (HCO_3^- form) column. A column was loaded with reaction mixture and washed thoroughly with water (until the eluate did not precipitate with $AgNO_3$ solution) to elute solvents and reagents that do not bind to the column. Then, nucleotides were eluted using 0–0.7 M, 0–1.0 M, 0–1.2 M or 0–1.4 M gradient of TEAB in deionized water for nucleoside mono-, di- and tri- and tetraphosphates, respectively. Collected fractions were analyzed spectrophotometrically at 260 nm and fractions containing the desired nucleotide were analyzed by RP HPLC and poured together. The yields were calculated on the basis of optical density miliunit measurements (mOD = absorbance of the solution · volume in mL) of isolated products and corresponding staring materials (nucleotides or nucleotide P-imidazolide derivatives). Optical unit measurements were performed in 0.1M phosphate buffer pH 7.0 at 260 nm for all nucleotides except 7-methylguanosine nucleotides, and in 0.1M phosphate buffer pH 6.0 at 260 nm for m^7G nucleotides. After evaporation under reduced pressure with repeated additions of 96% and then 99.8 % ethanol (to decompose TEAB and remove residual water, respectively), nucleotides were isolated as triethylammonium (TEA) salts. These were converted either into sodium salts by precipitation with acetone solution of $NaClO_4$ or into ammonium salts (especially if additional purification or diastereomer separation was required) by purification on semi-preparative RP HPLC (as described in paragraph 1.3). In the latter case, the products were isolated after repeated freeze-drying of the collected HPLC fractions.

1.3. Analytical and preparative reversed-phase (RP) HPLC

Analytical HPLC was performed using Supelcosil LC-18-T HPLC column (4.6 x 250 mm, flow rate 1.3 mL/min) with a linear gradient 0–25 % of methanol in 0.05 M ammonium acetate buffer (pH 5.9) in 15 min. Semi-preparative HPLC was performed on Discovery RP Amide C-16 HPLC column (25 cm x 21.2 mm, 5μm, flow rate 5.0 mL/min) with linear gradients of acetonitrile in 0.05 M ammonium acetate buffer (pH 5.9). In both cases UV-detection at 254 nm was used.

1.4. Spectroscopic analysis of the synthesized compounds

The structure and homogeneity of each final product was confirmed by re-chromatography on RP HPLC, high resolution mass spectrometry using negative electrospray ionization (HRMS(-)ES), and ^{1}H NMR, ^{31}P NMR and ^{19}F NMR spectroscopy. Intermediate compounds were characterized by RP HPLC and low resolution MS(-)ES. High resolution mass spectra were recorded on LTQ Orbitrap. NMR spectra were recorded on a 400 MHz or 500 MHz spectrometer equipped with a high stability temperature unit using 5mm 4NUC probe, at 399.94/500.61 MHz (^{1}H NMR), 376.28/471.00 MHz (^{19}F NMR) and 161.90/202.65 MHz (^{31}P NMR) and at 25 ^{o}C if not stated otherwise. The ^{1}H NMR chemical shifts were reported to sodium 3-(trimethylsilyl)-2,2′,3,3′-tetradeuteropropionate (TSP) (δ = 0 ppm) as an internal standard. The ^{31}P NMR chemical shifts were referenced to 20 % phosphorus acid in D_2O (δ_P 0 ppm) as an external standard. The ^{19}F NMR chemical shifts were reported to external 10 mM NaF in D_2O (δ_F -121.5 ppm). Typical parameters for proton spectra: pulse width 6.4 μs, acquisition time 2.5 s, equilibration delay 0.5 s, sweep width 4k, 20k points, resolution 0.25 Hz, number of transients -128. Typical parameters for phosphorus spectra: pulse width 15 μs, acquisition time 2.5 s, equilibration delay 0.5 s, sweep width 32k, 160k points, resolution 0.25 Hz, number of transients -512. Typical parameters for fluorine spectra: pulse width 27 μs, acquisition time 0.6 s, equilibration delay 4.0 s, sweep width 64k, 76k points, resolution 1.0 Hz, number of transients 4–128. ^{19}F NMR binding assays were recorded on a 600 MHz

spectrometer with BB-F/^{1}H Prodigy N_2 cryoprobe operating at 298 K using 5 mm diameter NMR tubes.

2. Preparation of fluorophosphate triethylammonium salt (**4**)**:**

The commercially available fluorophosphate sodium salt was converted before the synthesis into DMF-soluble triethylammonium salt. The sodium salt (5 g) dissolved in water (ca. 75 mL) was passed through Dowex 50 W x 8 cationite in the triethylammonium form. The collected eluate was evaporated under reduced pressure to dryness and then re-evaporated few times with ethanol, the residue was dried in vacuum over P_2O_5 to yield the fluorophosphate triethylammonium salt as a white solid (4.4–4.7 g, 0.022–0.024 mol).

3. Synthesis of fluorophosphate imidazolide (FPIm, **5**)

Fluorophosphate triethylammonium salt (**4**) (1.00 g, 4.95 mmol), imidazole (3.37 g, 49.5 mmol), 2,2′-dithiodipyridine (3.27 g, 14.8 mmol) were mixed in 5 mL of DMF. Triethylamine (9.9 mmol) and triphenylphosphine (3.89 mg, 14.8 mmol) were added and the mixture was stirred for 6–8 h. The product was precipitated from reaction mixture as a lithium salt by addition of solution of anhydrous $LiClO_4$ (2.64 g, 24.75 mmol) in dry acetonitrile (200 mL). After cooling at 4 ºC for 30 min, the precipitate was filtered off, washed repeatedly with cold, dry acetonitrile and dried *in vacuum* over P_4O_{10} to afford 750–800 mg of fluorophosphate lithium salt (90–100%). MS(-)ES *m/z* 148.5 calc. for $C_3H_3N_2O_2PF$: 148.9; ^{1}H NMR (400 MHz, D_2O) δ = 7.99 (s, 1 H), 7.36 (s, 1 H), 7.15 (s, 1 H); ^{31}P NMR (162 MHz, D_2O) δ = -12.61 (d, J=951 Hz ,1 P); ^{19}F NMR (376 MHz, D_2O) δ = -63.44 (d, J=951 Hz, 1 F)

4. Synthesis of nucleotide imidazolide derivatives **(2a–2l)**

All imidazolides except **2l** were prepared using triphenyl phospihine/dithiodipyride condensation system according to well-established procedures.[21] The commercially available nucleotides were converted before the synthesis into triethylammonium salts. GMP disodium salt, CMP disodium salt and UMP disodium salt were converted by passing their aqueous solutions (ca. 1 g/15 mL) through Dowex 50 W x 8 cationite in the triethylammonium form. In each case, the collected eluate was evaporated under reduced pressure with repeated additions of ethanol, the residue dried in vacuum over P_2O_5 to yield the nucleotide triethylammonium salt as a white solid. AMP (free acid) was suspended in water/ethanol mixture and neutralized to pH 7 by a stepwise addition of triethylamine under vigorous stirring. The resulting solution was evaporated and dried analogously as described for GMP, CMP and UMP. The nucleotides obtained by chemical synthesis, were isolated as triethylammonium salts after DEAE Sephadex purification and, hence, no conversion was required in their case. Then, an appropriate nucleotide (1 mmol, TEA salt), imidazole (8 mmol), 2,2′-dithiodipyridine (3 mmol) were mixed in DMF (~2.5 mL/100 mg of nucleotide). Triethylamine (2 mmol) and triphenylphosphine (3 mmol) were added and the mixture was stirred for 6–24 h. The product was precipitated from reaction mixture as a sodium salt by addition of a solution of anhydrous $NaClO_4$ (4 mmol) in dry acetone (~8 volumes of DMF volume). After cooling at 4 ºC, the precipitate was filtered off, washed repeatedly with cold, dry acetone and dried *in vacuum* over P_4O_{10}. Yields 90–100 %. Because of the chemically labile nature of the compounds, the purity of the products was checked only by RP HPLC and by measuring optical density miliunit per mg of dry compound. Compound **35** was prepared by a different route, a one-pot two-step procedure starting from adenosine as described earlier.[21] Compound **2a** sodium salt (AMPIm): analytical RP HPLC t_R 12.0 min, MS(-)ES 396.0; 29.0 mOD/mg; **2b** sodium salt (GMPIm): analytical RP HPLC t_R 9.2 min, MS(-)ES 412.1; 24.0 mOD/mg; **2d** sodium salt (UMPIm): analytical RP HPLC t_R 8.7 min, MS(-)ES

373.0; 22.0 mOD/mg; **2c** sodium salt (CMPIm): analytical RP HPLC t_R 7.2 min, MS(-)ES 372.1; 15.3 mOD/mg; **2l** disodium salt (cPAPIm): analytical RP HPLC t_R 7.5 min, MS(-)ES 458.0; 22.5 mOD/mg; **2h** disodium salt (ApCH_2p-Im): analitycal RP HPLC t_R 6.0 min, MS(-)ES 474.1; 23.5 mOD/mg; **2i** disodium salt (GpCH_2p-Im): analitycal RP HPLC t_R 5.5 min, MS(-)ES 490.1; 19.00 mOD/mg; **2j** disodium salt (ApBH_3p-Im): analitycal RP HPLC t_R 8.2 (D1) min and 9.1 (D2), MS(-)ES 474.1; 20.20 mOD/mg; **2e** sodium salt (m^7GMP-Im): analitycal RP HPLC Rt 9.0 min, MS(-)ES 426.1; 21.00 mOD/mg;

5. Synthesis of fluorophosphate nucleotide analogs

5.1. *General procedure A:* synthesis of fluorophosphate nucleotide analogs using TBAF as a nucleophile (GP A):

Aa appropriate volume of tetra-*n*-butylammonium fluoride (TBAF) solution in THF (containing 5–6 molar excess of TBAF to the nucleotide) was mixed with DMSO (1 mL/40 mg of nucleotide) and THF was removed under reduced pressure. To the resulting solution of TBAF in DMSO, nucleotide imidazolide (1 mmol) and $ZnCl_2$ (8–10 mmol) were added and the mixture was shaken vigorously until reagents dissolved. The reaction was stirred for 1–3 h and then quenched by addition of a mixture of Na_2EDTA (8–10 mmol) and $NaHCO_3$ (~35 mmol) in deionized water. The product was purified by DEAE Sephadex chromatography and converted into sodium salt as described in paragraph 1.2. If additional purification was necessary the product was purified by RP HPLC as described in paragraph 1.3.

5.2. *General procedure B:* synthesis of fluorophosphate nucleotide analogs using triethylammonium fluorophosphate as a nucleophile (GP B):

GP B1:

To a mixture of an appropriate nucleotide imidazolide derivative (1 mmol), fluoromonophosphate triethylammonium salt (2 mmol), DMF or DMSO (1 ml/40 mg of imidazolide) and $ZnCl_2$ (10 mmol) were added and the mixture was shaken vigorously until reagents dissolved. The reaction was stirred for 1–3 h and then quenched by addition of a mixture of Na_2EDTA (10 mmol) and $NaHCO_3$ (~35 mmol) dissolved in deionized water. The product was purified by DEAE Sephadex chromatography and converted into sodium salt as described in paragraph 1.2. If additional purification was necessary the product was purified by RP HPLC as described in paragraph 1.3.

GP B2:

To a mixture of an appropriate nucleotide imidazolide derivative (1 mmol) and fluoromonophosphate triethylammonium salt (2 mmol), DMF (1 mL/40 mg of imidazolide) and $MgCl_2$ (10 mmol) were added and the mixture was shaken vigorously until reagents dissolved. The reaction was stirred for 1–3 h and then quenched by addition of 10 volumes of deionized water. The product was purified by DEAE Sephadex chromatography and converted into sodium salt as described in paragraph 1.2. If additional purification was necessary the product was purified by RP HPLC as described in paragraph 1.3.

5.3. *General procedure C:* synthesis of fluorophosphate nucleotide analogs using fluorophosphate imidazolide lithium salt (*FPIm*) as an electrophile (GP C):

To a mixture of an appropriate nucleotide (1 mmol) and fluorophosphate imidazolide lithium salt (4–4.5 mmol), DMF (1.5 mL/100 mg of nucleotide) and $ZnCl_2$ (8 mmol) were added and

the mixture was shaken vigorously until reagents dissolved. The reaction was stirred for 1–3 h and then quenched by addition a mixture of Na_2EDTA (8 equiv.) and $NaHCO_3$ (~35 equiv.) dissolved in deionized water. The product was purified by DEAE Sephadex chromatography and converted into sodium salt as described in paragraph 1.2. If additional purification was necessary the product was purified by RP HPLC as described in paragraph 1.3.

5.5.1. Adenosine 5′-fluoromonophosphate (AMPF, **3a**):

Prepared according to GP A starting from **2a** (100 mg, 2900 mOD, 0.238 mmol), TBAF (1 M solution in THF, 1195 µl, 1.19 mmol), DMSO (1.5 mL) and $ZnCl_2$ (493 mg, 2.38 mmol) yielding 80 mg of **3a** sodium salt (2320 mOD, 0.205 mmol, 86%). Reaction time: 1 hour. HR MS(-) ES *m/z* 348.0510 calc. for $C_{10}H_{12}N_5O_6PF$: 348.0515; ^{1}H NMR (400 MHz, D_2O) δ = 8.52 (s, 1 H), 8.42 (s, 1 H), 6.20 (d, J=5.2 Hz, 1 H), 4.83-4.81 (dd overlapped with HDO, 1H), 4.56-4.54 (m, 1 H), 4.41 (m, 1 H), 4.26-4.29 (m, 2 H); ^{31}P NMR (162 MHz, D_2O) δ = -5.76 (d, J=934 Hz ,1 P); ^{19}F NMR (376 MHz, D_2O) δ = -79.65 (d, J=934 Hz, 1 F)

5.5.2. Guanosine 5′-fluoromonophosphate sodium salt (GMPF; **3b**)

Prepared according to GP A starting from **2b** (100 mg, 2400 mOD, 0.199 mmol), TBAF (1 M solution in THF, 1610 µl, 1.61 mmol), DMSO (1.8 mL) and $ZnCl_2$ (250 mg, 1.84 mmol) yielding 83 mg of **3b** (2105 mOD, 0.174 mmol, 88 %). Reaction time: 1.5 h. HR MS(-)ES *m/z* 364.04614 calc. for $C_{10}H_{12}N_5O_7PF$: 364.0464; ^{1}H NMR (400 MHz, D_2O) δ = 8.13 (s, 1 H), 5.94 (d, J=5.5 Hz, 1 H), 4.74 (t, J=5.5 Hz, 1 H) 4.49 (dd, J=5.5 Hz J=3.7 Hz, 1 H), 4.35 (m, 1 H), 4.25 (m, 2 H); ^{31}P NMR (162 MHz, D_2O) δ = -5.78 (d, J=934 Hz ,1 P); ^{19}F NMR (376 MHz, D_2O) δ = -78.98 (d, J=934 Hz, 1 F)

5.5.3. Cytidine 5′-fluoromonophosphate (CMPF, **3c**):

Prepared according to GP A starting from **2c** (100 mg, 1530 mOD, 0.253 mmol), TBAF (1 M solution in THF, 1265 µl, 1.27 mmol), DMSO (1.5 mL) and $ZnCl_2$ (524 mg, 2.53 mmol) yielding 73 mg of **3c** sodium salt (1117 mOD, 0.210 mmol, 83%). Reaction time: 1 hour. HR MS(-)ES *m/z* 324.0403 calc. for $C_9H_{12}N_3O_7PF$: 324.0402; ^{1}H NMR (400 MHz, D_2O) δ = 7.86 (d, J=7.6 Hz, 1 H), 6.09 (d, J=7.6 Hz, 1 H), 5.98 (d, J=3.7 Hz, 1 H), 4.34–4.24 (m, 5 H); ^{31}P NMR (162 MHz, D_2O) δ = -5.77 (d, J=934 Hz, 1 P); ^{19}F NMR (376 MHz, D_2O) δ = -79.17 (d, J=934 Hz, 1 F)

5.5.4. Uridine 5′-fluoromonophosphate (UMPF, **3d**):

Prepared according to GP A starting from **2d** (100 mg, 1760 mOD, 0.253 mmol), TBAF (1 M solution in THF, 1265 µl, 1.27 mmol), DMSO (1.5 mL) and $ZnCl_2$ (523 mg, 2.53 mmol) yielding 69 mg of **3d** sodium salt (1207 mOD, 0.197 mmol, 78%). Reaction time: 1.5 hours. HR MS(-) ES *m/z* 325.0243 calc. for $C_9H_{11}N_2O_8PF$: 325.0243; ^{1}H NMR (400 MHz, D_2O) δ = 7.85 (d, J=8.1 Hz, 1 H), 5.98 (d, J=4.2 Hz, 1 H), 5.94 (d, J=8.1 Hz, 1 H), 4.37–4.29 (m, 4 H), 4.25–4.21 (m, 1 H); ^{31}P NMR (162 MHz, D_2O) δ = -5.77 (d, J=934 Hz, 1 P); ^{19}F NMR (376 MHz, D_2O) δ = -79.15 (d, J=934 Hz, 1 F)

5.5.5. 7-methylguanosine 5′-fluoromonophosphate (m^7GMPF, **3e**):

Prepared according to GP A starting from **2e** (300 mg, 6300 mOD, 0.553 mmol), TBAF (1 M solution in THF, 5500 µl, 5.33 mmol), DMSO (10 mL) and $ZnCl_2$ (907 mg, 6.67 mmol) yielding **3e** triethylammonium salt (4446 mOD, 0.39 mmol). The compound was only 92% pure by RP HPLC. After additional HPLC purification 126 mg (0.320 mmol, 58%) of **3e** ammonium salt were obtained. Reaction time: 5 hours. HR MS(-)ES *m/z* 378.0619 calc. for $C_{11}H_{14}N_5O_7PF$: 378.0620; ^{1}H NMR (400 MHz, D_2O) δ = 6.06 (d, J=3.5 Hz, 1 H), 8.69 (dd, J=5.0 Hz J=3.5 Hz, 1 H), 4.46 (dd, J=5.5, 5.0 Hz, 1 H), 4.41 (dt, J=5.5, 2.5 Hz), 4.36 (ddd,

J=12.0, 5.2, 2.5, 1 H), 4.26 (ddd, J=12.0, 5.7, 2.5), 4.10 (s, 3 H); ^{31}P NMR (162 MHz, D_2O) δ = -5.62 (d, J=933, Hz ,1 P); ^{19}F NMR (376 MHz, D_2O) δ = -79.06 (d, J=933 Hz, 1 F)

5.5.6. Adenosine 5′-(2-fluorodiphosphate) (ADPF, **3f**):

Method 1: Prepared according to GP A starting from **2f** (100 mg, 2900 mOD, 0.200 mmol), TBAF (1M solution in THF, 960 µl, 0.96 mmol), DMSO (2 mL) and $ZnCl_2$ (260 mg, 1.92 mmol) yielding, after HPLC purification, 35 mg of **3f** diammonium salt (1207 mOD, 0.06 mmol, 42%). Reaction time: 1 hour.

Method 2: Prepared according to GP B starting from **2a** (150 mg, 4350 mOD, 0.357 mmol), **4** (59 mg, 0.714 mmol), DMF (2 mL) and $MgCl_2$ (339 mg, 3.57 mmol) yielding 102 mg of **3f** disodium salt (2958 mOD, 0.260 mmol, 73%). Reaction time: 2 hours.

Method 3: Prepared according to GP C starting from **1a** (20 mg, 0.045 mmol), **5** (31.27 mg, 0.203 mmol), DMF (0.5 mL) and $ZnCl_2$ (49 mg, 0.36 mmol). The reaction was analyzed over time by HPLC to determine the final conversion, but the product was not isolated on preparative scale. Reaction time: 30 minutes.

HR MS(-)ES m/z 428.0166 calc. for $C_{10}H_{13}N_5O_9P_2F$: 428.0178; ^{1}H NMR (400 MHz, D_2O) δ = 8.47 (s, 1 H), 8.25 (s, 1 H), 6.14 (d, J=6.1 Hz, 1 H), 4.76 (dd, J=6.1, 4.9 Hz, 1 H), 4.52 (dd, J=4.9, J=3.7 Hz 1 H), 4.40 (m, 1 H), 4.23 (dd, J=5.2, 3.0 Hz, 2 H); ^{31}P NMR (162 MHz, D_2O) δ = -11.33 (d, J=19.3 Hz, 1 P), -17.8 (dd, J=934 Hz J=19.3 Hz, 1P); ^{19}F NMR (376 MHz, D_2O) δ = -72.5 (d, J=934 Hz, 1 F)

5.5.7. Guanosine 5′-(2-fluorodiphosphate) (GDPF, **3g**):

Method 1: Prepared according to GP A starting from **2g** (120 mg, 2880 mOD, 0.220 mmol), TBAF (1M solution in THF, 1117 µl, 1.12 mmol), DMSO (2 mL) and $ZnCl_2$ (304 mg, 2.23

mmol) yielding 72 mg of **3g** disodium salt (1207 mOD, 0.15 mmol, 42%). Reaction time: 1 hour.

Method 2: Prepared according to GP B starting from **2b** (20 mg, 0.046 mmol), **4** (11 mg, 0.055 mmol), DMF (0.5 mL) and $ZnCl_2$ (41.3 mg, 0.304 mmol). The reaction was analyzed over time by HPLC to determine the final conversion, but the product was not isolated on preparative scale. Reaction time: 1.5 hours.

Method 3: Prepared according to GP C starting from **1b** (200 mg, 0.431 mmol), **5** (269 mg, 1.73 mmol), DMF (3.5 mL) and $MgCl_2$ (662 mg, 6.9 mmol) yielding 110 mg of **3g** disodium salt (3584 mOD, 0.297 mmol, 69 %). Reaction time: 2 hours.

HR MS(-)ES *m/z* 444.01248 calc. for $C_{10}H_{13}N_5O_{10}P_2F$: 444.0127; ^{1}H NMR (400 MHz, D_2O) δ = 8.08 (s, 1 H), 5.93 (d, J=6.2 Hz, 1 H), 4.76 (1H, overlapped with HDO), 4.51 (dd, J=5.0, 3.2 Hz, 1 H), 4.36 (m, 1 H), 4.22 (dd, J=5.5, 3.5 Hz, 2 H); ^{31}P NMR (162 MHz, D_2O) δ = -10.55 (d, J=20 Hz, 1 P), -17.00 (dd, J=934 Hz J=20 Hz, 1P); ^{19}F NMR (376 MHz, D_2O) δ = -72.7 (d, J=934 Hz, 1 F)

5.5.8. Adenosine 5′-(2-fluoro-1,2-methylenediphosphate) (ApCH_2pF, **3h**):

Prepared according to GP A starting from **2h** (120 mg, 2814 mOD, 0.188 mmol), TBAF (1 M solution in THF, 1400 µl, 1.4 mmol), DMSO (3.0 mL) and $ZnCl_2$ (251.6 mg, 1.85 mmol) yielding 132 mg of **3h** disodium salt (2302 mOD, 0.153 mmol, 82 %). Reaction time: 40 minutes. HR MS(-)ES *m/z* 426.03785 calc. for $C_{11}H_{15}N_5O_8P_2F$: 426.0385; ^{1}H NMR (400 MHz, D_2O) δ = 8.85 (s, 1 H), 8.25 (s, 1 H), 6.12 (d, J=5.7 Hz, 1 H), 4.8 (overlapped with HDO), 4.53 (dd, J=4.73 Hz J=4.0 Hz, 1 H), 4.38 (m, 1 H), 4.16 (m, 2 H), 2.30 (td, J=20.0 Hz J=5.0 Hz, 2 H); ^{31}P NMR (162 MHz, D_2O) δ = 17.70 (broad d, J=970 Hz, 1 P) 15.84 (broad s, 1 P); ^{19}F NMR (376 MHz, D_2O) δ= -53.98 (dt, J=970 Hz, J=4.5 Hz 1 F)

5.5.9. Guanosine 5′-(2-fluoro-1,2-methylenediphosphate) ($GpCH_2pF$, **3i**):

Prepared according to GP C starting from **2l** (80 mg, 1523 mOD, 0.126 mmol), TBAF (1 M solution in THF, 900 µl, 0.894 mmol), DMSO (1.2 mL) and $ZnCl_2$ (162 mg, 1.19 mmol) yielding 24 mg of **3i** disodium salt (777 mOD, 0.0643 mmol, 51 %). Reaction time: 3 hours. HR MS(-)ES *m/z* 442.03359 calc. for $C_{11}H_{15}N_5O_9P_2F$: 442.0335; ^{1}H NMR (400 MHz, D_2O) δ = 8.13 (s, 1 H), 5.93 (d, J=6.4 Hz, 1 H), 4.81 (dd, J=6.4, 5.2, 1 H) 4.52 (dd, J=5.2, 3.0 Hz, 1 H), 4.34 (m, 1 H), 4.16 (m, 2 H), 2.30 (td, J=20.4 Hz J=5.2 Hz, 2 H); ^{31}P NMR (162 MHz, D_2O) δ = 17.7 (m, 1 P), 15.8 (m, 1P); ^{19}F NMR (376 MHz,D_2O) δ = -54.0 (dt, J=973 Hz J=5.2, 1 F)

5.5.10. Adenosine 5′-(2-fluoroboranodiphosphate) ($ApBH_3pF$, **3j**):

Prepared according to GP A starting from **2j** (18 mg, 737 mOD, 0.049 mmol), TBAF (1 M solution in THF, 450 µl, 0.450 mmol), DMSO (1.0 mL) and $ZnCl_2$ (81.8 mg, 0.60 mmol) yielding 1:1.2 diastereomeric mixture **3j** triethylammonium salt (362 mOD, 0.024 mmol, 48.9 %). The diastereomers were separated by RP HPLC yielding 1.4 mg of **3j** D1 ammonium salt and 2.2 mg of **3j** D2 ammonium salt. Reaction time: 2 hours. HR MS(-)ES *m/z* 426.05595 calc. for $C_{10}H_{16}N_5O_8P_2BF$: 426.0557 D1: ^{1}H NMR (500 MHz, D_2O) δ = 8.55 (s, 1 H), 8.29 (s, 1 H), 6.16 (d, J=5.5 Hz, 1 H), 4.78 (t, J=5.5 Hz, 1 H), 4.55 (dd, J=5.5, 3.0 Hz, 1 H), 4.42 (m, 1 H), 4.21 (m, 2 H), 0.0-0.9 (broad m, 3H); ^{31}P NMR (203 MHz, D_2O) δ = 89.56 (m,1 P), 13.12 (dd, J=931, 30 Hz 1 P); ^{19}F NMR (471 MHz, D_2O) δ = -71.05 (d, J=931 Hz, 1 F); D2: ^{1}H NMR (500 MHz, D_2O) δ = 8.55 (s, 1 H), 8.29 (s, 1 H), 6.17 (d, J=5.7 Hz, 1 H), 4.78 (overlapped with HDO) 4.51 (dd, J=5.0, 3.5 Hz, 1 H), 4.42 (m, 1 H), 4.12-4.30 (broad m, 2 H), 0.0-0.9 (broad m, 3H); ^{31}P NMR (203 MHz, D_2O) δ = 89.71 (m,1 P) 13.11 (dd, J=931 Hz J=31 Hz 1 P); ^{19}F NMR (471 MHz, D_2O) δ = -70.94 (d, J=931 Hz 1 F)

5.5.11. Adenosine 5′-fluoroboranomonophosphate ($ApBH_3F$, **3k**):

Prepared according to the GP A starting from **2j** (18 mg, 737 mOD, 0.049 mmol), TBAF (1 M solution in THF, 294 µl, 0.294 mmol), DMSO (1.0 mL) and $ZnCl_2$ (54 mg, 0.40 mmol) yielding 1.0:1.2 diastereomeric mixture of **3k** triethylammonium salt (293 mOD, 0.019 mmol, 39 %). The diastereomers were separated by RP HPLC yielding 1.8 mg of **3k** D1 ammonium salt and 1.9 mg of **3k** D2 ammonium salt. Reaction time: 24 hours. HR MS(-)ES *m/z* 346.0893 calc. for $C_{10}H_{15}N_5O_5PBF$: 346.0893 D1: ^{1}H NMR (400 MHz, D_2O) δ = 8.47 (s, 1 H), 8.31 (s, 1 H), 6.16 (d, J=5.0 Hz, 1 H), 4.77 (overlapped with HDO), 4.52 (m, 1 H), 4.39 (m, 1 H), 4.24 (m, 2 H), 0.0-0.8 (broad m, 3H); ^{31}P NMR (162 MHz, D_2O) δ = 95.2 (bd, J=1140 Hz, 1 P); ^{19}F NMR (376 MHz, D_2O) δ= -52.75 (d, J=1140 Hz, 1 F) D2: ^{1}H NMR (400 MHz, D_2O) δ = 8.47 (s, 1 H), 8.32 (s, 1 H), 6.16 (d, J=5.0 Hz, 1 H), 4.79 (overlapped with HDO), 4.53 (t, J=4.35, 1 H), 4.39 (m, 1 H), 4.23 (m, 2 H), 0.0-0.8 (broad m, 3H); ^{31}P NMR (162 MHz, D_2O) δ = 94.78 (bd, J=1150 Hz, 1 P); ^{19}F NMR (376 MHz, D_2O) δ= -53.00 (d, J=1150 Hz, 1 F)

5.5.12. Guanosine 5′-(2-fluoro-1,2-imidodiphosphate) sodium salt (GpNHpF, **3l**):

Prepared according to the GP A starting from **2k** (93 mg, 0.177 mmol), tetra n-butyloamonium fluoride (1 M solution in THF, 1580 µl, 1.58 mmol), DMSO (3.3 mL) and $ZnCl_2$ (189 mg, 1.39 mmol) yielding 5.4 mg of **3l** (822.51 mOD, 0.068, 25 %). Reaction time: 24 h without microwave irradiation, 3 h using microwave irradiation as described in[10c]. HR MS(-)ES *m/z* 443.02913 calc. for $C_{10}H_{14}N_6O_9P_2F$: 443.0287; ^{1}H NMR (400 MHz, D_2O) δ = 8.22 (s, 1 H), 5.94 (d, J=6.0 Hz, 1 H), 4.98 (overlapped with HDO), 4.52 (dd, J=4.8, 3.5 Hz, 1 H), 4.35 (m, 1 H), 4.14 (m, 2 H); ^{31}P NMR (162 MHz, D_2O) δ = 3.16 (dd, J=914 Hz, J=6.2 Hz 1 P), 1.15 (d, J=6.2 Hz 1P); ^{19}F NMR (376 MHz, D_2O) δ= -61.5 (d, J=914 Hz 1 F)

5.5.13. Cytidine 5′-(2-fluorodiphosphate) (CDPF, **3m**):

Prepared according to GP B starting from **2c** (200 mg, 3060 mOD, 0.506 mmol), **4** (122 mg, 0.608 mmol), DMF (4.5 mL) and $MgCl_2$ (389 mg, 4.05 mmol) yielding 85 mg of **3m** disodium salt (1660 mOD, 0.235 mmol, 54 %). Reaction time: 2.5 h. HR MS(-)ES *m/z* 404.00658 calc. for $C_9H_{13}N_3O_{10}P_2F$: 404.0066 ; ^{1}H NMR (400 MHz, D_2O) δ = 7.91 (d, J=7.7 Hz, 1 H), 6.11 (d, J=7.7 Hz 1 H), 5.99 (d, J= 4.2 Hz, 1 H), 4.26-4.34 (m, 4 H), 4.18 (m, 1H) ; ^{31}P NMR (162 MHz, D_2O) δ = -10.55 (d, J=19.07 Hz ,1 P), -17.02 (dd. J=934 Hz J=19.07 Hz, 1 P); ^{19}F NMR (376 MHz, D_2O) δ= -73.70 (d, J=934 Hz, 1 F).

5.5.14. Uridine 5′-(2-fluorodiphosphate) (UDPF, **3n**):

Prepared according to GP B starting from **2d** (200 mg, 4400 mOD, 0.455 mmol), **4** (124 mg, 0.606 mmol), DMF (4.5 mL) and $MgCl_2$ (350 mg, 3.64 mmol) yielding 90 mg of **2n** disodium salt (3516 mOD, 0.364 mmol, 80 %). Reaction time: 5h. HR MS(-)ES *m/z* 404.9906 calc. for $C_9H_{12}N_2O_{11}P_2F$: 404.9906 ; ^{1}H NMR (400 MHz, D_2O) δ = 7.94 (d, J=8.2 Hz, 1 H), 6.00 (d, J=4.5 Hz, 1 H), 5.96 (d, J=8.2 Hz, 1 H), 4.36 (m, 2 H), 4.30 (m, 1 H), 4.24 (m, 1 H), 4.21 (m, 1H); ^{31}P NMR (162 MHz, D_2O) δ = -10.64 (d, J=19.71 Hz ,1 P), -14.08 (dd. J=934 Hz J=20.10, 1 P); ^{19}F NMR (376 MHz, D_2O) δ = -72.41 (d, J=934 Hz ,1 F)

5.5.15. 2′,3′-cyclic-phosphoadenosine 5′-(2-fluorodiphosphate) (cPADPF, **3o**):

Prepared according to GP B starting from **2l** (100 mg, 1900 mOD, 0.126 mmol), **4** (50.5 mg, 0.252 mmol), DMF (2.0 mL) and $MgCl_2$ (96 mg, 1.0 mmol), yielding 85 mg of **3o** triethylammonium salt (1240 mOD, 0.0822 mmol, 65 %). Reaction time: 2.5 h. Before the

reaction with RNAse T2 the product was additionally purified by RP phase HPLC and isolated as ammonium salt.

MS(-)ES m/z 489.8 calc. for $C_{10}H_{12}N_5O_{11}P_3F$: 489.9736; ^{1}H NMR (400 MHz, D_2O) δ = 8.37 (s, 1 H), 8.19 (s, 1 H), 6.32 (d, J=4.0 Hz, 1 H), 5.45 (ddd, J=10.8, 6.7, 4.0 Hz, 1 H), 5.24 (ddd, J=7.2, 6.7, 3.9 Hz, 1 H), 4.65 (m, 1 H), 4.27 (m, 1 H); ^{31}P NMR (162 MHz, D_2O) δ = 19.91 (dd, J=10.8 Hz, J=7.2 Hz, 1 P), -11.48 (d, J=19.0 Hz, 1 P); -17.78 (dd, J=934, 19.00 Hz, 1P); ^{19}F NMR (376 MHz, D_2O) δ = -72.67 (d, J=934 Hz, 1 F)

5.5.16. Adenosine 5′-(3-fluorotriphosphate) (ATPF; **3p**)

Method 1: Prepared according to GP C starting from **1d** (367 mg, 0.585 mmol), **5** (410 mg, 2.63 mmol), DMF (6 mL) and $ZnCl_2$ (636 mg, 4.68 mmol) yielding 202 mg of **3p** trisodium salt (5285 mOD, 0.352 mmol, 60 %). Reaction time: 5 hours. HR MS(-)ES *m/z* 507.9847 calc. for $C_{10}H_{14}N_5O_{12}P_3F$: 507.9841; ^{1}H NMR (500 MHz, D_2O) *δ* = 8.54 (s, 1 H), 8.29 (s, 1 H), 6.14 (d, J=6.0 Hz, 1 H), 4.79 (dd J=6.0, 5.2 Hz, 1 H), 4.56 (dd, J=5.2, 3.4 Hz, 1 H), 4.41 (m, 1 H), 4.26 (m, 2 H); ^{31}P NMR (203 MHz, D_2O) *δ* = -7.04 (d, J=19.0 Hz, 1 P), -13.58 (dd, J=934, 17.6 Hz, 1 P), -18.78 (dd, J=19.0, 17.6 Hz, 1 P); ^{19}F (471 MHz, D_2O) *δ*= -72.26 (d, J=934 Hz 1 F)

Method 2: Prepared according to GP B starting from **2f** (5 mg, 0.01 mmol), **4** (4 mg, 0.02 mmol), DMF (0.35 mL) and $ZnCl_2$ (10.44 mg, 0.08 mmol). The reaction was analyzed over time by HPLC to determine final conversion, but the product was not further purified. Reaction time: 2 hours.

5.5.17. Guanosine 5′-(3-fluorotriphosphate) (GTPF; **3q**)

Method 1: Prepared according to GP B starting from **2g** (20 mg, 0.037 mmol), **4** (9.0 mg, 0.044 mmol), DMF (0.5 mL) and $MgCl_2$ (28.2 mg, 0.296 mmol). The reaction was analyzed

over time by HPLC to determine final conversion, but the product was not further purified. Reaction time: 0.5 hours.

Method 2: Prepared according to GP C starting from **1e** (96 mg, 0.148 mmol), **5** (123 mg, 0.79 mmol), DMF (1.5 mL) and $MgCl_2$ (227 mg, 2.37 mmol) yielding 9.9 mg of **3q** trisodium salt (1003 mOD, 0.083 mmol, 56 %). Reaction time: 3 hours. HR MS(-)ES *m/z* 523.9799 calc. for $C_{10}H_{14}N_5O_{13}P_3F$: 523.9790; ^{1}H NMR (400 MHz, D_2O) δ = 8.10 (s, 1 H), 5.92 (d, J=6.7 Hz, 1 H), 4.82 (dd, J=6.7, 5.1, 1 H), 4.53 (dd, J=5.1, 2.7 Hz, 1 H), 4.34 (m, 1 H), 4.21 (m, 2 H); ^{31}P NMR (162 MHz, D_2O) δ = -10.83 (d, J=20.5 Hz, 1 P), -17.3 (dd, J=936 Hz, J=17.6 Hz, 1 P), -22.61 (t, J=19.07 Hz, 1 P); ^{19}F (376 MHz, D_2O) δ= -73.39 (d, J=936 Hz 1 F)

5.5.18. Deoxycytidine 5′-(2-fluorodiphosphate) (dCDPF, **3r**):

dCDPF was prepared according to GP C starting from **1c** (200 mg, 0.483 mmol), fluorophosphate imidazolide lithium salt (0.580 mmol), DMF (5 mL) and $MgCl_2$ (734 mg, 7.73 mmol) yielding 161 mg of **3r** disodium salt (2853 mOD, 0.402 mmol, 83 %). The compound was only 85% pure by ^{19}F NMR. After additional HPLC purification, 130 mg (0.307 mmol, 64%) **3r** diammonium salt were obtained. Reaction time: 24h. HR MS(-)ES *m/z* 388.0117 calc. for $C_9H_{13}N_3O_9P_2F$: 388.0117; ^{1}H NMR (400 MHz, D_2O) δ = 7.97 (d, J=7.6 Hz, 1 H), 6.33 (dd, J=7.5, 6.0 Hz, 1 H), 6.13 (d, J=7.6 Hz, 1 H), 4.58 (m, 1 H), 4.23 (m, 1 H), 4.19 (m, 2 H), 2.43 (ddd, J=14.0, 6.0, 3.5 Hz, 1H), 2.30 (ddd, J=14.0, 7.5, 6.1 Hz, 1H); ^{31}P NMR (162 MHz, D_2O) δ = -14.39 (d, J=19.84 Hz ,1 P), -20.88 (dd. J=934 Hz J=19.95 Hz, 1 P); ^{19}F NMR (376 MHz, D_2O) δ= -72.42 (d, J=934 Hz, 1 F)

5.5.19. Adenosine 5′-(4-fluorotetraphosphate) (Ap_4F, **3s**):

Prepared according to the GP C starting from **1f** (49 mg, 0.060 mmol), **5** (42 mg, 0.27 mmol), DMF (3 mL) and $ZnCl_2$ (65.3 mg, 0.48 mmol) yielding **3s** triethylammonium salt (255 mOD, 0.017 mmol, 28 %). After conversion into ammonium salt on RP HPLC 9.0 mg (0.014 mmol)

of **3s** tetraammonium salt were obtained. Reaction time: 1.5 hours. HR MS(-)ES *m/z* 587.9502 calc. for $C_{10}H_{15}N_5O_{15}P_4F$: 587.9505; ^{1}H NMR (400 MHz, D_2O) δ = 8.52 (s, 1 H) 8.25 (s, 1 H), 6.14 (d, J=6.5 Hz, 1 H), 4.79 (overlapped with HDO), 4.58 (dd, J=5.2, 3.2 Hz, 1 H), 4.41 (m, 1 H), 4.19-4.30 (m, 2 H); ^{31}P NMR (162 MHz, D_2O) δ = -11.37 (m, 1 P), -17.98 (dd, J=935 Hz J=17 Hz 1P) -23.31 (m, 2 P); ^{19}F NMR (376 MHz, D_2O) δ= -72.00 (d, J=935 Hz 1 F)

5.5.20. 7-methylguanosine 5′-(2-fluorodiphosphate) (m^7GDPF, **3t**):

Prepared according to GP C starting from **1g** (100 mg, 2620 mOD, 0.210 mmol), **5** (218 mg, 1.40 mmol), DMF (9.0 mL) and $ZnCl_2$ (342 mg, 2.51 mmol) yielding 1234 mg **3t** triethylammonium salt (1486.13 mOD, 0.130 mmol, 57 %). Reaction time: 48 hours. HR MS(-)ES *m/z* 458.0251 calc. for $C_{11}H_{15}N_5O_{10}P_2F$: 458.0284; ^{1}H NMR (400 MHz, D2O) δ = 6.06 (d, J=3.6 Hz, 1 H), 4.67 (dd, J=5.0, 3.5 Hz, 1 H), 4.48 (dd, J=5.2, 5.0 Hz, 1 H), 4.42 (m, 1H), 4.37-4.20 (m, 1 H), 4.11 (s, 3 H); ^{31}P NMR (162 MHz, D2O) δ = -11.10 (d, J=20 Hz ,1 P), -17.5 (dd, J=934, 20 Hz, 1 P); ^{19}F NMR (376 MHz,D2O) δ = -72.58 (d, J=934 Hz, 1 F)

5.5.21. 7-methylguanosine 5′-(3-fluorotriphosphate) (m^7GTPF, **3u**):

Prepared according to GP C starting from **1h** (120 mg, 2604 mOD, 0.178 mmol), **5** (115 mg, 0.767 mmol), DMF (3.5 mL) and $ZnCl_2$ (194 mg, 1.43 mmol) yielding 51.5 mg of **3u** trisodium salt (1560 mOD, 0.137 mmol, 60 %). Reaction time: 1.5 hours. HR MS(-)ES *m/z* 537.9923 calc. for $C_{11}H_{16}N_5O_{13}P_3F$: 537.9947; ^{1}H NMR (500 MHz, D_2O) δ = 9.20 (s, 1 H), 6.08 (d, J=3.7 Hz, 1 H), 4.69 (dd, J=4.8, 3.7 Hz 1 H), 4.52 (dd, J= 5.5, 4.8 Hz 1 H), 4.42 (dd, J=5.5, 2.6 Hz 1 H), 4.31 (m, 2 H), 4.13 (s, 3 H); ^{31}P NMR (203 MHz, D_2O) δ = -7.04 (d, J=19.5 Hz ,1 P), -13.56 (dd, J=934, 18.0 Hz 1 P), -18.69 (dd, J=19.5, 18.0 Hz 1 P); ^{19}F NMR (471 MHz, D_2O) δ = -72.28 (d, J=934 Hz, 1 F)

5.5.22. Adenosine 5′-(3-fluoro1,2-methylenetriphosphate) (ApCH_2ppF, **3v**):

Prepared according to GP C starting from **1i** (120 mg, 2440 mOD, 0.162 mmol), **5** (134.8 mg, 0.86 mmol), DMF (3.0 mL) and $ZnCl_2$ (208.9 mg, 1.54 mmol) yielding 63 mg of **3v** trisodium salt (1751 mOD, 0.117 mmol, 72 %). Reaction time: 1.5 hours. HR MS(-)ES *m/z* 506.00478 calc. for $C_{11}H_{16}N_5O_{11}P_3F$: 506.0049; ^{1}H NMR (400 MHz, D_2O) δ = 8.56 (s, 1 H), 8.25 (s, 1 H), 6.12 (d, J=5.7 Hz, 1 H), 4.80 (dd, J=5.5, 5.0, 1 H), 4.56 (dd, J=5.0, 3.7 Hz, 1 H), 4.38 (m, 1 H), 4.18 (m, 2 H), 2.37 (t, J=20.3 Hz, 2 H); ^{31}P NMR (162 MHz, D_2O) δ = -17.43 (dd, J=931 Hz J=25.7 Hz, 1 P), 8.7 (m, 1 P), 16.61 (m, 1 P); ^{19}F NMR (376 MHz, D_2O) δ= -71.26 (d, J=931 Hz, 1 F)

5.5.23. Guanosine 5′-(3-fluoro-1,2-methylenetriphosphate) ($GpCH_2ppF$, **3w**):

Prepared according to GP C starting from **1j** (120 mg, 0.186 mmol), **5** (183 mg, 1.17 mmol), DMF (3.5 mL) and $ZnCl_2$ (297 mg, 2.18 mmol) yielding 33 mg of **3w** trisodium salt (1411 mOD, 0.117, 63 %). Reaction time: 3 hours. HR MS(-)ES *m/z* 522.0003 calc. for $C_{11}H_{16}N_5O_{12}P_3F$: 521.9998; ^{1}H NMR (400 MHz, D_2O) δ = 8.24 (s, 1 H), 5.94 (d, J=6.0 Hz, 1 H), 4.82 (dd, J=6.0, 5.2 1 H), 4.56 (dd, J=5.2, 3.2 Hz, 1 H), 4.34 (m, 1 H), 4.18 (m, 2 H); 2.37 (t, J=20.5 Hz, 2 H); ^{31}P NMR (162 MHz, D_2O) δ = 17.38 (m, 1 P), 9.43 (dtd, J=26.4, 20.5, 9.0 Hz 1 P), -16.6 (dd, J=932 Hz J=26.4 Hz 1 P); ^{19}F NMR (376 MHz,D_2O) δ = -72.2 (d, J=932 Hz 1 F)

5.5.24. Guanosine 5′-(2-fluoro-1-thiodiphosphate) (Gp_SpF, **3x**):

Prepared according to GP C starting from **1k** (120 mg, 0.25 mmol), **5** (175.5 mg, 1.13 mmol), DMF (3.0 mL) and $ZnCl_2$ (272 mg, 1.99 mmol) yielding 1:1 P-diastereomeric mixture of **3x** triethylammonium salt (1744 mOD, 0.144 mmol, 74 %). The diastereomers (marked D1 and D2 according to the elution order RP HPLC column) were separated by RP HPLC yielding 25.9 mg of **3x** D1 ammonium salt and 27.8 mg of Gp_SpF D2 ammonium salt. Reaction time: 3 hours. HR MS(-)ES *m/z* 459.98970 calc. for $C_{10}H_{13}N_5O_9P_2SF$: 459.9899; D1: ^{1}H NMR (400

MHz, D_2O) δ = 8.19 (s, 1 H), 5.94 (d, J=6.2 Hz, 1 H), 4.79 (overlapped with HDO), 4.52 (dd, J=5.1, 3.1 Hz, 1 H), 4.38 (m, 1 H), 4.31-4.22 (m, 2 H); ^{31}P NMR (162 MHz, D_2O) δ = 44.3 (d, J=27.3 Hz ,1 P), -18.84 (dd, J=935 Hz J=27.3 Hz, 1 P); ^{19}F NMR (376 MHz, D_2O) δ = -72.46 (d, J=935 Hz, 1 F) D2: ^{1}H NMR (400 MHz, D_2O) δ = 8.15 (s, 1 H), 5.94 (d, J=6.5 Hz, 1 H), 4.80 (overlapped with HDO), 4.52 (dd, J=5.1, 3.1 Hz, 1 H), 4.38 (m, 1 H), 4.32-4.22 (m, 2 H); ^{31}P NMR (162 MHz, D_2O) δ = 44.25 (d, J=27.0 Hz ,1 P), -18.84 (dd, J=935 Hz J=27.0 Hz 1 P); ^{19}F NMR (376 MHz,D_2O) δ = -71.1 (d, J=935 Hz 1 F)

5.5.25. Guanosine 5′-(2-fluoro-1-boranodiphosphate) ($GpBH_3pF$, **3y**):

Prepared according to GP C starting from **1l** (300 mg, 0.58 mmol), **5** (176 mg, 1.12 mmol), DMF (3.0 mL) and $MgCl_2$ (191 mg, 2.0 mmol) yielding 1:1.3 diastereomeric mixture 18.2 mg of **3y** triethylammonium salt (740.65 mOD, 0.062 mmol, 11%). The diastereomers were separated by RP HPLC yielding 6.3 mg of $GpBH_3pF$ D1 ammonium salt and 11.9 mg of $GpBH_3pF$ D2 ammonium salt. Reaction time: 3 hours. HR MS(-) ES *m/z* 442.05048 calc. for $C_{10}H_{16}N_5O_9P_2BF$: 442.0506; D1: ^{1}H NMR (400 MHz, D_2O) δ = 8.16 (s, 1 H), 5.95 (d, J=6.0 Hz, 1 H), 4.76 (overlapped with HDO), 4.52 (dd, J=4.5, 3.5 Hz, 1 H), 4.36 (m, 1 H), 4.19 (m, 2 H), 0.0-0.9 (broad m, 3 H); ^{31}P NMR (162 MHz, D_2O) δ = 89.98 (m,1 P), 17.58 (dd, J=931, 30 Hz 1 P); ^{19}F NMR (376 MHz, D_2O) δ = -70.94 (d, J=931 Hz 1 F); D2: ^{1}H NMR (400 MHz, D_2O) δ = 8.13 (s, 1 H), 5.94 (d, J=6.2 Hz, 1 H), 4.76 (overlapped with HDO), 4.48 (dd, J=5.0, 3.2 Hz, 1 H), 4.36 (m, 1 H), 4.14-4.27 (broad m, 2 H), 0.0-0.85 (broad m 3 H); ^{31}P NMR (162 MHz, D_2O) δ = 82.75 (m,1 P), 17.57 (dd, J=931, 30 Hz 1 P); ^{19}F NMR (376 MHz, D_2O) δ = -70.92 (d, J=931 Hz, 1 F)

5.5.26. Adenosine 5′-(3-fluoroboranotriphosphate) ($ApBH_3ppF$, **3z**):

Prepared according to GP C starting from **1m** (60 mg, 1140 mOD, 0.096 mmol), **5** (67.8 mg, 0.43 mmol), DMF (6 mL) and $MgCl_2$ (73 mg, 0.77 mmol) yielding 1.0:1.3 diastereomeric

mixture of **3z** triethylammonium salt (938 mOD, 0.062 mmol, 82 %). The diastereomers were separated by RP HPLC yielding 9.3 mg of **3z** ammonium salt and 10.9 mg of **3z** D2 ammonium salt. Reaction time: 1 hours. HR MS(-) ES *m/z* 506.02203 calc. for $C_{10}H_{17}N_5O_{11}P_3BF$: 506.0221; ^{1}H NMR (400 MHz, D_2O) δ = 8.59 (s, 1 H), 8.29 (s, 1 H), 6.16 (d, J=5.7 Hz, 1 H), 4.80 (overlapped with HDO), 4.60 (dd, J=4.7, 3.5 Hz, 1 H), 4.42 (m, 1 H), 4.17-4.29 (broad m, 2 H), 0.0-0.9 (broad m, 3 H); ^{31}P NMR (162 MHz, D_2O) δ = 83.33 (m,1 P), -18.08 (dd, J=934, 18 Hz 1 P), -23.28 (dd, J=28, 18 Hz, 1 P); ^{19}F NMR (376 MHz, D_2O) δ = -72.12 (d, J=934 Hz, 1 F); D2: ^{1}H NMR (400 MHz, D_2O) δ = 8.57 (s, 1 H), 8.28 (s, 1 H), 6.14 (d, J=6.0 Hz, 1 H), 4.76 (overlapped with HDO), 4.52 (dd, J=4.8, 3.6 Hz, 1 H), 4.42 (m, 1 H), 4.17-4.32 (broad m, 2 H), 0.00-0.85 (broad m, 3 H); ^{31}P NMR (162 MHz, D_2O) δ = 84.26 (m,1 P), -18.06 (dd, J=933, 15 Hz 1 P) -23.26 (m, 1 P); ^{19}F NMR (376 MHz, D_2O) δ = -72.12 (d, J=933 Hz, 1 F)

5.5.27. Guanosine 5′-(3-fluoroboranotriphosphate) ($GpBH_3ppF$, **3aa**):

Prepared according to the GP C starting from **1n** (60 mg, 0.094 mmol), **5** (66.2 mg, 0.42 mmol), DMF (1 mL) and $MgCl_2$ (70.9 mg, 0.752 mmol) yielding 1.0:1.2 diastereomeric mixture of **3aa** triethylammonium salt (675 mOD, 0.056 mmol, 68 %). The diastereomers were separated by RP HPLC yielding 8.0 mg of **3aa** D1 ammonium salt and 3.7 mg **3aa** D2 ammonium salt. Reaction time: 1.5 hours. HR MS(-)ES *m/z* 522.0169 calc. for $C_{10}H_{17}N_5O_{12}P_3BF$: 522.0169; D1: ^{1}H NMR (400 MHz, D_2O) δ = 8.23 (s, 1 H), 5.95 (d, J=6.2 Hz, 1 H), 4.80 (overlapped with HDO), 4.57 (m, 1 H), 4.36 (m, 1 H), 4.14-4.27 (broad m, 2 H), 0.0-0.9 (broad m, 3 H); ^{31}P NMR (162 MHz, D_2O) δ = 83.78 (m,1 P), -18.10 (dd, J=934, 18 Hz 1 P), -23.35 (dd, J=30, 18 Hz, 1 P); ^{19}F NMR (376 MHz, D_2O) δ = -72.33 (d, J=934 Hz, 1 F); D2: ^{1}H NMR (400 MHz, D_2O) δ = 8.35 (s, 1 H), 5.96 (d, J=5.7 Hz, 1 H), 4.80 (overlapped with HDO), 4.50 (t, J=3.7 Hz, 1 H), 4.37 (m, 1 H), 4.21-4.27 (broad m, 2 H), 0.00-0.85 (broad m, 3 H); ^{31}P NMR (162 MHz, D_2O) δ = 83.88 (broad m,1 P), -15.22 (broad

d, J=930 Hz, 1 P) -23.33 (broad s, 1 P); ^{19}F NMR (376 MHz, D_2O) δ = -72.3 (d, J=930 Hz, 1 F)

5.5.28. Guanosine 5′-(3-fluoroimidotriphosphate) trisodium salt (GpNHppF, **3ab**):

Prepared according to the GP C starting from **1o** (100 mg, 1760 mOD, 0.146 mmol), **5** (169.6 mg, 1.09 mmol), DMSO (2 mL) and $ZnCl_2$ (168.9 mg, 1.24 mmol) yielding 61.4 mg of **3ab** (1214 mOD, 0.100 mmol, 70 %). Reaction time 2 hours. HR MS(-)ES *m/z* 522.99523 calc. for $C_{10}H_{15}N_6O_{12}P_3F$: 522.9950; ^{1}H NMR (400 MHz, D_2O) δ = 8.40 (s, 1 H), 5.96 (d, J=6.0 Hz, 1 H), 4.80 (overlapped with HDO), 4.55 (dd, J=4.7, 3.7 Hz, 1 H), 4.37 (m, 1 H), 4.16 (m, 2 H); ^{31}P NMR (162 MHz, D_2O) δ = -0.92 (s, 1 P), -10.87 (d, J=19 Hz, 1 P), -17.38 (dd, J=930 Hz J=19 Hz, 1 P); ^{19}F NMR (376 MHz, D_2O) δ= -72.12 (d, J=930 Hz 1 F)

5.5.29. Adenosine 5′-(3-thiofluorotriphosphate) (ATPFαS, **3ac**):

ATPFαS was prepared according to the GP C starting from **1p** (21.5 mg, 0.0334 mmol), **5** (23.45 mg, 0.15 mmol), DMF (3 mL) and $ZnCl_2$ (36.3 mg, 0.27 mmol) yielding 1:1.4 diastereomeric mixture **3ac** triethylammonium salt (302.3 mOD, 0.0201, 60 %). The diastereomers were separated by RP HPLC yielding 2.7 mg of **3ac** D1 ammonium salt and 4.8 mg **3ac** D2 ammonium salt. Reaction time: 3 hours. HR MS(-)ES *m/z* 523.9606 calc. for $C_{10}H_{14}N_5O_{11}P_3SF$: 523.9613; D1: ^{1}H NMR (400 MHz, D_2O) δ = 8.69 (s, 1 H), 8.30 (s, 1 H), 6.16 (d, J=6.2 Hz, 1 H), 4.81 (dd, J=6.2, 5.5 Hz, 1 H), 4.58 (dd, J=5.5 Hz J=3.2 Hz, 1 H), 4.43 (m, 1 H), 4.23-4.35 (broad m, 2 H); ^{31}P NMR (162 MHz, D_2O) δ = 43.60 (d, J=26 Hz ,1 P), -18.13 (dd, J=935, 18 Hz, 1 P), -24.30 (dd, J=26, 18 Hz, 1 P); ^{19}F NMR (376 MHz, D_2O) δ = -72.23 (d, J=935 Hz, 1 F) D2: ^{1}H NMR (400 MHz, D_2O) δ = 8.61 (s, 1 H), 8.28 (s, 1 H), 6.15 (d, J=6.6 Hz, 1 H), 4.80 (overlapped with HDO), 4.58 (m, 1 H), 4.43 (m, 1 H), 4.30 (m, 2 H);

^{31}P NMR (162 MHz, D_2O) δ = 43.41 (d, J=25 Hz ,1 P), -18.13 (dd, J=935, 17 Hz, 1 P), -24.35 (dd, J=25, 17 Hz, 1 P); ^{19}F NMR (376 MHz, D_2O) δ = -72.23 (d, J=935 Hz, 1 F)

5.5.30. 7-methylguanosine 5′-(2-fluoro-1,2-imidodiphosphate) (m^7GpNHpF, **3ad**):

To compound **3l** (22 mg, 0.034 mmol, 411 mOD) dissolved in deionized water (1.5 mL), acetic acid CH_3COOH (15 µl) and $(CH_3O)_2SO_2$ (200 µl, 2.1 mmol) were added. The mixture was stirred for 2 h at r.t. Over this period pH was carefully controlled and if necessary adjusted to 4.0–5.0 by addition of 5% NaOH. The product was purified by DEAE Sephadex and RP HPLC to afford 8.3 mg of **3ad** ammonium salt (318 mOD, 78 %).. HR MS(-)ES *m/z* 457.04422 calc. for $C_{11}H_{16}N_6O_9P_2F$: 457.0444; ^{1}H NMR (400 MHz, D_2O) δ = 6.08 (d, J=3.5 Hz, 1 H), 4.67 (dd, J=4.7, 3.5 Hz, 1 H), 4.51 (d, J=5.5, 4.7 Hz, 1 H), 4.41 (m, 1 H), 4.30-4.12 (m, 2H), 4.11 (s, 3 H); ^{31}P NMR (162 MHz, D_2O) δ = -0.90 (~d, J=5.5 Hz, 1 P), -3.00 (dd, J=915, 5.5 Hz, 1 P); ^{19}F NMR (376 MHz,D_2O) δ = -61.3 (d, J=915 Hz, 1 F)

5.5.31. Oligonucleotide 1: F-ON_1 **(6a)**:

p-ON_1 (3.87 mg, 125.16 mOD,1.25 µmol) was moisturized with 5 µl of deionized water. Then DMSO (300 µl), compound **5** (10 mg, 0.06 mmol), $ZnCl_2$ (12.5 mg, 0.092 mmol), were added and the mixture was stirred for 48 h (r.t.). After HPLC purification 25.5 mOD (ammonium salt) of F-ON_1 were obtained (20.4%). HR MS(-)ES *m/z* 1061.4887 calc. for $C_{98}H_{122}N_{37}O_{63}P_{11}F^3$: 1061.4864; ^{19}F NMR (376 MHz,D_2O) δ = -73.54 (d, J=933 Hz, 1 F)

5.5.32. Oligonucleotide 2: F-ON_2 **(6b)**:

p-ON_2 (3.78 mg, 118.73 mOD, 1.22 μmol) was moisturized with 5 μl of deionized water. Then DMSO (300 μl), compound **5** (10 mg, 0.06 mmol) and $ZnCl_2$ (13.09 mg, 0.096 mmol), were added and the mixture was stirred for 48 h (r.t.). After HPLC purification 25.18 mOD (ammonium salt) of F-ON_2 were obtained (21.2%). HR MS(-)ES *m/z* 1061.4889 calc. for $C_{98}H_{122}N_{37}O_{63}P_{11}F^{3-}$: 1061.4865; ^{19}F NMR (376 MHz,D_2O) δ = -72.59 (d, J=933 Hz, 1 F)

II. Biophysical and enzymatic experiments

II.1. Proteins

Mouse eukaryotic initiation factor eIF4E (residues 28-217) an human DcpS were expressed as described with minor modifications.[26] SVPDE, and RNAse T2 were purchased from Sigma-Aldrich (Poland) and Mo Bi Tec (Germany), respectively.

II.2. ^{19}F NMR monitored oligonucleotide hybridization

The concentrations of oligonucleotides (ON_1, F-ON_1, F-ON_2) were determined spectrophotometrically in 0.1 M phosphate buffer pH 7.0 taking an estimated extinction coefficient ε_{260}= 128000 M^{-1} cm^{-1}. For ^{19}F NMR measurements the oligonucleotides were dissolved in 500 μL SSC buffer (30 mM sodium citrate, 300 mM sodium chloride pH 7.0 and 10% D_2O) at the desired final concentration (80–240 μM). Titrations were performed by adding aliquots of complementary oligonucleotide from 100 mM stock solution. After each addition the sample was incubated at 95 °C for 3 min, left to cool down at r.t and then cooled for 30 min at 4 °C. ^{19}F NMR spectra were then recorded on 400 MHz spectrometer using 5mm 4NUC probe at 15 °C using standard puls sequence (usually 200 transients). A negative control sample performed by mixing the studied fluorinated oligo with an equal concentration of non-fluorinated nucleotide of the same sequence (no δ_F changes were observed).

II.3. ^{19}F NMR monitored enzymatic reactions

The enzymatic reactions were run in standard NMR tubes and contained a fluorophosphate-containing substrate or mixture of substrates at 200 μM–2 mM concentration dissolved in 600 μl of an appropriate buffer. The amount of enzyme added was adjusted empirically, usually, to achieve nearly complete substrate degradation within 30–120 min. Reactions were then monitored using a standard ^{19}F NMR pulse sequence (usually 8-32 transients) in 3–5 min intervals. The spectra were recorded on 400 MHz spectrometer using 5mm 4NUC probe. The reaction progress under the same conditions was independently analyzed using RP HPLC (to initially optimize the conditions and to confirm the structures of reaction products). Specific conditions were as follows. **SVPDE**: 1 mM AMPF, 1 mM ADPF, 1 mM ATPF, 1 mM Ap4F or their unequimolar mixtures at 37 °C in SVPDE buffer (50 mM Tris-CH_3COOH, pH 8.0 containing 14 mM $MgCl_2$ and 15% D_2O). **Human DcpS:** 1000 μM m^7GMPF, 500 μM m^7GDPF or 500 μM m^7GTPF at 30 °C in DcpS buffer (50 mM Tris-HCl pH 7.9 containing 150 mM NaCl, 2 mM DTT and 15% D_2O). **RNAse T2:** 2 mM cPAPPF at 37 °C in T2 buffer (100 mM CH_3COONH_4 pH 6.0 and 15 % D_2O).

II.4. Fluorescence quenching titration assay

The affinities for eIF4E and DcpS were studied by time-synchronized titration method.[27] Fluorescence data were collected using a quartz cuvette with optical path length of 4 mm for absorption and 10 mm for emission. The experiments were performed at 20 ˚C either in 50 mM Hepes/KOH buffer pH 7.20 containing 100 mM KCl, 0.5 mM EDTA and 1 mM DTT (for eIF4E) or in 50 mM Tris/HCl buffer pH 7.60 containing 200 mM KCl, 0.5 mM EDTA and 1 mM DTT (for DcpS). Titrations were carried out by adding 1 μl aliquots of tested ligand to 1400 μl solution of 0.1 μM eIF4E or 0.2 μM DcpS. eIF4E fluorescence signal was excited at 295 or 280 nm (slit 2.5 nm, auto cut-off filter) and monitored at 320 or 337 nm (slit

4 nm, 290 nm cut-off filter), respectively. DcpS fluorescence was excited at 280 nm (slit 2.5 nm, auto cut-off filter) and observed at 340 nm (slit 4 nm). Data correction for sample dilution and inner filter effect were applied. Association equilibrium constants (K_{AS}) were determined by fitting the theoretical dependence of fluorescence intensity in the function of total concentration of tested ligand as described earlier.[27] The final association constants K_{AS} were calculated from triplicates as weighted average, with the weights taken from reciprocal standard deviations squared. The dissociation constant values were calculated according to the equation $K_D = 1/K_{AS}$. The Gibbs free energies of binding were calculated from the K_{AS} values according to the standard equation $\Delta G° = -RT\ln K_{AS}$.

II.5. ^{19}F NMR binding assays

The PROJECT-CPMG sequence (90° x−[τ−180°y−τ−90°y−τ−180°y−τ]n−acq) was applied as described by Aguilar et al.,[28] using 40 ms filter, acquisition time 0.90 s and 128 transients. Samples typically contained 0.015 mM eIF4E and 0.05 mM m^{7}GTPF in HEPES (50 mM, pH 7.2) containing 100 mM KCl, 0.5 mM EDTA, 1mM DTT and 10% D_2O. Increasing amounts of displacing ligand (m^{7}GpppG or m^{7}GppCH$_2$ppG) were added from stock solutions. The protein-ligand complex concentration [PL], which is equal to the concentration of m^{7}GTPF displaced from the protein, was then plotted against ligand concentration (L_0). The apparent dissociation constant values K_{app} (defined as the concentration of the free ligand when 50% of the protein is bound to the ligand in the presence of the reporter ligand) were determined by fitting the experimental points to a theoretical curve $[PL]=P_0L_0/(K_{app}+L_0)$.

When concentration of the reporter ligand [R] and its dissociation constant (K_{rep}) are known, the correlation between apparent K_{app} of the ligand and the actual association constant is given by equation:[29]

$$K_D = K_{app}/([R_{50}]/K_{rep} + [P]_0/K_{rep}+1) \quad (7)$$

Where $[R_{50}]$ is the free reporter ligand concentration when 50% of the ligand is displaced from the enzyme and $[P]_0$ is the free enzyme concentration in the presence of reporter ligand.

$[R_{50}]$ is described as:

$$[R_{50}] = R_0 - 0.5(P_0 - [P]) \quad (8)$$

where P_0 is the initial concentration of enzyme and R_0 is the total concentration of the reporter ligand.

$[P]_0$ is given by equation:

$$[P]_0 = -0.5(K_{rep} + R_0 - P_0) + 0.5\ ((K_{rep} + R_0 - P_0)^2 + 4P_0K_{rep})^{0.5}$$

ASSOCIATED CONTENT

Supporting Information

Supporting Tables S1–S2 and Supporting Figures S1–S11; HPLC profiles for all the syntheses and purified products, NMR spectra and HRMS spectra for the products. This material is available free of charge via the Internet at http://pubs.acs.org.

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