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Synthesis of trifluoromethylated purine ribonucleotides and their evaluation as ^{19}F NMR probes

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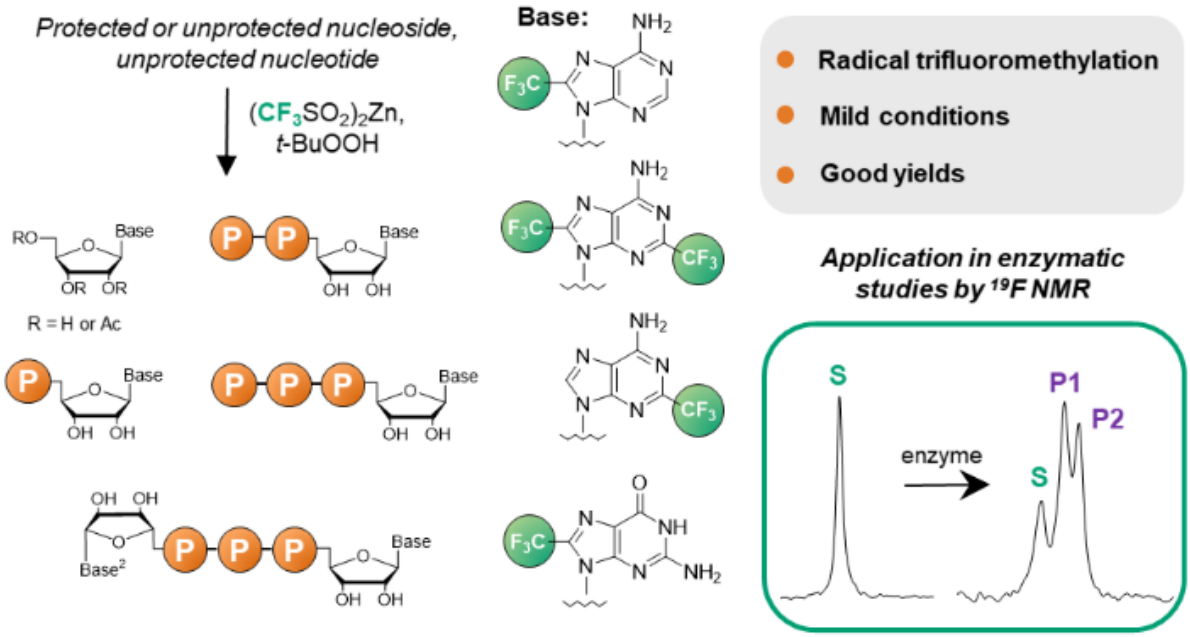


Abstract

Protected guanosine and adenosine ribonucleosides and guanine nucleotides are readily functionalized with CF_3 substituents within the nucleobase. Protected guanosine is trifluoromethylated

at the C-8 position under radical-generating conditions in up to 95% yield, and guanosine 5'-oligophosphates in up to 35% yield. In the case of adenosine, the selectivity of trifluoromethylation depends heavily on the functional group protection strategy and leads to a set of CF_3 modified nucleosides with different substitution patterns (C-8, C-2, or both) in up to 37% yield. Further transformations based on phosphorimidazolide chemistry afford various CF_3-substituted mono- and dinucleoside oligophosphates in good yields. The utility of the trifluoromethylated nucleotides as probes for ^{19}F NMR-based real-time enzymatic reaction monitoring is demonstrated with three different human nucleotide hydrolases (Fhit, DcpS, and cNIIIB). Substrate and product(s) resonances were sufficiently separated to enable effective tracking of each enzymatic activity of interest.

Introduction

Inserting a trifluoromethyl group (CF_3) into an organic molecule is a common and effective approach for fine-tuning the properties of drug candidates[1-3] and designing molecular probes for ^{19}F nuclear magnetic resonance (NMR) experiments.[4, 5] The placement of the CF_3 substituent in an organic compound can affect the neighboring functional groups and alter its overall polarity, acid-base properties, reactivity, and many other properties. Consequently, CF_3 functionalization has been explored in medicinal chemistry as a strategy for modulating the biological activities of drug candidates by influencing metabolic stability, conformational equilibrium, lipophilicity, pharmacodynamics, and pharmacokinetics.[1-3]

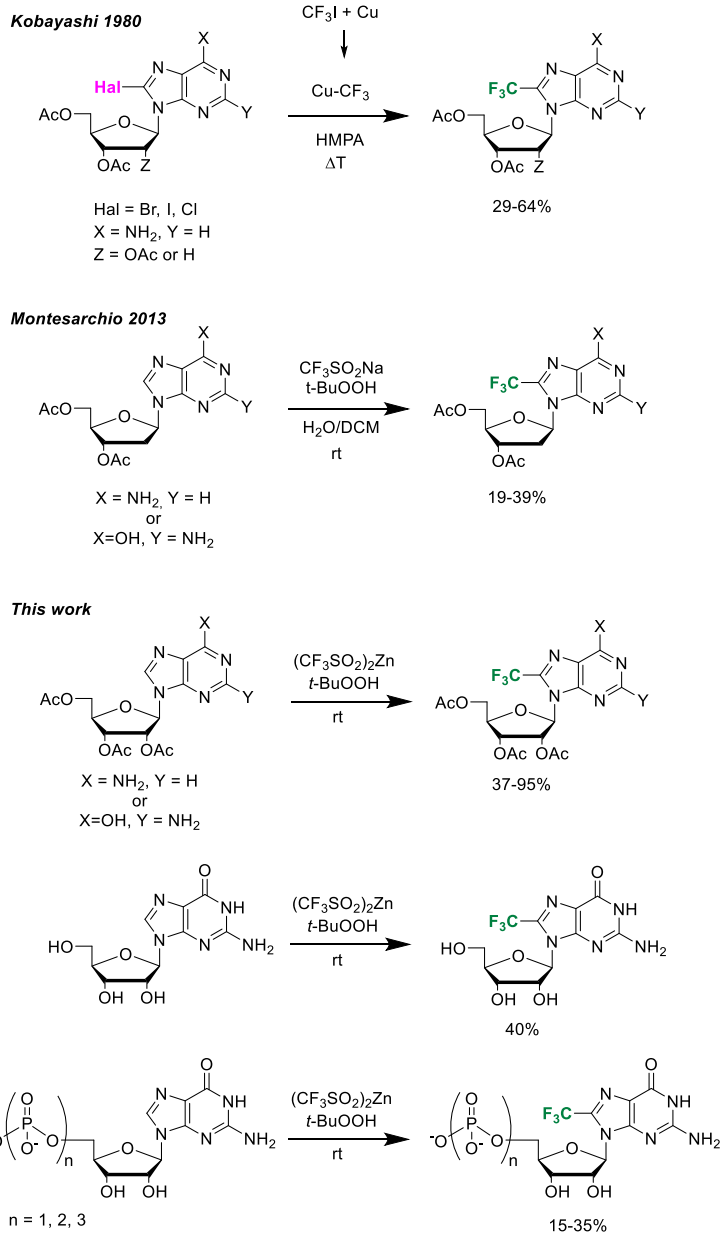
The ^{19}F nucleus has several properties that are beneficial for NMR spectroscopy, including spin of 1/2, one of the highest magnetic sensitivities (83% that of ^{1}H), and high abundance (100%). Moreover, the wide chemical shift range (up to 400 ppm) and the absence of fluorine in natural compounds (low physiological content) simplify ^{19}F NMR spectral analysis even for complex biomolecular mixtures. An additional advantage of using CF_3 group as a biomolecular NMR tag (instead of a single fluorine atom, for example) is the presence of three equivalent F atoms, which increases sensitivity. As such, fluorinated organic molecules and biopolymers have found application in NMR-based ligand screening

assays such as FAXS (fluorine chemical shift anisotropy and exchange for screening)[6, 7] and FABS (fluorine atoms for biochemical screening),[7, 8] enzymatic assays, protein and nucleic acid structure studies, and others.[9] Consequently, there is a high demand for synthetic transformations that assure the efficient and robust preparation of CF_3-containing building blocks, preferably through late stage, site-selective direct trifluoromethylation. Recent years have seen extensive developments in direct C-H trifluoromethylation methodologies, including electrophilic/nucleophilic reactions,[10-14] photoredox-based reactions,[15] metal-mediated reactions,[16] and radical reactions with various "CF_3" sources.[11, 17] Fluorinated nucleosides, nucleotides, and oligonucleotides[18] are of particular interest as anticancer and antiviral compounds or probes for studying nucleic acids structure, interactions, and biological transformations.[19-31] However, the synthesis of fluorinated nucleic acid components often poses complex challenges, especially in the case purine derivatives.

Consequently, few methods that access trifluoromethylated purine nucleosides have been reported; hence the properties of these compounds are underexplored. One of the early strategies involves the use of CF_3-containing building blocks for the de novo synthesis of the six-membered purine ring.[32] This approach has been applied by Langer[33] and Pankiewicz[34] for the preparation of CF_3-substituted purine nucleosides as enzymatic inhibitors. The trifuoromethylation of halogenated purine ribosides was introduced by Kobayashi (Scheme 1),[35] and requires the preparation of a CF_3-copper complex as a CF_3 source followed by reaction with an *O*-protected nucleoside bearing a halogen substituent at the C8-position. Although this process is multistep, operationally demanding, and afforded moderate yields, its modified variants have become methods of choice for preparing purine ribosides trifluoromethylated at positions C8,[36, 37] C2,[38-40] and C6.[41]

Montesarchio reported the direct trifluoromethylation of canonical nucleoside derivatives under mild conditions (Scheme 1)[42] in moderate yields by taking advantage of CF_3SO_2Na as a CF_3-radical precursor.[43] The substrate scope included deoxyguanosine, deoxyadenosine, and inosine.

Scheme 1. Synthetic approaches toward trifluoromethylated purines.

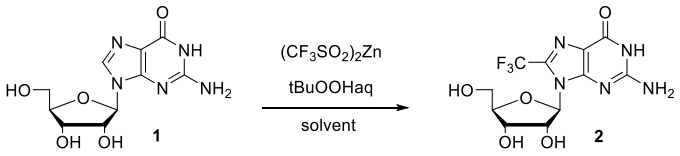


Baran reported $(CF_3SO_2)_2Zn$ as a versatile reagent for the C-H trifluoromethylation of heterocycles,[44] including drugs bearing the purine motif.[45] Inspired by this work, herein we aimed to develop $(CF_3SO_2)_2Zn$-based protocols for the synthesis of protected and unprotected purine ribonucleosides and ribonucleotides. As a result we report the synthesis of trifluoromethylated adenine and guanine nucleosides, nucleotides, and dinucleotides and evaluate their potential as ^{19}F NMR probes for enzymatic reactions.

Results and Discussion

In a pilot experiment, we subjected guanosine (**1**, Table1, entry 1) to the conditions described by Baran.[44] The reaction was selective, although the isolated yield of 8-trifluoromethylguanosine (**2**) was low (5%), even at elevated temperature (Table 1, entry 2). The effect of solvent mixtures (including two phase system) to the reaction was next evaluated.Application of two phase DCM/water system resulted in no product formation (Table 1, entry 3), while DMSO/water and DMSO/10% AcOH (which was recently reported by Parish and Krska to be optimal solvent for trifluoromethylation of complex compounds, such as peptides [46]) mixtures raised the yield to 20% and 25%, respectively (Table 1, entries 4 and 5). The highest conversion was achieved in 10% AcOH/DMSO with *t*-BuOOH added at 0 ° C (Table 1, entry 6); **2** was isolated in 40% yield. While the reaction was selective for the expected product, the starting material was not fully consumed.

Table 1. Optimisation of trifluoromethylation of guanosine[a]



Entry	Solvent	Time [h]	Temperature [°C]	Yield[d] [%]
1	DMSO	72	rt	5
2	DMSO	24	60[b]	7
3	DCM/water, 1/1	72	rt	0
4	DMSO/water, 1/1	72	rt	20
5	DMSO/10% AcOH, 1/1	72	rt	25
6	DMSO/10% AcOH, 1/1	72	0 to rt[c]	40

[a] General conditions: guanosine (0.2 mmol), solvent (2 ml), $(CF_3SO_2)_2Zn$ dihydrate (0.6 mmol), *t*-BuOOH (70% solution in water, 1 mmol).

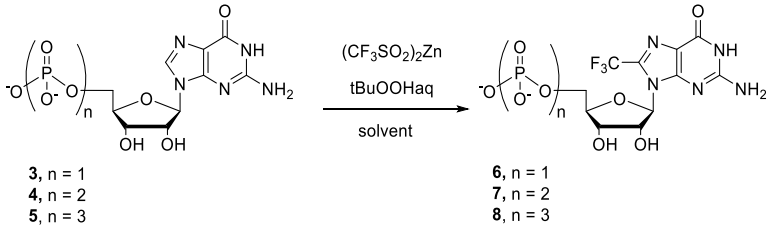
[b] The heating was started after addition of whole *t*-BuOOH solution.

[c] The solution of guanosine and $(CF_3SO_2)_2Zn$ was cooled to 0 °C prior addition of *t*-BuOOH solution. This temperature was maintained until whole *t*-BuOOH was added.

[d] Isolated yield.

We next examined the application of similar conditions to the trifluoromethylation of guanosine 5'-oligophosphates (**3-5**, Table 2). As observed for guanosine, higher yields were obtained in AcOH/DMSO at low temperatures (Table 2, entries 1-4), and increasing the AcOH concentration resulted in significant drop of yields (Table 2, entries 4-6). The optimized conditions afforded 8-trifluoromethyl-GMP (**6**) in 35% isolated yield (Table 2, entry 4), while GDP **4** and GTP **5** afforded the corresponding products **7** and **8** in 25% and 15% isolated yields, respectively (Table 2, entries 8 and 9). Prolonged reaction times led to the decomposition of products **7** and **8** through pyrophosphate bond hydrolysis. It is worth to mention that the reaction outcome was insensitive towards the counter ion of the nucleotide (Table 2, entry 4 vs entry 7). Surprisingly, adenosine, adenosine monophosphate, and diadenosine 5',5'-triphosphate gave only traces of the trifluoromethylated compounds under the conditions examined.

Table 2. Optimisation of trifluoromethylation of guanosine 5'-oligophosphates[a]



3, n = 1
4, n = 2
5, n = 3

6, n = 1
7, n = 2
8, n = 3

Entry	Substrate	Solvent	Temperature [°C]	Product	Yield[d] [%]
1	**3**	DMSO	rt	**6**	traces
2	**3**	DMSO/water, 1/1	rt	**6**	< 5
3	**3**	DMSO/10% AcOH, 1/1	rt	**6**	15
4	**3**	DMSO/10% AcOH, 1/1	0 to rt	**6**	35
5	**3**	DMSO/20% AcOH, 1/1	0 to rt	**6**	20
6	**3**	DMSO/30% AcOH, 1/1	0 to rt	**6**	10
7[b]	**3**	DMSO/10% AcOH, 1/1	0 to rt	**6**	35
8[c]	**4**	DMSO/10% AcOH, 1/1	0 to rt	**7**	25

9[c]	**5**	DMSO/10% AcOH, 1/1	0 to rt	**8**	15

[a] General conditions: triethyl ammonium salt of respective nucleotide (0.1 mmol), solvent (1 ml), $(CF_3SO_2)_2Zn$ dihydrate (0.3 mmol), *t*-BuOOH (70% solution in water,0.5 mmol).

[b] sodium salt of **3** was used.

[c] reaction was stopped after 24 h due to the detection of decomposition products in the reaction mixture (HPLC analysis).

[d] Isolated yield.

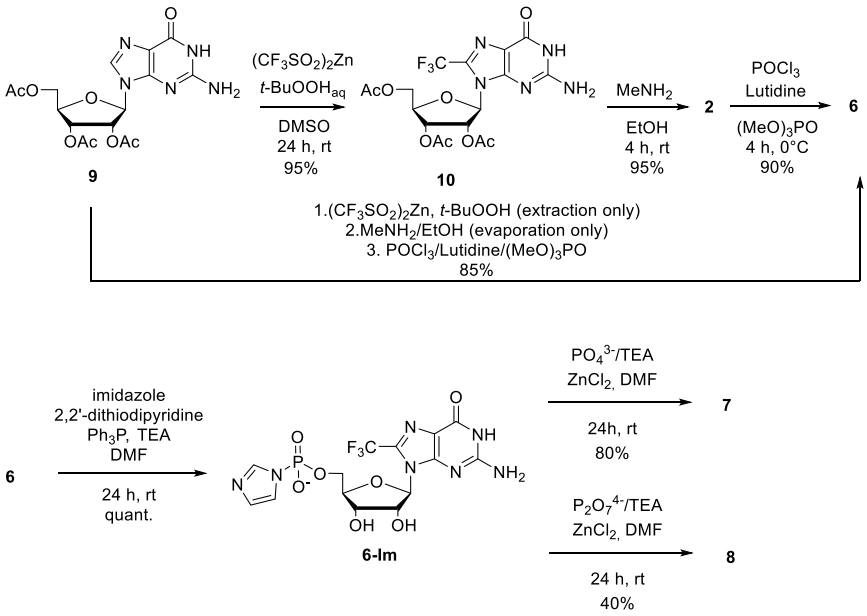
Although the conditions developed for guanosine and its derivatives appear acceptable, incomplete conversion led to tedious product isolation. Moreover, the adenosine problem remained unsolved. To improve conversions and to simplify isolation procedures, we examined the reactions of protected nucleosides, since this strategy has been reported effective for purine 2' -deoxynucleosides[42].

2',3',5'-Tri-*O*-acetylguanosine (**9**, Scheme 2) turned out to be an excellent starting material for this transformation, giving **10** in 95% isolated yield. The product was easily isolated by extraction followed by simple silica-gel chromatography to remove trace impurities. Treatment of **10** with $MeNH_2$/EtOH solution afforded pure **2** in nearly quantitative yield by solvent evaporation; **2** was then transformed into monophosphate **6** under Yoshikawa conditions[47] in the presence of lutidine (to avoid depurination). This reaction provided **6** in 90% yield after ion-exchange chromatography. Since all reactions in this sequence were selective and high yielding, we decided to repeat it without isolating the intermediates by chromatography, which resulted in 85% overall yield of **6** from **9** over three steps (Scheme 2), which is practical from a preparative perspective.

Monophosphate **6** was converted into its P-imidazolide derivative **6-Im** by reacting it with imidazole in the presence of 2,2' -dithiodipyridine/triphenylphosphine (Scheme 2);[48] **6-Im** decomposed upon storage, but when used directly after preparation, it afforded good yields in further transformations. For example, when **6-Im** was reacted with triethylammonium phosphate or pyrophosphate in the

presence of $ZnCl_2$, diphosphate **7** or triphosphate **8** were produced in satisfactory yields, respectively (Scheme 2).

Scheme 2. The synthesis of 8-trifluoromethylguanosine and subsequent transformations.

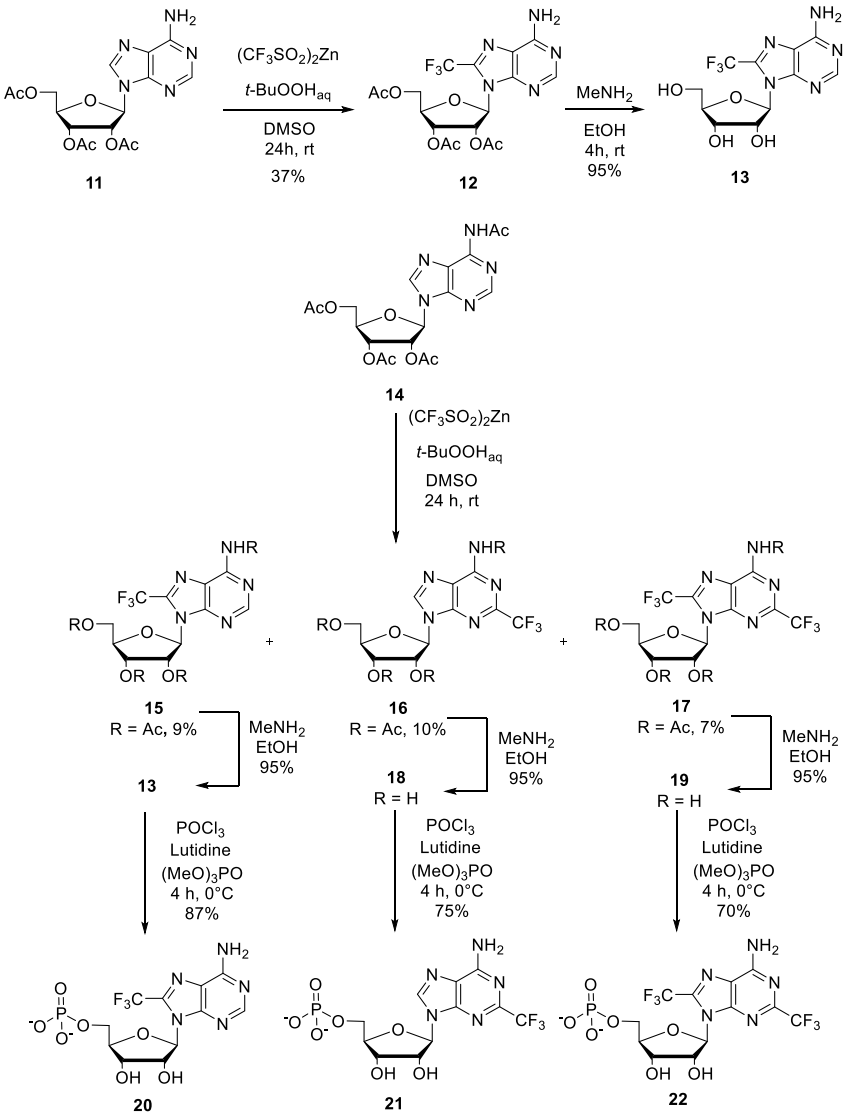


Protecting the OH groups of guanosine significantly improved the yield and simplified product isolation; consequently, we applied the same approach for adenosine. Indeed, when 2',3',5'-tri-*O*-acetyladenosine (**11**, Scheme 3) was subjected to similar conditions, the 8-trifluoromethylated product **12** was obtained in 37% yield. Further manipulation of the reaction conditions did not improve this result.

A complex mixture of products was obtained when N^6,2',3',5'-tri-*O*-tetraacetyladenosine **14** was subjected to similar conditions (Scheme 3). Chromatographic separation and analysis revealed the presence of three trifluoromethylated products: the C8- and C2-trifluoromethyladenosine derivatives **15** (9%) and **16** (10%), and the C2,C8-disubstituted adenosine derivative **17** (7%). To the best of our knowledge, **17** is the first reported example of an adenosine derivative bearing two trifluoromethyl substituents on the purine ring. Yields were not improved nor was the product distribution significantly altered by changing the reaction conditions (temperature, solvent). Treating compounds **12**, and **15**-

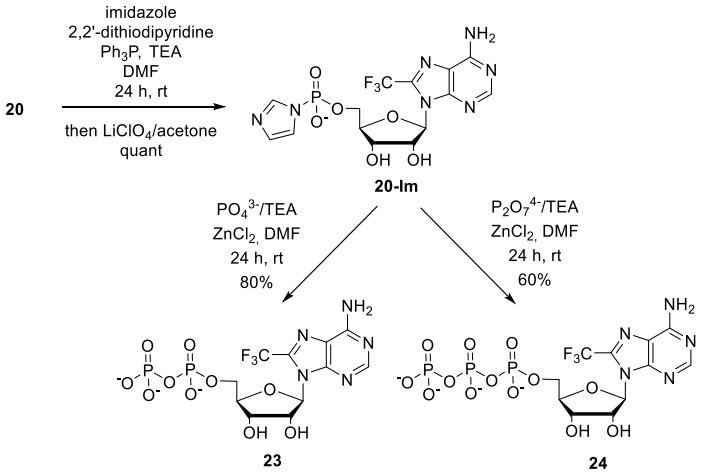
17 with 33% $MeNH_2$/EtOH afforded nucleosides **13**, **18**, and **19**, which were phosphorylated to their respective monophosphates **20**–**22** in good yields (Scheme 3).

Scheme 3. The synthesis of trifluoromethyladenosine 5' -monophosphates.



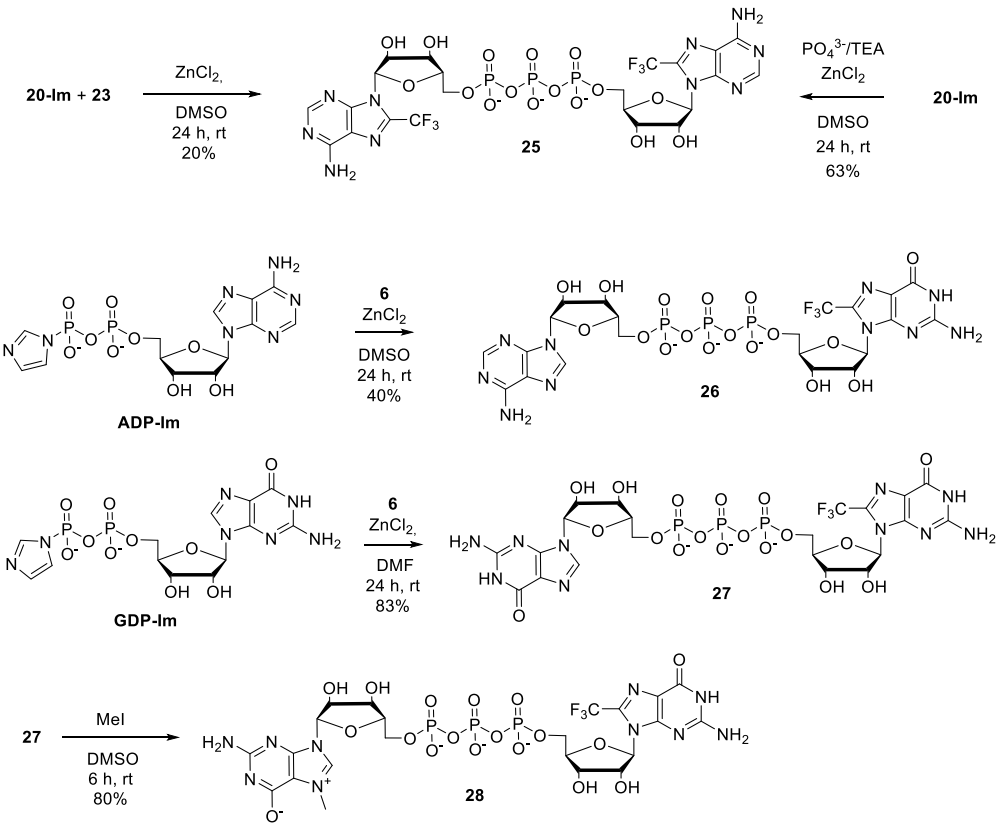
Monophosphate **20** was converted into its P-imidazolide derivative **20-Im** (Scheme 4), which enabled the efficient elongation of the phosphate chain to give 8-CF_3 adenosine diphosphate **23** and triphosphate **24** in good yields.

Scheme 4. The synthesis of 8-triflormethyladenosine oligophosphates.



Finally, we prepared a series of trifluoromethylated dinucleoside 5',5'-triphosphates using some of the synthesized mononucleotides (Scheme 5). This was achieved by coupling together two nucleotide building blocks, one of which is activated as a phosphorimidazolide, in the presence of excess $ZnCl_2$ to form a new pyrophosphate bond. The reaction of imidazolide **20-Im** with diphosphate **23** led to a complex reaction mixture containing the desired dinucleotide **25**, the monophosphate **20** (from the hydrolysis of **20-Im**), and the coupling product of **20-Im** and **20**. Nevertheless, we isolated the desired dinucleotide **25** in 20% yield. The reaction of **20-Im** with a sub-equimolar amount of triethylammonium phosphate proved to be more efficient; the *in situ* formed diphosphate **23** readily reacted with excess **20-Im** present in the reaction mixture to yield **25** in 63%. The reaction of imidazole-activated adenosine monophosphate **ADP-Im** with monophosphate **6** led to dinucleotide **26** in 40% yield, which could not be improved by reacting **6-Im** with adenosine diphosphate. On the other hand, **6** reacted smoothly with **GDP-Im** to give dinucleotide **27** composed of two guanosines, one of which bears an 8-CF_3 group. The presence of the electron-withdrawing 8-CF_3 group in the guanosine structure dramatically decreased the nucleophilicity of the neighboring (N7) nitrogen. The drop in reactivity was evident when **27** was treated with iodomethane affording exclusively mRNA 5' cap analogue **28**, which was site-selectively methylated at the guanosine rather than 8-CF_3-guanosine.

Scheme 5. The synthesis of trifluoromethylated dinucleotides.



To evaluate the usefulness of trifluoromethylated purine nucleotides as probes for enzymatic activity monitoring by ^{19}F NMR, we subjected select compounds to three nucleotide-specific phosphohydrolases, hFhit, hDcpS, and hcNIIIB. These enzymes are of interest due to their function in regulation of endogenous nucleotide metabolism and links to disease development, and as such, assays have been developed that allow monitoring their activity and the discovery of inhibitors. These include assays involving radioactivity,[49, 50,] fluorogenic probes,[31] malachite green (MG) assay,[51](2) and fluorescent FRET probes.[52, 53](3) However, these assays have some limitations such as discontinuity, susceptibility to interference from UV-VIS absorbing and emitting inhibitors, or high structural complexity of the probes. Thus, methods that enable robust and straightforward real-time monitoring of activity these enzymes are still desired.

We first used compounds **25**–**27** to monitor the activity of the human fragile histidine triad (hFhit) pyrophosphatase, which unsymmetrically cleaves diadenosine 5',5'-triphosphate (Ap_3A) and other purine dinucleotides.[54] hFhit is considered to be a tumor suppressor, and its function has been linked to substrate binding. Hence, efforts have been made to identify hFhit inhibitors,[52, 53] but not by ^{19}F NMR spectroscopy. We verified that hFhit accepts bis(trifluoromethylated) Ap_3A analog **25** as a substrate. Compound **25** exhibits a single narrow signal at -61.38 ppm in its ^{19}F NMR spectrum, which corresponds to six equivalent fluorine atoms (Figure 1A). A decrease in intensity of this resonance was observed after adding 20 nM hFhit to a 100 µM buffered solution of **25**, and two new slightly upfield-shifted signals appeared, consistent with substrate hydrolysis to give **20** (8-CF_3-AMP, δ_F -61.39) and **23** (8-CF_3-ADP, δ_F -61.40; Figure S1A). The signals of **20** and **23** were assigned on the basis of the ^{19}F NMR spectra of synthetic references (Figure S1B-D). Despite the small differences in chemical shifts from the substrate, it was convenient to monitor the reaction progress by ^{19}F NMR and observe the inhibitory effect of a previously identified compound[53] (Figure 1A). Similar experiments were performed for unsymmetrical 8-trifluoromethylguanosine-containing dinucleotides **26** and **27** (100 µM each in the presence of 20 nM or 25 nM hFhit, respectively; Figure S2). ^{19}F NMR spectroscopy revealed the formation of two trifluoromethylated products from each compound, namely 8-CF_3-GDP (**7**; δ_F -61.28) and 8-CF_3-GMP (**6;** δ_F -61.27), albeit in different proportions (Figures S3). Under these comparable conditions, **25** and **26** are preferred substrates of hFhit, rather than **27**, consistent with the known preference of the enzyme for adenine-containing dinucleotides.

We next investigated decapping scavenger (hDcpS) and cytosolic nucleotidase IIIB (hcNIIIB), two enzymes involved in the cellular metabolism of N7-methylguanine nucleotides. hDcpS degrades cap moieties (m^7GpppN_n) released during 3'-to-5' mRNA degradation[55] and has been identified as a therapeutic target for spinal muscular atrophy and acute myeloid leukemia, which created demand for inhibitors.[49, 50, 56, 57] To verify that trifluoromethylated nucleotides can be used to study hDcpS activity, mRNA cap analog **28** (100 µM;) was incubated with 80 nM enzyme in the absence and presence of RG3039, a potent inhibitor. ^{19}F NMR analysis revealed that the substrate (δ_F -61.22) is site-selectively

cleaved to release **7** (8-CF_3-GDP; δ_F -61.25), consistent with the high specificity of DcpS for 7-methylguanosine, which controls the regioselectivity; inhibition by RG3039 was also clearly visible (Figure 1B).

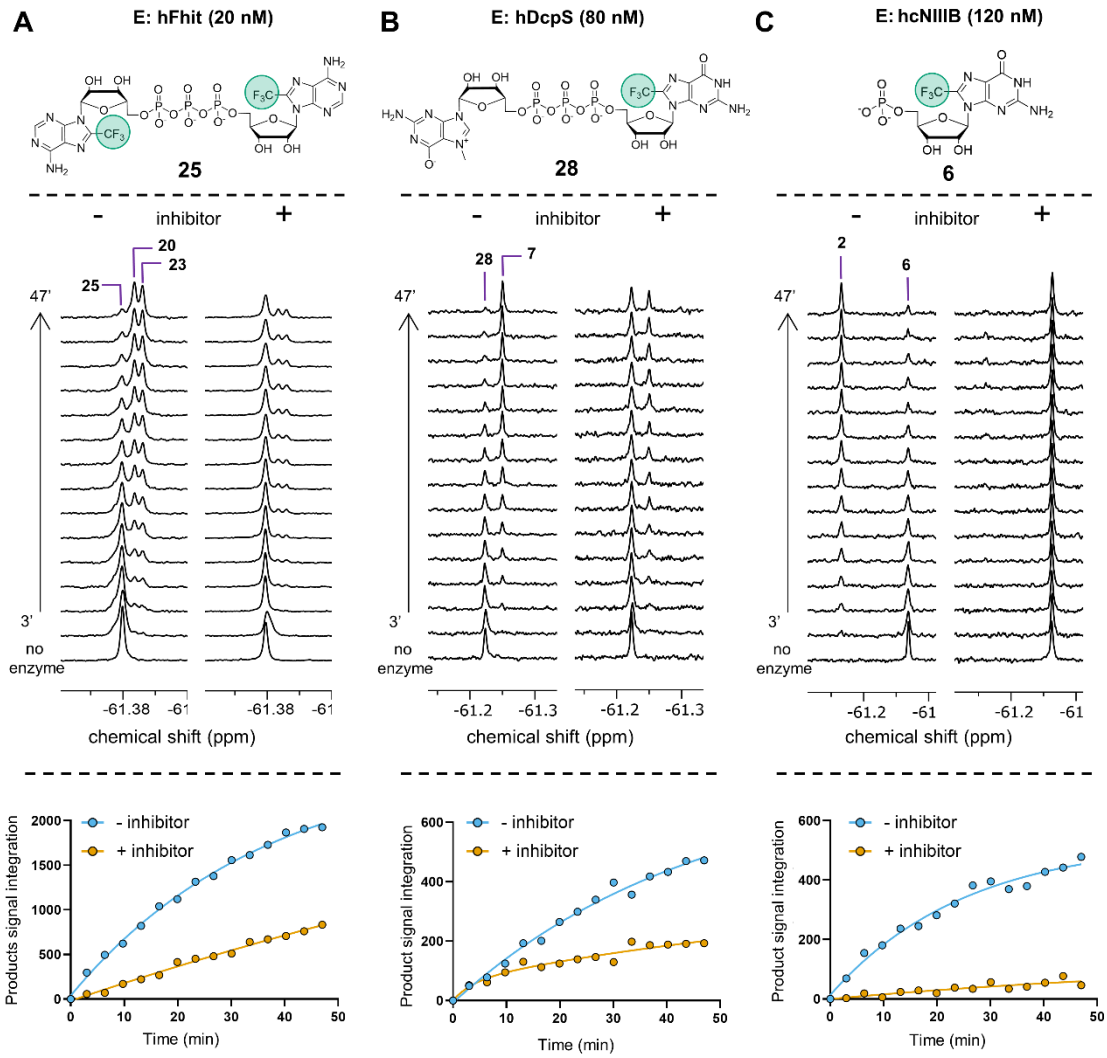


Figure 1. Monitoring enzymatic activity by ^{19}F NMR spectroscopy. (A) **25** with hFhit, (B) **28** with hDcpS, and (C) **6** with hcNIIIB. For experimental details see Supporting Information.

hcNIIIB dephosphorylates m^7GMP to 7-methylguanosine[58] and its inhibitors are potential modulators of mononucleotide metabolism and downstream RNA degradation pathways.[51] hcNIIIB also hydrolyses electron-poor pyrimidine nucleotides, whereas GMP and AMP are very poor substrates.[59] Since trifluoromethylation decreases the electron density in the purine, we tested 8-CF_3-GMP (**6**) as an

artificial substrate for hcNIIIB. Indeed, the substrate peak (δ_F -61.27) was observed to disappear when **6** at 100 μM was incubated with 120 nM hcNIIIB, and a product signal (δ_F -61.17) emerged, which was independently confirmed to be 8-trifluoromethylguanosine (**2**). The reaction was almost completely stopped by hcNIIIB-specific inhibitor (Figure 1C).

Conclusion

In summary, we optimized the conditions for the synthesis of trifluoromethylated purine nucleotides and nucleosides using $(CF_3SO_2)_2Zn$ as a source of CF_3 radicals. The synthe-sized compounds include trifluoromethylguanosine and trifluormethyladenosine, their 5'-mono, di-, and triphos-phates, as well as several dinucleoside 5',5'-triphosphates. The synthetized trifluoromethylated (di)nucleotides were successfully used as molecular probes to monitor the activ-ities of three enzymes (hFhit and hDcpS pyrophosphatases and hcNIIIB phosphatase) by ^{19}F NMR spectroscopy. The introduction of CF_3 moieties into the purines in dinucleotide analogs does not prevent specific recognition by either hFhit or hDcpS. Interestingly, 8-CF_3-GMP (**6**) acted as a m^7GMP mimic, as manifested by its efficient dephosphory-lation by cNIIIB. Substrate and product(s) resonances were sufficiently separated to enable effective monitoring of the enzymatic activity of interest, which opens possibilities for the development of ^{19}F NMR-based inhibitor-discovery and evaluation assays. We envisage that the higher syn-thetic availability of trifluoromethylated guanine- and adenine-derived building blocks offered by our work will also pave the way for their use in more-complex biomolecular systems, such as oligonucleotides and nucleic acids, thereby facilitating studies on nucleic acid structure and function.

Experimental Section

General information. All commercial reagents and solvents were used as received without additional purification. Guanosine and adenosine were purchased from Carbosynth. $(CF_3SO_2)_2Zn$ dihydrate and tBuOOH (70% in water) were purchased from TCI. Anhydrous solvents were purchased from Sigma-Aldrich. Thin Layer Chromatography (TLC) analysis was carried out on pre-coated Silica Gel 60 Å on aluminum foil with fluorescence indicator (Sigma-Aldrich) and visualised under UV lamp (254 nm).

Preparative chromatography. Preparative chromatography (SiO_2 and RP C18) was performed using Reveleris X2 flash chromatography system (BUCHI) with FlashPure cartridges (4 g, 12 g, 24 g, 40 g). Conditioning methods, loading and flow rates were set according to producers guidelines. UV detection was performed at 3 wavelengths (254 nm, 265 nm and 280 nm) simultaneously.

Ion-exchange chromatography. The synthesized nucleotides were purified by ion-exchange chromatography on DEAE Sephadex A-25 (HCO^{3-} form) column. After loading the column with reaction mixture and washing it with water, the products were eluted using different linear gradients of triethylammonium bicarbonate (TEAB) in deionized water: 0–0.7 M for nucleoside monophosphates, 0–1.0 M for nucleoside diphosphates and dinucleotides or 0–1.2 M for nucleoside triphosphates. Fractions containing desired product were collected together after RP HPLC and spectrophotometric (at 260 nm) analysis. Evaporation under reduced pressure with repeated additions of 96% and then 99.8% ethanol resulted in isolation of nucleotide analogues as triethylammonium salts.

Analytical and preparative HPLC. Analytical HPLC was performed on Agilent Tech. Series 1200 using Supelcosil LC-18-T HPLC column (4.6 x 250 mm, flow rate 1.3 mL/min) with linear gradients of methanol in 0.05 M ammonium acetate buffer and UV-detection at 254 nm. Analytical HPLC programs included in supporting informations. Semi-preparative HPLC was performed on the same apparatus equipped with Discovery RP Amide C-16 HPLC column (25 cm x 21.2 mm, 5µm, flow rate 5.0 mL/min) with linear gradients of MeCN in 0.05 M ammonium acetate buffer (pH 5.9) and UV-detection at 260 nm.

Spectroscopic analysis of the synthesized compounds. The structure and purity of compounds product were confirmed high resolution mass spectrometry using electrospray ionization (HRMS ESI), and NMR spectroscopy. Purity of water soluble compounds was additionally confirmed by RP HPLC. Mass spectra were recorded on Thermo Scientific LTQ OrbitrapVelos (high resolution spectra) and AB Sciex API 3200 (low resolution spectra) spectrometers. NMR spectra were recorded on a Varian INOVA 400 MHz or 500 MHz spectrometer equipped with a high stability temperature unit using 5 mm 4NUC probe at 25 °C. The chemical shifts were reported in ppm with residual solvent peak as internal

standard. The ^{31}P NMR chemical shifts were reported in ppm and referenced to 20% phosphoric acid in D_2O as an external standard. The ^{19}F chemical shifts were reported in ppm and referenced to $CFCl_3$ (for spectra recorded in $CDCl_3$ and DMSO-d6 , 0.65, -0.24 ppm respectively) or NaF (for spectra recorded in D_2O, -121.50 ppm) as external standard. Signal assignments of compounds **2**, **13**, **16** and **17** were based on COSY, HSQC and HMBC spectra analysis

Optimisation of trifluoromethylation of guanosine, general procedure. Guanosine (56 mg, 0.2 mmol) was dissolved/suspended in the solvent/solvent mixture (2 ml) followed by addition of $(CF_3SO_2)_2Zn$ dihydrate (219 mg, 0.6 mmol). To this mixture *t*-BuOOH (70% solution in water, 130 μl, 1 mmol) in 10 aliquots (13 μl each) in 20 min. intervals was added upon vigorous stirring. The reaction mixture was stirred at room temperature. The progress of the reaction was monitored by TLC analysis (10% MeOH in DCM) until no further progress could be detected. After the indicated time the reaction mixture was diluted with water (approx. 20 ml) and extracted with DCM (3 x 10 ml). The combined organic fractions were washed with water (approx. 20 ml), brine (approx. 20 ml) and dried over Na_2SO_4. The drying agent was filtered off, washed with DCM and the filtrate concentrated *in vacuo*. To the residue 10 ml of MeOH and 0.5 g of silica was added. The slurry was concentrated *in vacuo* and the dry residue was loaded on the preconditioned silica gel column (4 g). The product was eluted with the mixture of MeOH in DCM (0 to 10% linear gradient). The fractions containing the desired product were combined, concentrated *in vacuo*, co-evaporated with diethyl ether (approx. 5 ml) and dried overnight under high vacuum giving **2** as off-white solid. Isolated yields and additional information are summarised in Table 1. ^1H NMR (400 MHz, DMSO-d_6) $\delta =$ 11.00 (bs, 1H, NH), 6.71 (bs, 2H, -NH_2), 5.63 (d, *J* = 6.2 Hz, 1H, C1'), 5.49 (d, *J* = 6.22 Hz, 1H, C2'-OH), 5.11 (d, *J* = 4.9 Hz, 1H, C3'-OH), 5.05 (d, *J* = 5.9 Hz, 11.7 Hz, 1H, C2'), 4.91 (t, *J* = 6.0 Hz, 1H, C5'-OH), 4.16 (m, 1H, C3'), 3.89 (m, 1H, C4'), 3.71-3.63 (m, 1H, C5'), 3.57-3.49 (m, 1H, C5'); ^{13}C {H} NMR (100 MHz, DMSO-d_6) $\delta =$ 156.5 (C6), 154.4 (C2), 152.5 (C4), 133.3 (q, *J* = 39.0 Hz, C8), 118.6 (q, *J* = 269.9 Hz, CF_3), 116.5 (C5), 89.4 (C1'), 86.4 (C4'), 70.7 (C2'), 70.6 (C3'), 61.9 (C5'); ^{19}F NMR (376 MHz, DMSO-d_6) $\delta =$ -59.87; HRMS (+) ESI m/z: $[M+H]^+$ calcd for $C_{11}H_{13}F_3N_5O_5^+$ 352.0863 found 352.0861,.

Optimisation of trifluoromethylation of guanosine phosphates, general procedure. A triethyl ammonium salt of respective nucleotide (0.1 mmol) was dissolved in the indicated solvent (1 ml) followed by addition of $(CF_3SO_2)_2Zn$ dihydrate (110 mg, 0.3 mmol). To this mixture *t*-BuOOH (70% solution in water, 65 μl, 0.5 mmol) in 10 aliquots (6.5 μl each) in 20 min intervals was added under vigorous stirring. The progress of the reaction was monitored by RP HPLC analysis until no further progress could be detected (usually 72 h) or decomposition of the starting material/products was detected. The reaction mixture was diluted with EDTA solution (100 mg in 10 ml of water) and neutralised with 10% $NaHCO_3$. The resulting mixture was loaded on DEAE Sephadex A-25 column (HCO^{3-} form, 10 g), the column was washed with water (50 ml) and then eluted using TEAB in deionized water (400 ml, linear gradient). The fractions containing the mixture of the desired product and starting material (UV and RP HPLC analysis) were combined, concentrated *in vacuo*, co-evaporated with 96% EtOH (approx. 50 ml), co-evaporated with MeCN (approx. 50 ml) and the residue was dried overnight under high vacuum. The product was separated from starting material and possible by products using preparative RP HPLC chromatography with linear gradient of MeCN in 0.05 M ammonium acetate buffer (pH = 5.9). The fractions containing pure product were concentrated *in vacuo,* co-evaporated with 96% EtOH (approx. 50 ml) and co-evaporated with MeCN (approx. 50 ml). The residue was dissolved in MQ water and freeze-dried. After repeated freeze-drying, product was isolated as ammonium salt. Isolated yields and additional information are summarised in Table 2.

8-trifluoromethylguanosine 5'-monophosphate (**6**). White foam. ^{1}H NMR (400 MHz, D_2O) δ = 5.89 (d, *J* = 6.1 Hz, 1H), 5.33 (t, *J* = 5.8 Hz, 1H), 4.59 (dd, *J* = 3.6 Hz, 5.7 Hz, 1H), 4.31-4.23 (m, 1H), 4.22-4.09 (m, 1H); ^{13}C {H} NMR (125 MHz, D_2O) δ = 161.0, 156.7, 155.5, 138 (q, *J* = 40.1 Hz), 120.6 (q, *J* = 270.3 Hz), 118.1, 91.9, 86.7, 73.7, 72.8, 67.20; ^{19}F NMR (376 MHz, D_2O) δ = -61.23; ^{31}P NMR (162 MHz, D_2O) δ = 1.75; HRMS (-) ESI m/z: $[M-H]^-$ calcd for $C_{11}H_{12}F_3N_5O_8P^-$ 430.0381; found 430.0380,.

8-trifluoromethylguanosine 5'-diphosphate (**7**). White foam. ^{1}H NMR (400 MHz, D_2O) δ = 5.90 (d, *J* = 6.2 Hz, 1H), 5.38 (t, *J* = 5.9 Hz, 1H), 4.67 (dd, *J* = 3.1 Hz, 5.5 Hz, 1H), 4.35-4.27 (m, 2H), 4.26-4.17 (m,

1H); ^{19}F NMR (376 MHz, D_2O) δ -61.21; ^{31}P NMR (162 MHz, D_2O) δ = -9.85 (d, *J* = 20.7 Hz, 1P), -10.29 (dt, *J* = 5.9 Hz, 20.7 Hz, 1P); HRMS (-) ESI m/z: $[M-H]^-$ calcd for $C_{11}H_{13}F_3N_5O_{11}P_2^-$ 510.0044; found: 510.0041,.

8-trifluoromethylguanosine 5'-triphosphate (**8**). White foam. ^{1}H NMR (400 MHz, D_2O) δ = 5.90 (d, *J* = 6.2 Hz, 1H), 5.39 (t, *J* = 5.9 Hz, 1H), 4.69 (dd, *J* = 2.3 Hz, 5.3 Hz, 1H), 4.38-4.22 (m, 3H); ^{19}F NMR (376 MHz, D_2O) δ = -61.20; ^{31}P NMR (162 MHz, D_2O) δ = -9.82 (d, *J* = 19.3 Hz, 1P), -10.35 (d, *J* = 19.7 Hz, 1P), -22.10 (t, *J* = 19.5 Hz, 1P); HRMS (-) ESI m/z: $[M-H]^-$ calcd for $C_{11}H_{14}F_3N_5O_{14}P_3^-$ 589.9708; found 589.9708.

2',3',5'-tri-O-acetyl-8-trifluoromethylguanosine (**10**). 2',3',5'-tri-*O*-acetyl-guanosine (**9**, 818 mg, 2 mmol) was dissolved in DMSO (12 ml) followed by addition of $(CF_3SO_2)_2Zn$ dihydrate (2200 mg, 6 mmol) upon vigorous stirring at room temperature. When the clear solution was formed (15-20 min), *t*-BuOOH (70% solution in water, 1.3 ml, 10 mmol) in 10 aliquots (130 μl each) in 20 min. intervals. During addition of *t*-BuOOH the reaction mixture started to became yellow. The reaction mixture was stirred for 24h after which TLC analysis (3% MeOH in DCM) indicated full consumption of the starting material. The reaction mixture was poured into 200 ml of water and extracted with DCM (3 x 50 ml). The combined organic layers were washed with water (3 x approx. 100 ml), brine (approx. 100 ml) and dried over Na_2SO_4. The drying agent was filtered off, washed with DCM and the filtrate was concentrated *in vacuo*. The oily residue was dissolved in small amount of DCM and loaded on the preconditioned silica gel column (12 g). The product was eluted with the mixture of MeOH in DCM (5%, v/v). The fractions containing the product were combined, concentrated *in vacuo*, co-evaporated with diethyl ether (approx. 10 ml) and dried overnight under high vacuum giving **10** as off-white solid (906 mg, 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 6.36 (bs, 1H), 5.95 (d, *J* = 4.6 Hz, 1H), 5.94-5.89 (m, 1H), 4.56-4.50 (m, 1H), 4.47-4.36 (m, 2H), 2.15 (s, 3H), 2.11 (s, 3H), 2.04 (s, 3H); ^{19}F NMR (376 MHz, DMSO-d_6) δ = - 61.19; HRMS (+) ESI m/z: $[M+H]^+$ calcd for $C_{17}H_{19}F_3N_5O_8^+$ 478.1180; found 478.1187.

Deprotection of 10. 10 (906 mg, 1.9 mmol) was placed in 50 ml round bottom flask equipped with rubber septum and flushed with stream of argon. Then $MeNH_2$ (33% in EtOH, 10 ml) was added under gentle flow of argon and the resulting mixture was stirred for 4 h at room temperature after which TLC analysis (5% MeOH in DCM) indicated full consumption of the starting material. The reaction mixture was concentrated *in vacuo.* To the residue approx. 20 ml of MeOH and 2 g of silica was added. The slurry was concentrated *in vacuo* and the dry residue was loaded on the preconditioned silica gel column (10 g). The product was eluted with the mixture of MeOH in DCM (10%, v/v). The fractions containing the desired product were combined, concentrated *in vacuo*, co-evaporated with diethyl ether (approx. 10 ml) and dried overnight under high vacuum giving **2** as yellowish solid (634 mg, 95%). The analytical data matched those obtained for **2** synthesized by direct trifluoromethylation of guanosine.

8-trifluoromethylguanosine 5'-monophosphate (**6**). 8-trifluoromethylguanosine (**2**, 351 mg, 1 mmol) was dissolved in anhydrous $(MeO)_3PO$ (10 ml) under gentle flow of argon. The resulting solution was cooled below 0°C (ice/brine bath) followed by addition of 2,6-lutidine (350 μl, 3 mmol) and dropwise addition of freshly distilled $POCl_3$ (280 μl, 3 mmol). During the reaction white precipitate was formed. The reaction mixture was stirred below 0°C for 4 h after which RP HPLC analysis indicated full consumption of the starting material. The reaction mixture was poured to cold, deionised water (approx. 100 ml) and neutralised with 10% $NaHCO_3$. The resulting mixture was loaded on DEAE Sephadex A-25 column (HCO^{3-} form, 100 g), the column was washed with thoroughly with water and then eluted using TEAB in deionized water (3600 ml, 0 to 0.7 M linear gradient). The fractions containing pure product (UV and RP HPLC analysis) were combined, concentrated *in vacuo*, co-evaporated with 96% EtOH (approx. 50 ml), co-evaporated with MeCN (approx. 50 ml). The residue was dissolved in MQ water, loaded on RP C18 column (20g) and eluted with 20% MeCN in 0.05 M ammonium acetate buffer (pH 5.9). The fractions containing the desired product were combined, concentrated *in vacuo* co-evaporated with 96% EtOH (approx. 50ml), co-evaporated with MeCN. The residue was dissolved in MQ water and freeze-dried. After repeated freeze-drying, product was

isolated as ammonium salt (419 mg, 90%). The analytical data matched those obtained for **6** synthesized by direct trifluoromethylation of guanosine monophosphate.

2',3',5'-tri-O-acetyl-8-trifluoromethyl-adenosine (**12**). 2',3',5'-tri-*O*-acetyl-adenosine (**11**, 393 mg, 1 mmol) was dissolved in DMSO (6 ml) followed by addition of $(CF_3SO_2)_2Zn$ dihydrate (1100 mg, 3 mmol) upon vigorous stirring at room temperature. When the clear solution was formed (15-20 min), *t*-BuOOH (70% solution in water, 0.65 ml, 5 mmol) in 10 aliquots (65 μl each) in 20 min intervals. During addition of *t*-BuOOH the reaction mixture started to became yellow. The reaction mixture was stirred for 24 h after which TLC analysis (3% MeOH in DCM) indicated no further progress of the reaction. The reaction mixture was poured into 100 ml of water and extracted with DCM (3 x 30 ml). The combined organic layers were washed with water (3 x approx. 100 ml), brine (approx. 100 ml) and dried over Na_2SO_4. The drying agent was filtered off, washed with DCM and the filtrate was concentrated *in vacuo*. To the oily residue approx. 20 ml of DCM and 2 g of silica was added. The slurry was concentrated *in vacuo* and the dry residue was loaded on the preconditioned silica gel column (24 g). The product was eluted with the mixture of MeOH in DCM (0 to 5% linear gradient). The fractions containing the product were combined, concentrated *in vacuo*, co-evaporated with diethyl ether (approx. 10 ml) and dried overnight under high vacuum giving **12** as off-white solid (171 mg, 37%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.42 (s, 1H), 6.41 (dd, *J* = 4.7 Hz, 6.1 Hz, 1H), 6.09 (d, *J* = 4.7 Hz, 1H), 5.90 (m, 3H), 4.55 (m, 1H), 4.44-4.36 (m, 2H), 2.16 (s, 3H), 2.09 (s, 3H), 2.06 (s, 3H); ^{19}F NMR (376 MHz, $CDCl_3$) δ = - 61.39; HRMS (+) ESI m/z: $[M+H]^+$ calcd for $C_{17}H_{19}F_3N_5O_7^+$ 462.1231; found 462.1225.

Trifluoromethylation of N^6,2',3',5'-tri-O-tetraacetyladenosine. *N*^{6},2',3',5'-tri-*O*-tetraacetyladenosine (**14**, 870 mg, 2 mmol) was dissolved in DMSO (12 ml) followed by addition of $(CF_3SO_2)_2Zn$ dihydrate (2200 mg, 6 mmol) upon vigorous stirring at room temperature. When the clear solution was formed (15-20 min), *t*-BuOOH (70% solution in water, 1.3 ml, 10 mmol) in 10 aliquots (135 μl each) in 20 min. intervals. During addition of *t*-BuOOH the reaction mixture started to became yellow. The reaction mixture was stirred for 24 h after which TLC analysis (3% MeOH in DCM) indicated no further progress

of the reaction. The reaction mixture was poured into 100 ml of water and extracted with DCM (3 x 30 ml). The combined organic layers were washed with water (3 x approx. 100 ml), brine (approx. 100 ml) and dried over Na_2SO_4. The drying agent was filtered off, washed with DCM and the filtrate was concentrated *in vacuo*. To the oily residue approx. 20 ml of DCM and 2 g of silica was added. The slurry was concentrated *in vacuo* and the dry residue was loaded on the preconditioned silica gel column (40 g). The column was eluted with the mixture of MeOH in DCM by stepwise changing of the MeOH concentration (0 to 3%). The products and remaining starting material were washed from the column in the following order: **17**, **15**, **16**, **14** (see figure S4). The fractions containing each product were combined, concentrated *in vacuo*, co-evaporated with diethyl ether (approx. 10 ml) and dried overnight under high vacuum.

N^6,2',3',5'-tri-O-tetraacetyl-8-trifluoromethyl-adenosine (**15**). White solid (91 mg, 9%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.78 (s, 1H), 8.73 (bs, 1H), 6.40 (dd, *J* = 4.9 Hz, 6.1 Hz, 1H), 6.11 (d, *J* = 4.9 Hz, 1H), 5.86 (dd, *J* = 5.2 Hz, 6.1 Hz, 1H), 4.45 (dd, *J* = 3.5 Hz, 11.4 Hz, 1H), 4.47-4.36 (m 2H), 2.65 (s, 3H), 2.18 (s, 3H), 2.08 (s, 3H), 2.06 (s, 3H); ^{13}C {H} NMR (100 MHz, $CDCl_3$) δ = 170.6, 170.4, 169.6, 169.4, 154.6, 151.7, 151.0, 140.7 (q, *J* = 40.7 Hz) 120.2, 118.2 (q, *J* = 272.4 Hz), 88.1 (q, *J* = 2.4 Hz), 80.9, 72.0, 70.5, 62.86, 26.0, 20.8, 20.6, 20.4; ^{19}F NMR (376 MHz, $CDCl_3$) δ = -61.63; HRMS (+) ESI m/z: $[M+H]^+$ calcd for $C_{19}H_{21}F_3N_5O_8^+$ 504.1337; found 504.1336.

N^6,2',3',5'-tri-O-tetraacetyl-2-trifluoromethyl-adenosine (**16**). White solid (101 mg, 10%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.50 (bs, 1H), 8.48 (s, 1H), 6.26 (d, *J* = 5.3 Hz, 1H), 5.87 (dd, *J* = 5.5 Hz, 5.9 Hz, 1H), 5.68 (dd, *J* = 4.5 Hz, 5.6 Hz, 1H), 4.53-4.47 (m, 1H), 4.46-4.38 (m, 2H), 2.76 (s, 3H), 2.19 (s, 3H), 2.11 (s, 3H), 2.09 (s, 3H); ^{13}C {H} NMR (100 MHz, $CDCl_3$) δ = 171.9, 170.5, 169.7, 169.6, 150.9, 150.01 (q, *J* = 37.4 Hz), 149.9, 144.0, 122.7, 119.7 (q, *J* = 275.1 Hz), 87.1, 80.9, 73.6, 70.8, 63.2, 26.4, 20.8, 20.7, 20.4; ^{19}F NMR (376 MHz, $CDCl_3$) δ = -69.37; HRMS (+) ESI m/z: $[M+H]^+$ calcd for $C_{19}H_{21}F_3N_5O_8^+$ 504.1337; found 504.1333.

N^6,2',3',5'-tri-O-tetraacetyl-2,8-ditrifluoromethyl-adenosine (**17**). Yellowish solid (80 mg, 7%). ^{1}H NMR (400 MHz, $CDCl_3$) $\delta =$ 8.55 (bs, 1H), 6.21 (dd, *J* = 4.6 Hz, 6.2 Hz, 1H), 6.11 (d, *J* = 4.6 Hz, 1H), 5.83 (dd, *J* = 5.8 Hz, 6.2 Hz, 1H), 4.55 (dd, *J* = 4.0 Hz, 11.8 Hz, 1H), 4.48-4.42 (m, 1H), 4.41-4.35 (m, 1H), 2.77 (s, 3H), 2.18 (s, 3H), 2.08 (s, 3H), 2.04 (s, 3H); ^{19}F NMR (376 MHz, $CDCl_3$) $\delta =$ - 61.79, -69. 71; HRMS (+) ESI m/z: $[M+H]^+$ calcd for $C_{20}H_{20}F_6N_5O_8^+$ 572.1211; found 572.1200.

14 was recovered in 33% yield.

General procedure for deprotection of 12, 15, 16 and 17, the synthesis of 13, 18 and 19. Protected nucleoside was placed in round bottom flask equipped with rubber septum and flushed with stream of argon. Then $MeNH_2$ (33% in EtOH, 100 μl per each 10 mg of the starting material) was added under gentle flow of argon ant the resulting mixture was stirred for 4 h at room temperature after which TLC analysis (5% MeOH in DCM) indicated full consumption of the starting material. The reaction mixture was concentrated *in vacuo.* To the residue a small amount of MeOH and silica was added. The slurry was concentrated *in vacuo* and the dry residue was loaded on the preconditioned silica gel column. The product was eluted with the mixture of MeOH in DCM (5%, v/v). The fractions containing the desired product were combined, concentrated *in vacuo*, co-evaporated with diethyl ether (approx. 10 ml) and dried overnight under high vacuum.

8-trifluoromethyl-adenosine (**13**). White solid (64 mg, starting from 92 mg (0.20 mmol) of **12**, 95%),. ^{1}H NMR (400 MHz, DMSO-d_6) $\delta =$ 8.26 (s, 1H, C2), 8.12-7.79 (m, 2H, $-NH_2$), 5.79 (d, *J* = 6.8 Hz, 1H, C1'), 5.54 (dd, *J* = 3.8 Hz, 8.8 Hz, 1H, C5'-OH), 5.50 (d, *J* = 6.2 Hz, 1H, C2'-OH), 5.27 (d, *J* = 4.4 Hz, 1H, C3'-OH), 5.11 (m, 1H, C2'), 4.22 (m, 2H, C3'), 4.02 (m, 1H, C4'), 3.75-3.67 (m, 1H, C5'), 3.60-3.51 (m, 1H, C5'); ^{13}C {H} NMR (100 MHz, DMSO-d_6) $\delta =$ 157.33 (C6), 154.6 (C2), 150.0 (C4), 136.8 (q, *J* = 39.1 Hz, C8), 118.7 (q, *J* = 271.4 Hz, CF_3), 118.0 (C5), 89.7 (C1'), 87.2 (C4'), 71.5 (C2'), 70.9 (C3'), 62.0 (C5'); ^{19}F NMR (376 MHz, DMSO-d_6) $\delta =$ -59.95; HRMS (+) ESI m/z: $[M+H]^+$ calcd for $C_{11}H1_3F_3N_5O_4^+$ 336.0914; found 336.0911.

2-trifluoromethyl-adenosine (**18**). White solid (32 mg, starting from 50 mg (0.10 mmol) of **16**, 95%). ^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.57 (s, 1H, C8), 7.97 (bs, 1H, -NH_2), 5.91 (d, *J* = 6.1 Hz, 1H, C1'), 5.49 (d, *J* = 6.2 Hz, 1H, C2'-OH), 4.92 (d, *J* = 4.9 Hz, 1H, C3'-OH), 4.99 (dd, *J* = 5.5 Hz, 5.7 Hz, 1H, C5'-OH), 4.60 (m, 1H, C2'), 4.15 (m, 1H, C3'), 3.95 (m, 1H, C4'), 3.71-3.62 (m, 1H, C5'), 3.59-3.51 (m, 1H, C5'); ^{13}C {H} NMR (100 MHz, DMSO-d_6) δ = 156.3 (C6), 149.2 (q, *J* = 34.7 Hz, C2), 148.9 (C4), 141.7 (C8), 120.0 (q, *J* = 275.0 Hz, CF_3), 119.85 (C5), 87.3 (C1'), 85.9 (C4'), 73.6 (C2'), 70.5 (C3'), 61.4 (C5'); ^{19}F NMR (376 MHz, DMSO-d_6) δ = -68.03 ppm. HRMS (+) ESI m/z: $[M+H]^+$ calcd for $C_{11}H_{13}F_3N_5O_4^+$ 336.0914; found 336.0910.

2,8-ditrifluoromethyl-adenosine (**19**) Off-white solid (38 mg, starting from 57 mg (0.10 mmol) of **17**, 95%). ^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.64-8.38 (m, 2H, -NH_2), 5.82 (d, *J* = 6.2 Hz, 1H, C1'), 5.53 (d, *J* = 5.9 Hz, 1H, C2'-OH), 5.32 (d, *J* = 4.8 Hz, 1H, C3'-OH), 5.09 (m, 1H, C2'), 4.77 (dd, *J* = 5.6 Hz, 6.6 Hz, 1H, 5'-OH), 4.25 (m, 1H, C3'), 3.96 (m, 1H, C4'), 3.77-3.67 (m, 1H, C5'), 3.59-3.49 (m, 1H, C5'); ^{13}C {H} NMR (100 MHz, DMSO-d_6) δ = 157.5 (C6), 151.1 (q, *J* = 35.7 Hz, C2), 149.8 (C4), 138.3 (q, *J* = 39.3 Hz, C8), 119.7 (q, *J* = 275.7 Hz, C2-CF_3), 118.7 (C5), 118.3 (q, *J* = 271.0 Hz, C8-CF_3), 89.9 (C1'), 86.7 (C4'), 71.4 (C2'), 70.6 (C3'), 61.6 (C5'); ^{19}F NMR (376 MHz, DMSO-d_6) δ = -60.23, -68.66; HRMS (+) ESI m/z: $[M+H]^+$ calcd for $C_{12}H_{12}F_6N_5O_4^+$ 404.0788; found 404.0784.

General procedure for phosphorylation of 13, 18 and 19, the synthesis of 20, 21 and 22. Nucleoside (1 equiv.) was placed in round bottom flask equipped with rubber septum and flushed with stream of argon. $(MeO)_3PO$ (0.1 M) was added under gentle flow of argon and the resulting solution was cooled below 0°C (ice/brine bath) followed by addition of 2,6 lutidine (3 equiv.) and dropwise addition of freshly distilled $POCl_3$ (3 equiv.). The reaction mixture was stirred below 0 °C until RP HPLC analysis indicated full consumption of the starting material (usually 3-4 h). The reaction mixture was poured to cold, deionised water (10 times the volume of the solvent used) and neutralised with 10% $NaHCO_3$. The resulting mixture was loaded on DEAE Sephadex A-25 column (HCO^{3-} form, 10 g), the column was washed with thoroughly with water and then eluted using TEAB in deionized water (400 ml, 0 to 0.7 M

linear gradient). The fractions containing pure product (UV and RP HPLC analysis) were combined, concentrated *in vacuo*, co-evaporated with 96% EtOH (approx. 50 ml), co-evaporated with MeCN (approx. 50 ml). The residue was dissolved in MQ water and freeze-dried. The products were purified using preparative RP HPLC chromatography with linear gradient of MeCN in 0.05 M ammonium acetate buffer (pH = 5.9). The fractions containing pure product were concentrated *in vacuo,* co-evaporated with 96% EtOH and co-evaporated with MeCN (approx. 50 ml). The residue was dissolved in MQ water and freeze-dried. After repeated freeze-drying, products were obtained as ammonium salt.

8-trifluoromethyl-adenosine 5'-monophosphate (**20**). White foam (58.6 mg, starting from 50.3 mg (0.15 mmol) of **13,** 87%),. ^{1}H NMR (400 MHz, D_2O) δ = 8.25 (s, 1H), 6.02 (d, *J* = 5.8 Hz, 1H), 5.28 (dd, *J* = 5.9 Hz, 6.1 Hz, 1H), 4.59 (dd, *J* = 4.7 Hz, 6.1 Hz, 1H), 4.26 (m, 1H), 4.16-4.01 (m, 2H); ^{13}C {H} NMR (125 MHz, D_2O) δ = 158.6, 157.2, 152.6, 140.8 (q, *J* = 40.3 Hz), 120.3 (q, *J* = 271.1 Hz), 120.0, 91.5, 86.4, 73.5, 72.1, 66.4; ^{19}F NMR (376 MHz, D_2O) δ = -61.32; ^{31}P NMR (162 MHz, D_2O) δ = 0.95; HRMS (-) ESI m/z: $[M-H]^-$ calcd for $C_{11}H_{12}F_3N_5O_7P^-$ 414.0432; found 414.0432.

2-trifluoromethyl-adenosine 5'-monophosphate (**21**). White foam (23.6 mg, starting from 23.5 mg (0.07 mmol) of **18**, 75%). ^{1}H NMR (400 MHz, D_2O) δ = 8.54 (s, 1H), 6.16 (d, *J* = 5.3 Hz, 1H), 4,73 (m, 1H), 4.51 (m, 1H), 4.38 (m, 1H), 4.14 (m, 2H); ^{13}C {H} NMR (125 MHz, D_2O) δ = 158.4, 152.9 (q, *J* = 36.0 Hz) , 151.5, 144.1, 122.2 (q, *J* = 274.6 Hz), 122.0, 90.1, 86.5, 77.2, 72.9, 67.0; ^{19}F NMR (376 MHz, D_2O) δ = -68.98; ^{31}P NMR (162 MHz, D_2O) δ = 1.15; HRMS (-) ESI m/z: $[M-H]^-$ calcd for $C_{11}H_{12}F_3N_5O_7P^-$ 414.0432; found 414.0432.

2,8-ditrifluoromethyl-adenosine 5'-monophosphate (**22**) White foam (7.2 mg, starting from 8.1 mg (0.02 mmol) of **19**, 70%). ^{1}H NMR (400 MHz, D_2O) δ = 6.12 (d, *J* = 4.9 Hz, 1H), 5.27 (dd, *J* = 5.3 Hz, 5.9 Hz, 1H), 4.73 (dd, *J* = 5.1 Hz, 6.0 Hz, 1H), 4.33 (m, 1H), 4.27-4.09 (m, 2H); ^{13}C {H} NMR (125 MHz, D_2O) δ = 159.7, 154.5 (q, *J* = 36.4 Hz), 152.4, 142.7 (q, *J* = 40.8 Hz), 123.1 (q, *J* = 274.8 Hz), 121.3, 120.5 (q, *J* = 271.2 Hz),92.5, 86.9, 74.8, 72.9, 67.5; ^{19}F NMR (376 MHz, D_2O) δ = - 61.53, -69.61; ^{31}P NMR (162 MHz, D_2O) δ = 1.44; HRMS (-) ESI m/z: $[M-H]^-$ calcd for $C_{12}H_{11}F_6N_5O_7P^-$ 482.0306; found 482.0305.

General procedure for the synthesis of 6-Im and 20-Im. Monophosphate **6** or **20** (1 equiv.) was dissolved in anhydrous DMF (0.05 M) followed by addition of imidazole (20 equiv.), 2,2'-dithiodipyridine (5 equiv.) and trimethylamine (5 equiv.). The resulting solution was stirred at room temperature for 15 min and then triphenylphosphine (5 equiv.) was added in one portion. The solution turned immediately deep yellow. The reaction mixture was stirred overnight at room temperature. Addition of a solution of anhydrous $LiClO_4$ (5 equiv.) in anhydrous acetone (10 times volumes of DMF used) resulted in precipitation of the product as lithium salt. The suspension was cooled at 4 °C for approx. 2 h and the precipitate was centrifuged, washed repeatedly with cold acetone until the supernatant was colourless. The resulting solid was additionally washed with cold diethyl ether and dried overnight under high vacuum. Yields + 95%**. CAUTION**: A substantial level of decomposition of **6-Im** and **20-Im** was observed while storage, even at - 18°C. To assure good yields of further reactions they should be used immediately after preparation.

General procedure for the synthesis of 7, 8, 23 and 24. Freshly prepared P-imidazolide of the respective nucleotide (1 equiv.) was dissolved in anhydrous DMF (0.05 M). Triethyl ammonium phosphate or triethylammonium pyrophosphate (8 equiv.) was added followed by addition of anhydrous $ZnCl_2$ (8 equiv.) The reaction mixture was stirred at room temperature until HPLC analysis showed full consumption of the starting material (usually 24-30 h). The reaction mixture was diluted with EDTA solution (8.1 equiv. in water, 10 times volume of DMF used) and neutralised with 10% $NaHCO_3$. The resulting mixture was loaded on DEAE Sephadex A-25 column (HCO^{3-} form, 10 g), the column was washed with water (approx. 50 ml) and then eluted using TEAB in deionized water (400 ml, linear gradient). The fractions containing the mixture of the desired product and starting material (UV and RP HPLC analysis) were combined, concentrated *in vacuo*, co-evaporated with 96% EtOH (approx. 50 ml), co-evaporated with MeCN (approx. 50 ml) and the residue was dried overnight under high vacuum. The product was purified using RP HPLC chromatography with linear gradient of MeCN in 0.05 M ammonium acetate buffer (pH = 5.9). The fractions containing pure product were concentrated *in vacuo,* co-evaporated with 96% EtOH (approx. 50 ml) and co-evaporated with MeCN

(approx. 50 ml). The residue was dissolved in MQ water and freeze-dried. After repeated freeze-drying, product was obtained as ammonium salt.

7 was isolated in 80% yield (9.4 mg starting from 10.0 mg (0.021 mmol) of **6-Im**). **8** was isolated in 40% yield (5.5 mg starting from 10.0 mg (0.021 mmol) of **6-Im**). For **7** and **8** the analytical data matched those obtained from the **7** and **8** synthesized by direct trifluoromethylation of guanosine diphosphate and triphosphate respectively.

8-trifluoromethyl-adenosine 5'-diphosphate (**23**). White foam (9.2 mg starting from 10.0 mg (0.021 mmol) of **20-Im**, 80%). ^{1}H NMR (400 MHz, D_2O) δ = 8.36 (s, 1H), 6.05 (d, *J* = 5.6 Hz, 1H), 5.28 (dd, *J* = 5.7 Hz, 6.2 Hz, 1H), 4.65 (dd, *J* = 4.3 Hz, 5.8 Hz, 1H), 4.35-4.18 (m, 3H);^{19}F NMR (376 MHz, D_2O) δ = - 61.43; ^{31}P NMR (162 MHz, D_2O) δ = - 9.91 (d, *J* = 20.1 Hz, 1p), -10.34 (d, *J* = 20.3 Hz, 1P); HRMS (-) ESI m/z: $[M-H]^-$ calcd for $C_{11}H_{13}F_3N_5O_{10}P_2^-$ 494.0095; found 494.0095.

8-trifluoromethyl-adenosine 5'-triphosphate (**24**). White foam (7.7 mg starting from 10.0 mg (0.021 mmol) of **20-Im**, 60%).^{1}H NMR (400 MHz, D_2O) δ = 8.37 (s, 1H), 6.08 (d, *J* = 6.0 Hz, 1H), 5.39 (dd, *J* = 6.0 Hz, 6.7 Hz, 1H), 4.68 (m, 1H), 4.40-4.19 (m, 3H); ^{19}F NMR (376 MHz, D_2O) δ = - 61.31; ^{31}P NMR (162 MHz, D_2O) δ = -9.37- -9.99 (m, 1P), -10.36 (d, *J* = 19.8 Hz, 1P), -22.2 (t, *J* = 19.8 Hz, 1P); HRMS (-) ESI m/z: $[M-H]^-$ calcd for $C_{11}H_{14}F_3N_5O_{13}P_3^-$ 573.9759; found: 573.9761.

The synthesis of dinucleotide 25. Freshly prepared **20-Im** (10.0 mg, 0.021 mmol) was dissolved in anhydrous DMSO (0.5 ml) followed by addition of triethyl ammonium phosphate (6.0 mg, 0.015 mmol). To this solution anhydrous $ZnCl_2$ was added (32.0 mg, 0.235 mmol). After approx. 2h HPLC analysis indicated formation of substantial amounts of **23** and the presence of **20** (formed by the hydrolysis of the starting material). Additional portion of **20-Im** (10.0 mg, 0.021 mmol) was added and the mixture was stirred overnight at room temperature. The reaction was stopped by addition of EDTA solution (80 mg, 0.238 mmol in 5 ml of water) and neutralised with 10% $NaHCO_3$. The resulting mixture was loaded on DEAE Sephadex A-25 column (HCO^{3-} form, 10 g), the column was washed with water (approx. 50 ml) and then eluted using TEAB in deionized water (400 ml, 0 to 1 M linear gradient). The fractions

containing the mixture of the desired product and traces of by-products (UV and RP HPLC analysis) were combined, concentrated *in vacuo*, co-evaporated with 96% EtOH (approx. 50 ml), co-evaporated with MeCN (approx. 50 ml) and the residue was dried overnight under high vacuum. The product was purified using RP HPLC chromatography with linear gradient of MeCN in 0.05 M ammonium acetate buffer (pH = 5.9). The fractions containing pure product were concentrated *in vacuo,* co-evaporated with 96% EtOH (approx. 50 ml) and co-evaporated with MeCN (approx. 50 ml). The residue was dissolved in MQ water and freeze-dried. After repeated freeze-drying, product was obtained as ammonium salt (white foam, 8.9 mg, 63%). ^{1}H NMR (400 MHz, D_2O) δ = 8.05 (s, 2H), 5.77 (d, J = 6.0 Hz, 2H), 5.06 (dd, J = 5.8 Hz, 6.1 Hz, 2H), 4.47 (dd, J = 3.6 Hz, 5.7 Hz, 2H), 4.25-4.15 (m, 2H), 4.14-4.01 (m, 4H);^{19}F NMR (376 MHz, D_2O) δ = -61.28; ^{31}P NMR (162 MHz, D_2O) δ = -10.46 (d, J = 17.7 Hz, 2P), -22.01 (t, J = 17.8 Hz, 1P); HRMS (-) ESI m/z: $[M-H]^-$ calcd for $C_{22}H_{24}F_6N_{10}O_{16}P_3^-$ 891.0494; found 891.0502.

The synthesis of dinucleotide 26. ADP-Im (sodium salt, 27.0 mg, 0.051 mmol) and **6** (ammonium salt, 17.0 mg, 0.036 mmol) were dissolved in anhydrous DMSO (2 ml) followed by addition of anhydrous $ZnCl_2$ (86.0 mg, 0.632 mmol). The reaction mixture was stirred at room temperature for 72h at which time HPLC analysis no further progress of the reaction. The reaction mixture was diluted with EDTA solution (235 mg, 0.642 mmol in 20 ml of water) and neutralised with 10% $NaHCO_3$. The resulting mixture was loaded on DEAE Sephadex A-25 column (HCO^{3-} form, 10 g), the column was washed with water (approx. 50 ml) and then eluted using TEAB in deionized water (400 ml, 0 to 1 M linear gradient). The fractions containing the mixture of the desired product and traces of starting material (UV and RP HPLC analysis) were combined, concentrated *in vacuo*, co-evaporated with 96% EtOH (approx. 50 ml), co-evaporated with MeCN (approx. 50 ml) and the residue was dried overnight under high vacuum. The product was purified using RP HPLC chromatography with linear gradient of MeCN in 0.05 M ammonium acetate buffer (pH = 5.9). The fractions containing pure product were concentrated *in vacuo,* co-evaporated with 96% EtOH (approx. 50 ml) and co-evaporated with MeCN (approx. 50 ml). The residue was dissolved in MQ water and freeze-dried. After repeated freeze-drying, product was obtained as ammonium salt (white foam, 12.8 mg, 40%). ^{1}H NMR (400 MHz, D_2O) δ = 8.46 (s, 1H), 8.27

(s, 1H), 6.00 (d, *J* = 5.5 Hz, 1H), 5.77 (d, *J* = 6.4 Hz, 1H), 5.30 (dd, *J* = 5.8 Hz, 6.2 Hz, 1H), 4.63 (dd, *J* = 5.4 H, 5.7 Hz, 1H), 4.59 (dd, *J* = 2.6 Hz, 5.4Hz, 1H), 4.50 (m, 1H), 4.42-4.16 (m, 6H); ^{19}F NMR (376 MHz, D_2O) δ = - 61.14; ^{31}P NMR (162 MHz, D_2O) δ = - 10.46 (t, *J* = 17.0 Hz, 2P), -22.16 (t, *J* = 18.9 Hz, 1P); HRMS (-) ESI m/z: $[M-H]^-$ calcd for $C_{21}H_{25}F_3N_{10}O_{17}P_3^-$ 839.0570; found 839.0571.

The synthesis of dinucleotide 27. GDP-Im (sodium salt, 34.0 mg, 0.051 mmol) and **6** (ammonium salt, 17.0 mg, 0.036 mmol) were dissolved in anhydrous DMSO (2 ml) followed by addition of anhydrous $ZnCl_2$ (86.0 mg, 0.632 mmol). The reaction mixture was stirred at room temperature for 48h at which time HPLC analysis no further progress of the reaction. The reaction mixture was diluted with EDTA solution (235 mg, 0.642 mmol in 20 ml of water) and neutralised with 10% $NaHCO_3$. The resulting mixture was loaded on DEAE Sephadex A-25 column (HCO^{3-} form, 10 g), the column was washed with water (approx. 50 ml) and then eluted using TEAB in deionized water (400 ml, 0 to 1 M linear gradient). The fractions containing the mixture of the desired product and traces of starting material (UV and RP HPLC analysis) were combined, concentrated *in vacuo*, co-evaporated with 96% EtOH (approx. 50 ml), co-evaporated with MeCN (approx. 50 ml) and the residue was dried overnight under high vacuum. The product was purified using RP HPLC chromatography with linear gradient of MeCN in 0.05 M ammonium acetate buffer (pH = 5.9). The fractions containing pure product were concentrated *in vacuo,* co-evaporated with 96% EtOH (approx. 50 ml) and co-evaporated with MeCN (approx. 50 ml). The residue was dissolved in MQ water and freeze-dried. After repeated freeze-drying, product was obtained as ammonium salt (white foam, 27.1 mg, 83%). ^{1}H NMR (400 MHz, D_2O) δ = 8.11 (s, 1H), 5.82 (d, *J* = 6.5 Hz, 1H), 5.79 (d, *J* = 5.8 Hz, 1H), 5.36 (dd, *J* = 5.8 Hz, 6.6 Hz, 1H), 4.66 (dd, *J* = 5.5 Hz, 6.0 Hz, 1H), 4.59 (m, 1H), 4.49 (m, 1H), 4.39-4.17 (m, 6H); ^{19}F NMR (376 MHz, D_2O) δ = - 61.06; ^{31}P NMR (162 MHz, D_2O) δ = -10.41 (t, *J* = 6.8 Hz, 1P), -10.50 (t, *J* = 6.5 Hz, 1P), -23.14 (t, *J* = 19.0 Hz, 1P); HRMS (-) ESI m/z: $[M-H]^-$ calcd for $C_{21}H_{25}F_3N_{10}O_{18}P_3$ 855.0519; found 855.0524.

The synthesis of dinucleotide 28. 27 (9.0 mg, 0.01 mmol) was dissolved in anhydrous DMSO (0.5 ml) followed by addition of MeI (40 μl, excess). The reaction was stirred at room temperature until HPLC

analysis indicated full consumption of the starting material (approx. 6 h). The reaction mixture was diluted with water (5 ml) and washed with diethyl ether (3 x 5 ml). The aqueous phase was freeze-dried and the residue was purified using RP HPLC chromatography with linear gradient of MeCN in 0.05 M ammonium acetate buffer (pH = 5.9). The fractions containing pure product were concentrated *in vacuo,* co-evaporated with 96% EtOH (approx. 50 ml) and co-evaporated with MeCN (approx. 50 ml). The residue was dissolved in MQ water and freeze-dried. After repeated freeze-drying, product was obtained as ammonium salt (white foam, 7.2 mg, 80%). ^{1}H NMR (400 MHz, D_2O) δ =5.79-5.71 (m, 2H), 5.16 (m, 1H), 4.55 (m, 1H), 4.47 (m, 1H), 4.44-4.32 (m, 4H), 4.31-4.16 (m, 3H), 4.06 (bs, 3H); ^{19}F NMR (376 MHz, D_2O) δ = -61.17; ^{31}P NMR (162 MHz, D_2O) δ = -10.36 - -10.69 (m, 2P), -22.15 (t, *J* = 19.4 Hz, 1P); HRMS (-) ESI m/z: $[M-H]^-$ calcd for $C_{22}H_{27}F_3N_{10}O_{18}P_3^-$ 869.0675; found: 869.0681.

Stock solutions of studied (di)nucleotides. Compounds **6**, **20**, **23**, **25**, **26**, **27** and **28** were dissolved in pure water (200-300 µL), **13** was dissolved in DMSO (200 µL) and concentrations were estimated spectrophotometrically by measurement of absorbance at 260 nm in 0.1 M phosphate buffer pH 6.0 (**6**, **28**) or 7.0 (**23**, **25**, **26**, **27**). To calculate the exact concentrations the following molar extinction coefficients [M^{-1} cm^{-1}] were employed: ε = 11400 (**6**, **13**, **20**), ε = 15020 (**23**), ε = 27036 (**25**, **26**), ε = 22600 (**27**), ε = 21132 (**28**).

Samples preparation for enzymatic studies. For studies with human Fhit compounds **26** or **27** were diluted in buffer containing 50 mM MES KOH pH 6.50, 1 mM $MgCl_2$ and 10% D_2O to a final concentration of 100 µM. For studies with human DcpS compound **28** was diluted in buffer containing 50 mM Tris HCl pH 7.60, 0.2 M KCl, 0.5 mM EDTA and 10% D_2O to final concentration of 100 µM. For studies with human cNIIIB compound **6** was diluted in buffer containing 20 mM HEPES KOH pH 7.50, 50 mM KCl, 5 mM $MgCl_2$, 10% D_2O to final concentration of 100 µM. RG3039 was purchased from KareBay Biochem. Human Fhit inhibitor (IN-A, *7,8-Dihydro-7,7-dimethyl-10-(4-chlorophenyl)-5H-indeno[1,2-b]quinoline-9,11(6H,10H)-dione*) was synthesized as previously described.[60] All inhibitors were dissolved in DMSO and the concentrations were established by mass (RG3039, IN-A) or

spectrophotometrically by absorbance measurement at 260 nm in 0.1 M phosphate buffer pH 6.0 and by using molar extinction coefficient equal 11400 M^{-1} cm^{-1} (cNIIIB-specific inhibitor). To samples without inhibitor 0.3-0.5% DMSO was added (v/v) to match the solvent composition of all samples. All enzymatic reactions were performed at 30°C.

^{19}F NMR spectroscopy. ^{19}F NMR spectra were recorded on a Bruker Avance III HD 500 MHz spectrometer equipped with 5 mm PABBO BB/19F-1H/D Z-GRD probe at a frequency of 470.67 MHz in 5 mm NMR samples. Typical experimental parameters were chosen as follows: ^{19}F excitation pulse, 15.1 µs; acquisition time, 1.2 s; relaxation delay, 1.0 s; number of scans, 32; spectral width, 32.8 ppm; spectral resolution, 0.83 Hz. The ^{19}F NMR chemical shifts were reported to 0.1 M NaF in D_2O (δF = −121.5 ppm) as external standard. Before each enzymatic experiment the sample without enzyme was incubated inside magnet at 30°C for 5 min, then locked, tuned, shimmed and initial 32 scans were recorded (See Figure 1 and S2, no enzyme spectra). To perform kinetic experiment the multi_zgvd command was applied with fixed delays (120 s) and number of experiments set to 14. The data were analyzed by MestReNova 12.0 and GraphPad Prism 8.0.

Protein expression and purification. The plasmids for expression of human Fhit, pSGA02_hFhit, and Arabidopsis Thaliana Fhit, pSGA02_AtFhit, were kindly provided by Dr Pawel Bieganowski (Mossakowski Medical Research Centre, Polish Academy of Sciences).

Human Fhit. Full length human Fhit was produced in E. coli BL21(DE3) RIL strain in LB medium with ampicillin (100 µg/mL). Bacterial culture was grown to OD_{600} 0.4 and protein expression was induced by 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 30 °C during 6 h. For the protein purification, cells were lysed with 0.1 mg/mL lysozyme during 20 min on ice followed by sonication in a buffer A containing 20 mM Tris/HCl pH 7.5, 100 mM NaCl and 2 mM dithiothreitol (DTT) with addition of protease inhibitors (10 µM leupeptin, 0.3 µM aprotinin, 1 µM pepstatin A and 1 mM phenylmethylsulfonyl fluoride (PMSF)). The lysate was clarified by centrifugation at 35 000 x g for 40 min. at 4 °C. Then nucleic acids were removed from supernatant by precipitation using 0.1%

polyethyleneimine (PEI) and protein was clarified by multistep precipitation in ammonium sulfate. Initial precipitation in 20% ammonium sulfate removed non-soluble proteins and double precipitation in 70% ammonium sulfate isolated the main protein fraction. Finally, pelleted protein was resuspended in buffer A, desalted on HiPrep 26/10 column and polished by gel filtration on HiLoad 26/600 Superdex 75 pg column filled with buffer B containing 20 mM HEPES/NaOH pH 7.0, 150 mM NaCl, and 2 mM DTT. This multistep procedure allows to gain more than 90% pure protein assessed by SDS–PAGE gel electrophoresis. The absorbance ratioA_{260}/A_{280} for final protein sample was 0.73. Human Fhit samples were concentrated on Amicon® Ultra-15 10K filters up to 8.4 mg/mL or 16.8 g/mL, flash-frozen in liquid nitrogen and stored at -80 °C in 50 µL or 100 µL aliquots in the presence of 10% glycerol. Molecular mass of the human Fhit monomer is 16 800 Da and the protein concentration was determined spectrophotometrically using the extinction coefficient calculated from amino acid composition, ε_{280} = 8480 M^{-1} cm^{-1} (Expasy Server).

Human DcpS preparation. Human DcpS (hDcpS) was expressed as previously described, but with minor modifications.[61] The concentration of the protein was determined spectrophotometrically by assuming ε_{280} = 30 400 M^{-1} cm^{-1}. The enzyme was stored at −80 °C in a storage buffer (50 mM Tris-HCl, pH 7.6, 200 mM NaCl, 1 mM DTT, 10% glycerol).

Human cNIIIB preparation. Human cNIIIB (^{Hs}cNIIIB) was expressed as previously described.[57]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

^{1}H NMR, ^{13}C NNMR, ^{19}F NMR, ^{31}P NMR, HR MS spectra, HPLC profiles of new compounds, supporting figures and additional experimental details.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interests.

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