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Structural-based design and synthesis of novel 9-deazaguanine derivatives having a phosphate mimic as multi-substrate analogue inhibitors for mammalian PNPs

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ABSTRACT

9-(5′,5′-Difluoro-5′-phosphonopentyl)-9-deazaguanine (**DFPP-DG**) was designed as a multi-substrate analogue inhibitor against purine nucleoside phosphorylase (PNP) on the basis of X-ray crystallographic data obtained for a binary complex of 9-(5′,5′-difluoro-5′-phosphonopentyl)guanine **(DFPP-G)** with calf-spleen PNP. **DFPP-DG** and its analogous compounds were synthesized by the Sonogashira coupling reaction between a 9-deaza-9-iodoguanine derivative and ω-alkynyldifluoromethylene phosphonates as a key reaction. The experimental details focused on the synthetic chemistry along with some insights into the physical and biological properties of newly synthesized **DFPP-DG** derivatives are disclosed.

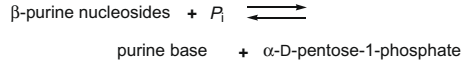
1. Introduction

Phosphorylation and dephosphorylation of biological molecules including proteins, sugars, and nucleosides is one of the most fundamental processes in the mammalian organism. Molecular design to regulate the activity of enzymes which catalyze the phosphorylation/dephosphorylation processes has a bright future in the development of functional molecules as therapeutic reagents and biological tools.[1] Purine nucleoside phosphorylase (PNP, E.C. 2.4.2.1), a nucleoside processing enzyme, is ubiquitous and essential in providing precursors for RNA and DNA synthesis and energy metabolism.[2] PNP catalyzes the reversible phosphorolytic cleavage of the glycosidic bond of ribo- and deoxyribonucleosides, in the presence of inorganic orthophosphate (Pi) as a second substrate, to generate purine bases and ribose(deoxyribose)-1-phosphate (Scheme 1).

In mammals, PNP is crucial for the proper functioning of cellular immune systems, since PNP deficiency in humans leads to the impairment of T-cell function, usually with no apparent effects on B-cell function.[2] Consequently, PNP inhibition has become a target for drug design on selective immunosuppressive agents.

PNP accomplishes the reversible phosphorylation of purine nucleosides via a ternary complex of enzyme, nucleoside, and orthophosphate. Compounds that contain covalently linked elements of both substrates (nucleoside and orthophosphate) in their structure are expected to act as a 'multi-substrate analogue' inhibitor of PNP. Therefore, a number of metabolically stable acyclic nucleotides containing a purine and phosphate-like moiety connected by a linker have been synthesized.[3] Of the PNP inhibitors reported, 9-(5′,5′-difluoro-5′-phosphonopentyl)guanine **(DFPP-G)** developed by Halazy et al., is one of the most potent and structurally simple multi-substrate analogue inhibitors of PNP.[4]

During our previous studies directed toward the design and synthesis of a multi-substrate analogue inhibitor of trimeric PNP based on the use of difluoromethylenephosphonic acid as a phosphate mimic,[5] we have recently succeeded in crystallizing a binary complex with **DFPP-G** and calf-spleen PNP.[6] High-resolution X-ray differentiation data confirmed for the first time that **DFPP-G** acts as a multi-substrate analogue inhibitor as it binds to both nucleoside- and phosphate-binding sites of PNP. In addition, the putative hydrogen bonds identified in the base-binding site indicate that the contact of guanine O^6 with Asn243 O^{d1} is not a direct contact but is mediated by a water molecule (Fig. 1 and 2). The bridging water molecule is entropically disfavored, and exclusion of the water molecule from the complex may induce a tighter bounding to PNP. On the basis of these findings and hypothesis, we designed a new candidate, 9-(5′,5′-difluoro-5′-phosphonopentyl)-9-deaza-



Scheme 1. PNP-catalyzed reversible phosphorylation of purine nucleosides.

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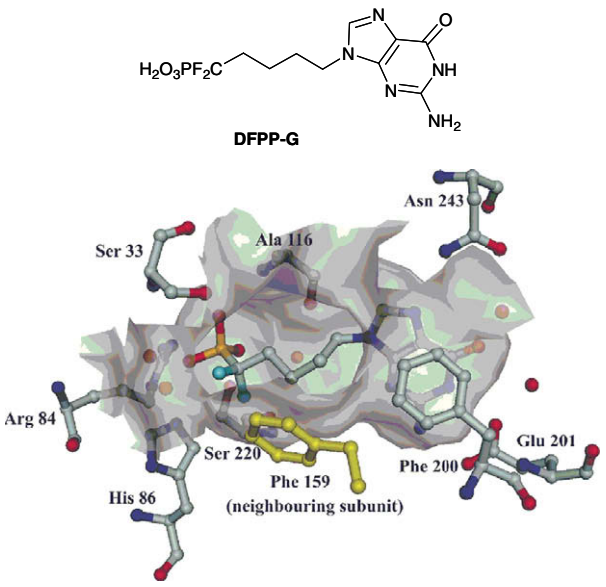


Figure 1. Structure of **DFPP-G** and its interactions with calf-spleen PNP in the binary complex (figure from Ref. 6).

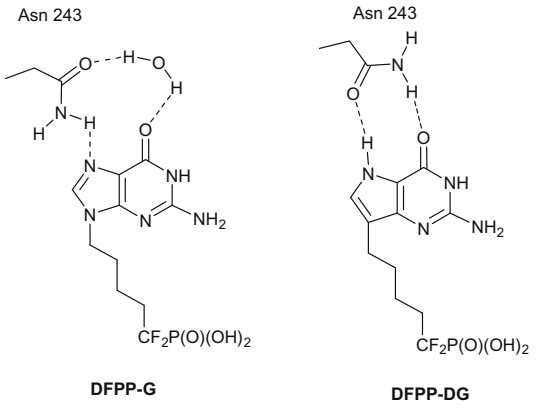


Figure 2. Binding patterns of **DFPP-G** and expected binding patterns of **DFPP-DG** with the base-binding site of PNP.

guanine (**DFPP-DG**) as an inhibitor against PNP, in which the guanine base of **DFPP-G** is replaced by 9-deazaguanine (Fig 2).

Newly designed **DFPP-DG** is expected to bind to the nucleoside-binding site through direct hydrogen-bonds to Asn243 as shown in Figure 2. Such hydrogen-bond patterns in which *N*(7) of the base is protonated and donates a hydrogen to Asn243O^{d1}, while the Asn243N^{d1} donates a hydrogen to the exocyclic O^6 of the base, are generally observed with binary complexes with PNP and ground-state PNP inhibitors such as 9-arylmethy-9-deazaguanine derivatives.[2,7] In addition, similar hydrogen-bond patterns were detected in a ternary complex with the enzyme, phosphate, and immucillin G, a transition-state analogue inhibitor of PNP.[8] Synthetic and biological studies of **DFPP-DG** and the related analogues have been reported in a preliminary communication.[9] In this paper, we now disclose the experimental details focused on the synthetic chemistry along with additional insights into the physical and biological properties of newly synthesized **DFPP-DG** derivatives as an extension of our previous works. We also disclose an improved synthesis of a ω-alkynyldifluoromethylenephosphonate unit, which are previously synthesized in a very low yield.

2. Results and discussion

2.1. Synthesis

2.1.1. Synthetic strategies

To synthesize **DFPP-DG** derivatives, a strategy which has a high diversity for adjusting the length of the linker is desirable. In this context, we consider Pd-catalyzed cross-coupling reactions between base unit **1** and CF_2-phosphonate units **2–4** under Heck, Still and Sonogashira conditions. This strategy would lead to a variety of analogues of **DFPP-DG** upon adjusting the number of carbon atoms (n) in compounds **2–4** (Scheme 2).

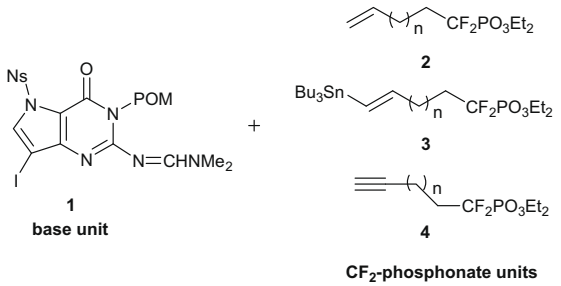
Preliminary studies of the coupling reaction of **1** with simple linker units such as 3-buten-1-ol, *tri-n*-butylvinylstanane, and 3-butyn-1-ol under Heck, Still, and Sonogashira reaction conditions,[10] respectively, revealed that only the Sonogashira reaction with 3-butyn-1-ol gave the corresponding coupling-product in good yield, while the Still and Heck reactions gave no desired products. Therefore we decided to examine the cross-coupling reaction of **1** with ω-alkynyldifluoromethylenephosphonate units **4** under Sonogashira conditions for the synthesis of **DFPP-DG** derivatives.

2.1.2. Synthesis of base unit 1

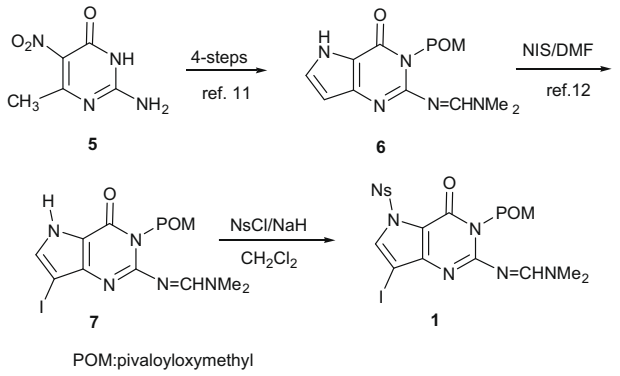
A literature review revealed that suitably protected 9-deazaguanine derivative **6** has been previously prepared from readily accessible pyrimidinone **5**,[11] and compound **6** was transformed to 9-iodo-9-deazaguanine derivative **7** by NIS-mediated selective iodination at the 9-position.[12] Then, according to the method in literature, we were able to obtain multi-grams of **7** without any difficulty. To protect the acidic proton at the 7-position, **7** was nosylated by a conventional method to give **1** in a quantitative yield (Scheme 3).

2.1.3. Preparation of CF_2-phosphonate units 4

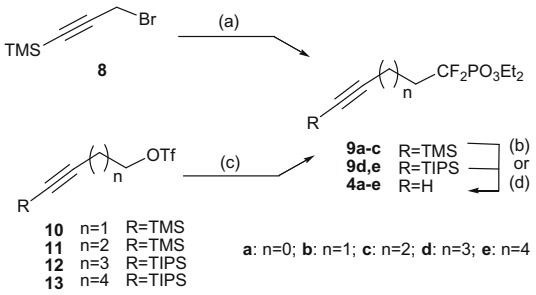
Treatment of TMS-protected bromopropyne **8** with $BrZnCF_2PO_3Et_2$ in the presence of CuBr in DMF according to our previously reported protocols[13] gave **9a** in a 70% yield. Tetrabutylammonium floride (TBAF)-induced desilylation of **9a** in THF in the presence of AcOH gave ω-alkynyldifluoromethylene phosphonate unit **4a** (C3-phosphonate unit) in a 97% yield. To synthesize C4–C7-phosphonate units **4b–e**, alkylation of $LiCF_2PO_3Et_2$ with readily available triflates **10–13** was applied according to the method described by Berkowitz.[14] While the reaction with **11–13** under the conditions proceeded smoothly to give **9c–e** in high yields, the reaction with **10** was problematic due to the competitive formation of large amounts of enyne products by β-elimination of HF under the conditions. However, **9b** was isolated in a very low yield (8%). Compounds **9b–e** were treated with TBAF in THF to give the requisite C4–C7-phosphonate units **4b–e** in modest and good yields (Scheme 4).



Scheme 2. Cross-coupling strategy for synthesis of **DFPP-DG** analogues.



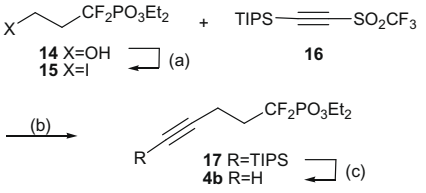
Scheme 3. Preparation of 9-iodo-9-deazaguanine derivatives.



Scheme 4. Synthesis of ω-alkynyldifluoromethylenephosphonate units. Reagents and conditions: (a) $BrZnCF_2PO_3Et_2$, CuBr, DMF, rt; (b) TBAF, THF, AcOH; (c) $LiCF_2PO_3Et_2$, THF, −78 °C; (d) TBAF, THF.

2.1.4. Improved synthesis of CF_2-phosphonate 4b

As described in the previous section, we encountered a significant difficulty in preparation of CF_2-phosphonate unit **4b** via a conventional alkylation reaction of $LiCF_2PO_3Et_2$ with the corresponding triflate **10**. The difficulty attributes to extremely unstable of triflate **10** and the alkylation product **9b** under the basic conditions. Therefore, to obtain **4b** in a reasonable yield and quantities, a new reaction, which proceeds under a neutral condition, should be developed as a key reaction. Fuchs has reported that organic iodides undergo chemospecific alkynylation with acetylenic triflones through photochemical irradiation.[15,16] The Fuch method possess an attractive feature and would be applicable to the synthesis of **4b**, since the reaction medium is almost neutral. Then, we examined alkynylation reaction of 3-iodo-1,1-difluorophosphonate **15**, readily prepared from the corresponding alcohol **14**, with triisopropylsilylacetylenic triflone **16** (Scheme 5).[16] According to the reported method, a 0.15 M solution of **15** (1.0 equiv) and **16** (1.2 equiv) in benzene was irradiated by 500 W Hg-lamp (Pyrex filter) in the presence of a catalytic amount of hexabutyldistannane for 16 h at ambient temperature. As expected, this reaction provided the desired adduct **17** in 42% yield. Compound **17** was desilylated with TBAF in THF to give **4b** in 67% yield. Using this sequence, required **4b** was obtained in modest overall yield (26 %).



Scheme 5. Improved synthesis of phosphonate unit **4b**. Reagents and conditions: (a) I_2, PPh_3, imidazole, CH_2Cl_2; (b) $(n\text{-}Bu_3Sn)_2$, hν, benzene; (c) TBAF, THF.

2.1.5. Sonogashira reaction with phosphonate units 4b-e

The Sonogashira coupling reaction between base unit **1** and C5-phosphonate unit **4c** was first examined to verify proper reaction conditions, since **4c** was readily available in a large scale. These results are summarized in Table 1.

When the Sonogashira reaction of **1** was carried out with a slight excess of **4c** in Et_3N in the presence of $PdCl_2(PPh_3)_2$ (2 mol %) and CuI (1 mol %) at 60 °C for 15 h according to the procedure reported by Larock,[17] desired coupling product **18c** was obtained in a 58% yield along with a large amount of recovered **1** (36%) (entry 1). The yield of **18c** was not increased upon using the Pd(0) catalysis under the same conditions (entry 2). In an effort to increase the yield, modified conditions using representative solvents composed by 2 equiv of Et_3N were examined (entries 4–9). This survey identified MeCN was a good solvent to induce a good yield (69%) of **18c** and minimize the recovered yield of **1** (entry 6).

The findings were applicable to the Sonogashira reactions with phosphonate units **4b**, **4d**, and **4e**. The results are also included in Table 1. It should be noted that the prolonged reaction time (15 h) of the reactions with phosphonate units **4d** and **4e** resulted in low yields and modest yields were obtained upon diminishing the reaction time to 3–4 h (entries 8 and 9).

2.1.6. The Sonogashira reaction of 1 with phosphonate unit 4a

The Sonogashira reaction of **1** with phoshonate unit **4a** was examined according to the optimized conditions described in the previous section (Table 1, entry 6). However, this reaction yielded the desired coupling product **18a** in a low yield along with the de-nosyl derivative **19a** and de-nosyl enyne product **20** in a comparative yield (Scheme 5 and Table 2, entry 1).

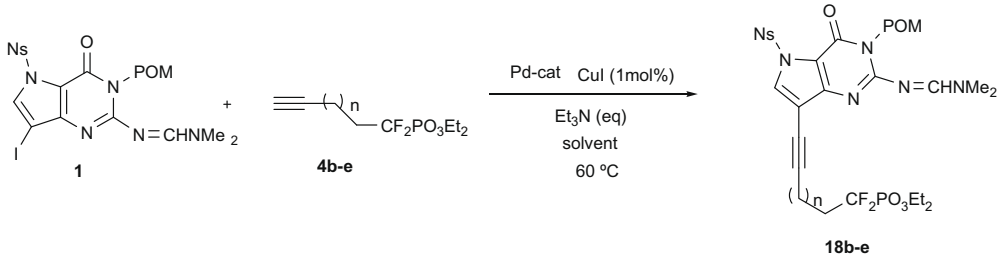
To gain an insight into a mechanism of the formation of **19a** and **20**, isolated **18a** was treated with Et_3N in MeCN at 60 °C for 22 h. This reaction gave **19a** and **20** in 8% and 38% yields, respectively. However, phosphonate unit **4a** was inert to the same conditions. On the basis of these findings, the mechanism shown in Figure 3 is proposed for the formation of the by-products. In this mechanism, the initial-formed cross-coupling product **18a** is transformed to enyne derivative **21** through Et_3N-induced β-elimination of HF, since the acidity of propalgyl protons increase upon incorporation of the heteroaromatic. Enyne **21** is rapidly de-nosylated to give **20** by action of the floride ion derived from triethylammonium floride ($Et_3N^+HF^-$),[18] that is produced in the first-step reaction. Alternatively, the route where compound **19a** is first de-nosylated, followed by β-elimination of HF to give **20**, is also predictable.

Keeping the above mechanistic prediction in mind, the cross-coupling reaction between **1** and **4a** was carried out in MeCN in the presence of a slight excess of Et_3N for diminishing reaction time (1 h) to suppress the formation of the by-products (Table 2, entry 2). This reaction gave the desired coupling product **18a** in a 59% yield, along with recovering **1** in a 41% yield. As expected, the formation of the by-products was significantly suppressed under the conditions.

2.1.7. Preparation of DFPP-DG and its derivatives

Compounds **18a–e**, thus obtained, were readily transformed to **DFPP-DG** and its analogues (Scheme 6). Thiophenol-mediated removal of the nosyl protecting group for **18b**, followed by hydrogenation, gave **22b** in a 90% yield. Compound **22b** was treated with NaOMe in MeOH to give **23b** in an 82% yield. Removal of the ethyl protecting group and the dimethylaminomethylene group for **23b** was achieved in concd HCl at reflux (20 h) to give **DFPP-DG** (**25**) as a white solid in a 93% yield. Compounds **18a** and **18c–e** were, respectively, transformed to ***nor*-DFPP-DG** (**24**), ***homo*-DFPP-DG** (**26**), ***6C*-DFPP-DG** (**27**), and ***7C*-DFPP-DG** (**28**) by the same procedure as a series of **DFPP-DG** in good overall yield.

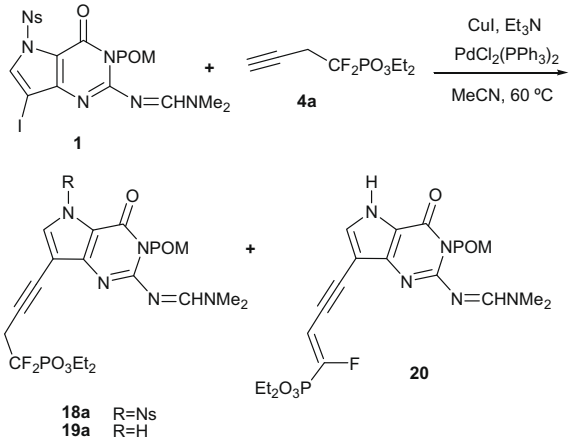
Table 1
Sonogashira coupling between **1** and **4b–e**[a]



Entry	Phosphonate Unit	Pd-cat (mol %)	Et_3N (equiv)	Time (h)	Solvent	Yield (%)	
						18	**1**
1	**4c** (n = 2)	$PdCl_2(PPh_3)_2$ (2)	>60	15	None	58	36
2	**4c** (n = 2)	$Pd(PPh_3)_4$ (2)	>60	15	None	22	78
3	**4c** (n = 2)	$PdCl_2(PPh_3)_2$ (20)	>60	15	None	40	49
4	**4c** (n = 2)	$PdCl_2(PPh_3)_2$ (2)	2	15	THF	15	69
5	**4c** (n = 2)	$PdCl_2(PPh_3)_2$ (2)	2	15	DMF	41	59
6	**4c** (n = 2)	$PdCl_2(PPh_3)_2$ (2)	2	15	MeCN	64	6
7	**4b** (n = 1)	$PdCl_2(PPh_3)_2$ (2)	2	15	MeCN	66	8
8	**4d** (n = 3)	$PdCl_2(PPh_3)_2$ (2)	2	3	MeCN	75	21
9	**4e** (n = 4)	$PdCl_2(PPh_3)_2$ (2)	2	4	MeCN	68	19

[a] All reactions were carried out in the presence of 1 mol % of CuI.

Table 2
Sonogashira reaction of **1** with phosphonate unit **4a**[a]



Entry	Et_3N (equiv)	Time (h)	Yield (%)			
			18a	**19a**	**20**	**1**
1	2	14	15	17	11	ND[b]
2	1.2	1	59	ND[b]	ND[b]	41

[a] All reactions were carried out in the presence of 1 mol % of CuI and 2 mol % of $PdCl_2(PPh_3)_2$.
[b] Not detected.

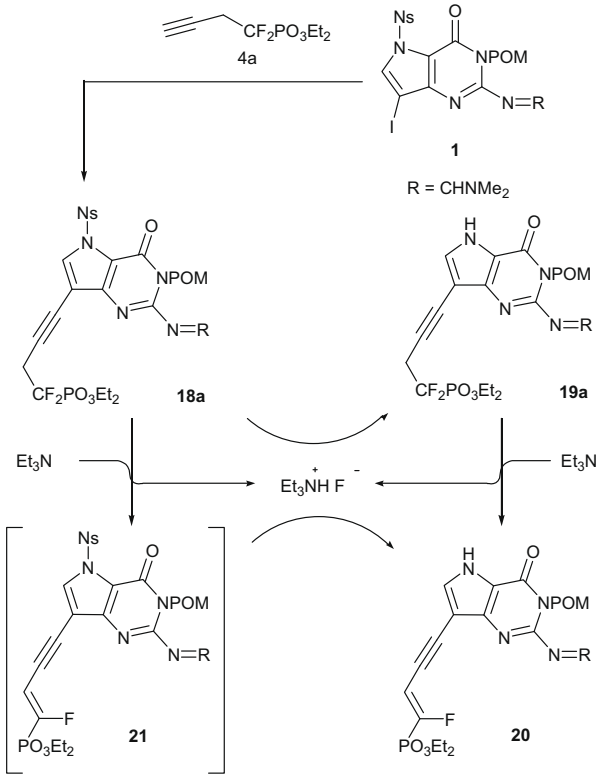


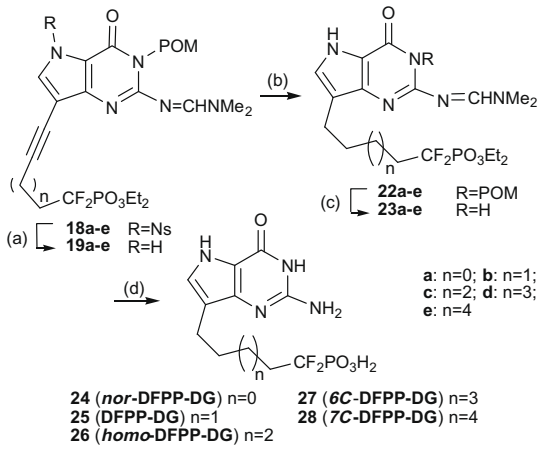
Figure 3. The predictive mechanism of the formation of by-products in Sonogashira reaction with phosphate unit **4a**.

2.2. Biophysical/biochemical results and discussion

2.2.1. Spectral data for the new analogues

Spectrophotometric titrations of the **DFPP-DG** analogues and 9-deazaguanine were carried out in the pH range 1–14. These titrations indicated that all **DFPP-DG** analogues show two ionizable sites at the heterocyclic base: one in the pH range 4–5 and the second one about pH 11 (Fig. 4). These were attributed, by comparison with the spectra of three model compounds, 9-deazaguanine, 1-methyl-9-deazaguanine and 1,7-dimethyl-9-deazaguanine (data not shown), to cation formation, most probably as a result of protonation at nitrogen N(3) and anion formation resulting in deprotonation of N(1)-H, as shown in Scheme 7.

On the basis of the pK_{a1} and pK_{a2} values, the active form of **DFPP-DG** derivative is most probably the neutral form under the physiological conditions. Some time ago it was hypothesized, on the basis of unusual enhancement of the fluorescence in the PNP/guanine complex that trimeric PNPs bind preferentially to the



Scheme 6. Synthesis of **DFPP-DG** analogues. Reagents and conditions: (a) thiophenol, K_2CO_3, DMF; (b) H_2, Pd–C, MeOH–$CHCl_3$; (c) NaOMe, MeOH; (d) concd HCl.

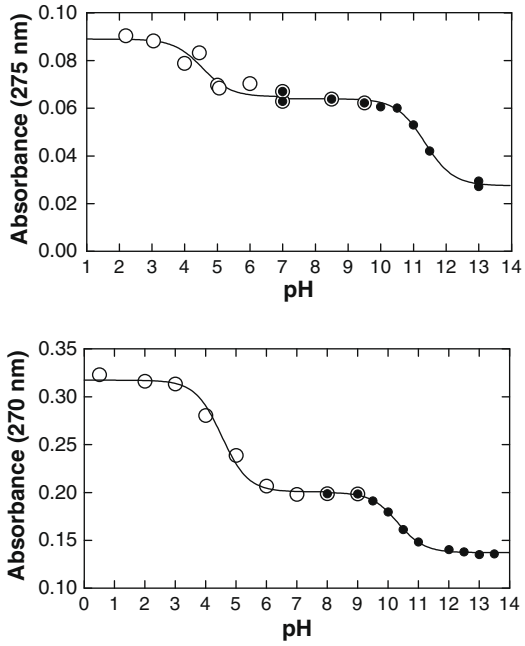
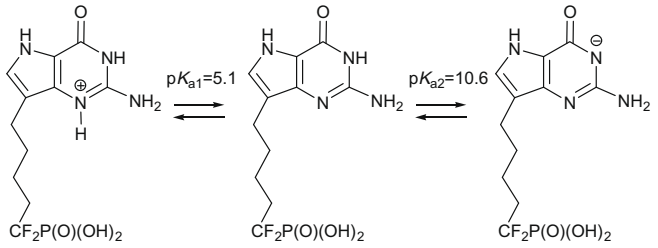


Figure 4. Spectrophotometric titrations of **DFPP-DG** (upper panel) and 9-deazaguanine (bottom panel) showing two ionization sites in the pH range 1–14. The solid curves are the least-squared fits to the points of titration, conducted separately in the pH range 1–9 and 7–14 assuming a single proton ionization site in the particular pH range examined.



Scheme 7. Chemical structures of neutral and ionic forms of **DFPP-DG** and pK_a assignments that were done by comparison with UV spectra and pK_a of model compounds, 1-methyl-9-deazaguanine and 1,7-dimethyl-9-deazaguanine.

anion of guanine.[19] However, subsequent crystallographic[20] and solution studies[21] have shown unequivocally that this is not the case.

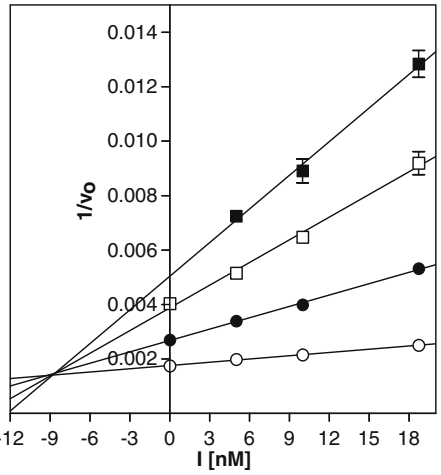


Figure 5. Inhibition of human erythrocyte PNP by **DFPP-DG**, (○) 0 μM (●) 4.99 μM, (□) 10.98 μM, (■) 18.71 μM, shown in Dixon plot form. Reactions were carried out in 50 mM hepes buffer pH 7.0, at 25 °C, with 7-methylguanosine as a variable substrate, and in the presence of 1 mM phosphate. Kinetic data are presented in the form of the Dixon plot only to visualize the type of inhibition. Data (v_o, c_o and [I]) were analyzed and kinetic constants were obtained with the use of the weighted least-squares non-linear regression using the program Leonora.[21]

2.2.2. Inhibition data

The inhibitory properties of new compounds with calf-spleen and human erythrocyte PNPs were determined with 7-methylguanosine (m^7Guo) as a variable substrate using methods previously described for other inhibitors of trimeric PNPs.[22] The substrate employed was m^7Guo, because in the case of trimeric PNPs, its kinetics, in striking contrast to those observed in the natural substrates inosine and guanosine, is fairly well described by the Michaelis–Menten model.[22a] With fixed concentrations of one substrate—inorganic phosphate—apparent inhibition constants (K_i^{app}) were determined from initial velocity data with variable concentrations of the inhibitor and the second substrate—m^7Guo. Dixon plots displayed a competitive mode of inhibition as shown in Figure 5 for **DFPP-DG** and human erythrocyte PNP. Kinetic data are presented in the form of the Dixon plot only to visualize the type of inhibition. Data sets were analyzed and the apparent inhibition constants were calculated with the use of the weighted least-squares non-linear regression using the program Leonora[23] as described in the Experimental section. The results obtained for **DFPP-DG** and analogues are summarized in Table 3.

Not only the apparent inhibition constants but also IC_{50}s were calculated form the experimental data (see Experimental section). This allowed for a more reliable comparison with literature data available for previously synthesized PNP inhibitors. For a comparison of the inhibitory activity of **DFPP-G**[22a] and a transition state-analogue inhibitor—immucillin H[24]—are also included in Table 3.

All compounds studied were found to be very potent inhibitors of 7-methylguanosine (m^7Guo) phosphorolysis with apparent inhibition constants, K_i^{app}, in the nM range. Inhibition is competitive versus nucleoside (m^7Guo), and apparent K_i values decrease with decreasing phosphate (the fixed substrate) concentration (see Table 3). This indicates that the inhibitors studied bind to both nucleoside- and phosphate-binding sites, hence they act as multi-substrate analogue inhibitors.

As predicted by previous structural studies,[6] **DFPP-DG** allows for more favorable interactions with the base-binding site of calf spleen and human erythrocyte PNPs when compared with **DFPP-G**, and therefore, yields K_i^{app} and IC_{50}s lower than those observed for **DFPP-G** (Table 3). Other derivatives with shorter and longer linkers exhibited weaker inhibitory effects, except for

Table 3
Inhibitory properties of **DFPP-G**, **DFPP-DG** and their analogues vs calf-spleen and human erythrocyte PNPs[a]

Compound	Pi concd (mM)	IC_{50}[b] (nM) for calf PNP	IC_{50}[b] (nM) for human PNP	K_i^{app} (nM) for calf PNP	K_i^{app} (nM) for human PNP	K_i^{eq} (nM) for calf PNP
DFPP-G	1	18.7	20.2	6.9 ± 0.7[c]	10.8 ± 0.7	0.72 ± 0.13
DFPP-G	0.025	—	—	2.7 ± 0.2	—	
DFPP-DG	1	10.2	20.4	4.4 ± 0.6	8.1 ± 0.6	0.085 ± 0.013
DFPP-DG	0.025	—	—	1.0 ± 0.2	1.0 ± 0.2	
***nor*-DFPP-DG**	1	—	170	—	—	
***homo*-DFPP-DG**	1	10.2	9.5	5.7 ± 0.6	5.3 ± 0.4	0.36 ± 0.04
***6C*-DFPP-DG**	1	42	22	21 ± 2	13 ± 1	0.23 ± 0.03
***7C*-DFPP-DG**	1	—	—	6.1 ± 0.9	5.6 ± 0.8	0.94 ± 0.10
Immucillin H	50	—	—	41 ± 8[d]	—	0.023 ± 0.05[d]

[a] All reactions were carried out in 50 mM hepes buffer pH 7.0, at 25 °C, with 7-methylguanosine as a variable substrate, in the presence of a fixed concentration of phosphate.
[b] With 25 μM of 7-methylguanosine.
[c] Data from Iwanow et al. (Ref. 22b).
[d] Data from Miles et al. (Ref. 24).

***homo*-DFPP-DG** vs human erythrocyte PNP, which exhibited even better binding properties than those observed for **DGPP-DG** (K_i^{app} = 5.3 nM as compared with 8.1 nM, see Table 3).

These inhibition constants, K_i^{app}, determined by the classical approach and shown in Table 3 should be treated as apparent values since the reaction rates observed in the presence of **DFPP-DG** and analogues exhibit some inhibition in the initial velocity experiments, with increasing inhibition as a function of time (Fig. 6). Therefore, the inhibition constant for binding of **DFPP-DG** and analogues to calf-spleen PNP was also determined at equilibrium, as described in Experimental section. Approach to equilibrium was followed by measuring the velocity observed after various times of incubation (0.5–120 min), and for various inhibitor concentrations (in the range 1–50 nM), $v(t, [I])$. Steady-state velocities, v_s were determined as shown in Figure 6, upper panels, by fitting Eq. 1 to the $v(t)$ dependence, separately for each inhibitor concentration [I]. From the set of v_s obtained for various inhibitor concentrations, the inhibition constant at equilibrium, K_i^{eq}, was determined by fitting Eq. 2 to the $v_s[I]/k_c$ dependence, and for **DFPP-DG** was found to be K_i^{eq} = 85 ± 13 pM (Table 3), hence two orders of magnitude lower than the apparent inhibition constant determined in the standard initial velocity experiment, K_i^{app} = 4.4 nM (Table 3). For other compounds K_i^{eq} was also at least 10-fold lower that the apparent inhibition constant, K_i^{app} determined by the standard initial velocity procedure (se Table 3).

The time-dependence of inhibition may be due to low enzyme and inhibitor concentrations leading to slow-binding inhibition caused by the fact that equilibrium may not be reached in the time scale of the initial velocity studies.[25] However, it is also possible that the time-dependence of inhibition of **DFPP-DG** is due to the slow-binding of the inhibitor with the enzyme (one step mechanism) or slow-onset (i.e., two step) mechanism. In the two-step mechanism binding involves the rapid formation of the enzyme/inhibitor collision complex followed by a slow conformational change leading to a more tightly bound enzyme/inhibitor complex, as observed for immucillin H and analogues.[24] Studies that aim to answer the question, which of the two mechanisms is responsible for the time-dependence of inhibition of **DFPP-DG**, were undertaken and the results obtained are more consistent with the two-step mechanism.[26]

3. Conclusion

In summary, we have designed and synthesized 9-(5′,5′-difluoro-5′-phosphonopentyl)-9-deazaguanine (**DFPP-DG**) and its analogues as new multi-substrate analogue inhibitors against PNPs on the basis of the structure for the binary complex of **DFPP-G**/calf-spleen PNP. The preparation of these nucleotide analogues was achieved using the Sonogashira reaction between a 9-iodo-9-deazaguanine derivative and ω-alkynyldifluoromethylenephosphonate units as a key reaction. The apparent inhibition constants of **DFPP-DG**, determined by the initial velocity experiments, proved to be more potent than those of **DFPP-G** and immucillin H. The equilibrium inhibition constant for **DFPP-DG** is K_i^{eq} = 85 ± 13 pM and those of **DFPP-DG** analogues are also in the pM range. Hence these compounds are almost as potent inhibitors as the transition state inhibitors, immucillins.

DFPP-G[4] and some of its analogues with cyclic and acyclic linkers between the purine base and difluoromethylene phosphonic acid groups,[5,22b] were synthesized following a suggestion, derived from electronic and steric considerations,[27] that α-fluoro and α,α-difluoro alkanephosphonates should better mimic phosphate esters than the corresponding phosphonates, hence they are expected to better interact with the phosphate-binding site of PNPs. As expected, on the basis of these findings, such derivatives proved to be competitive inhibitors of trimeric PNPs exhibiting inhibitory activity in pM concentrations. Some of them also showed interesting biological properties, for example, they significantly slow down proliferation of T-lymphocytes isolated from patients with autoimmune thyroid disease—Hashimoto's thyroiditis, compared to the inhibitory effects on the growth of human blood T-lymphocytes isolated from healthy donors.[22b] It is therefore very likely that the newly synthesized series of **DFPP-DG** and its analogues are even more promising candidates as in vivo PNP inhibitors. Detailed studies of their effect on the proliferation of various cell lines are now in progress.

4. Experimental section

4.1. Chemicals

4.1.1. 2*N*-(*N*,*N*-Dimethylaminomethylidene)-9-iodo-1*N*-(pivaloyloxy)methyl-9-deazaguanine (7)

To a stirred solution of **6**[10] (1.88 g, 5.89 mmol) in DMF (59 mL) was added NIS (1.32 g, 5.89 mmol) at room temperature. After being stirred for 30 min, the solvent was evaporated in vacuo. The residue was treated with MeOH and the resulting precipitates were filtered to give **7** (2.56 g, 98%) as a white solid: mp >228 °C (colored); ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.25 (br s, 1H, NH), 8.50 (s, 1H, C*H*NMe$_2$), 7.45 (d, 1H, 8-H, J = 2.2 Hz), 6.17 (s, 2H, $CH_2OC(O)^tBu$), 3.16, 2.97 (each s, 6H, CHN(CH_3)$_2$), 1.08 (s, 9H, ^{t}Bu); ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.64, 156.68, 153.65, 153.44, 144.56, 132.08, 113.73, 65.49, 57.57, 40.56, 38.22, 34.50, 26.69 (3 carbons); ESI-MS (LR) *m/z* 446 (MH^+); ESI-MS (HR) calcd for $C_{15}H_{21}N_5O_3I$ (MH^+): 446.0689, found: 446.0677.

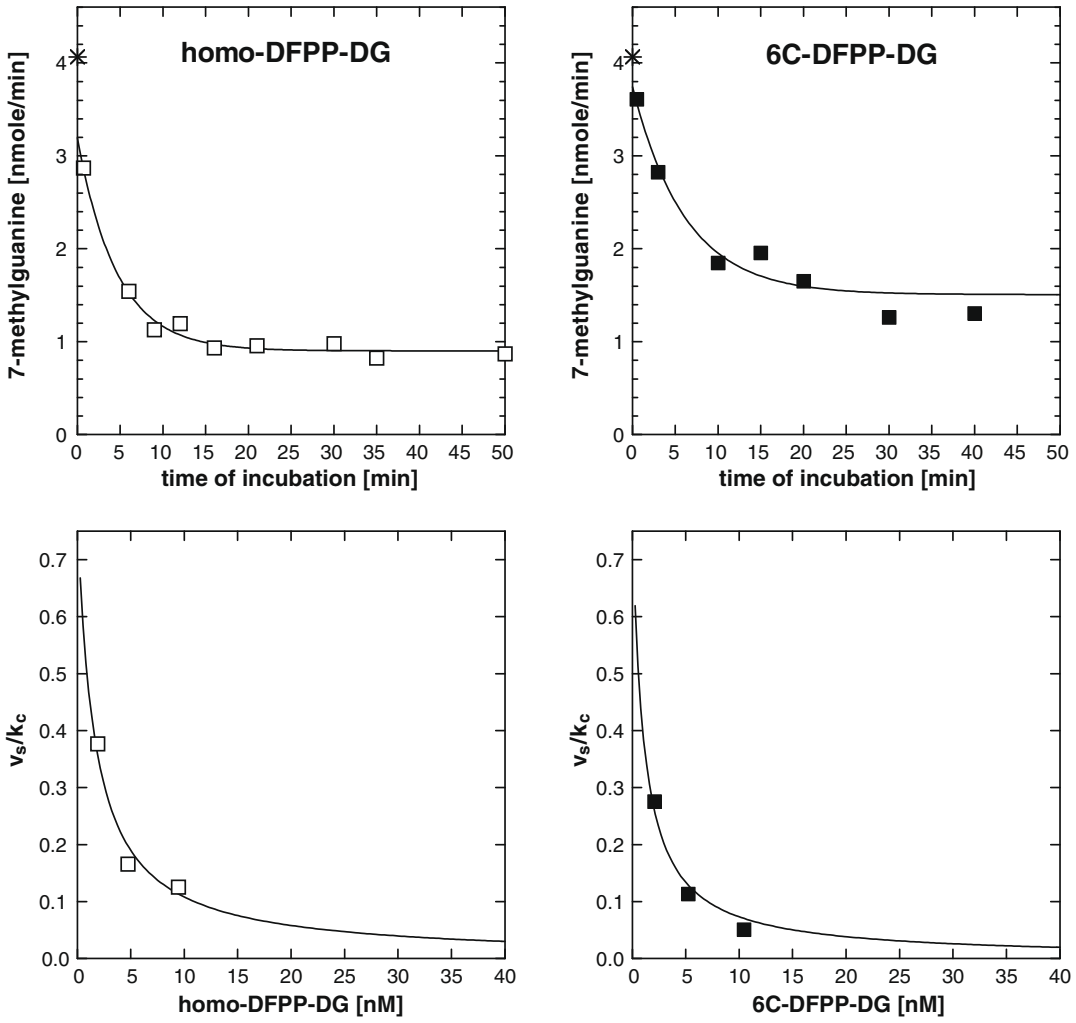


Figure 6. Time-dependence of inhibition of (upper panels) and inhibition in equilibrium (lower panels) of calf spleen PNP by ***homo*-DFPP-DG** (left panels) and ***6C*-DFPP-DG** (right panels). Time-dependence of inhibition: 4 73 nM of ***homo*-DFPP-DG** (□) and 2.09 nM of ***6C*-DFPP-DG** (■) were incubated with 3.95 nM and 2.45 nM of calf PNP (in terms of subunits), respectively, and initial velocity was measured as described in Experimental section. Similar experiments were repeated for various inhibitor concentrations (not shown here) and velocity in equilibrium, v_s, was determined by fitting Eq. 1 to the time-dependence observed. The equilibrium velocities, v_s were plotted as a function of the inhibitor concentration (lower panels). The inhibition constants at equilibrium, K_i^{eq}, were obtained by fitting Eq. 2 to the dependence of equilibrium velocities v_s versus inhibitor concentration, [I]. The K_i^{eq} value obtained from these data is 0.36 ± 0.04 nM in the case of ***homo*-DFPP-DG**, and 0.23 ± 0.03 nM in the case of ***6C*-DFPP-DG**.

4.1.2. 2*N*-(*N*,*N*-Dimethylaminomethylidene)-9-iodo-7*N*-(2-nitrobenzene)sulfonyl-1*N*-(pivaloyloxy)methyl-9-deazaguanine (1)

To a stirred suspension of **7** (2.33 g, 5.23 mmol) in CH_2Cl_2 (106 mL) was added NaH (50% purity, 276 mg, 5.75 mmol) at room temperature. After being stirred for 10 min, the mixture was treated with *o*-nitrobenzenesulfonyl chloride (1.39 g, 6.28 mmol). The mixture was stirred for 6 h at room temperature. The reaction was quenched with MeOH, poured into water and extracted with $CHCl_3$. The organic extracts were washed in brine, dried over $MgSO_4$ and evaporated. The resulting residue was chromatographed on silica gel (hexane/$CHCl_3$ = 1:1) to give **1** (3.51 g, ca. 100%) as a pale yellow solid: mp 238–240 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.57 (s, 1H, C*H*NMe$_2$), 8.30 (dd, 1H, CH of Ns, J = 1.3 Hz, 7.9 Hz), 8.10 (dd, 1H, CH of Ns, J = 1.2 Hz, 7.8 Hz), 8.00–7.92 (m, 3H, CH of Ns × 2, 8-H), 6.01 (s, 2H, C*H*$_2$OC(O)^{t}Bu), 3.20, 2.99 (each s, 6H, CHN(C*H*$_3$)$_2$), 1.03 (s, 9H, ^{t}Bu); ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.42, 157.70, 156.55, 151.80, 150.37, 147.29, 136.28, 135.08, 132.99, 132.59, 130.08, 125.22, 112.19, 67.34, 65.56, 40.93, 38.18, 34.79, 26.61 (3 carbons); ESI-MS (LR) *m/z* 631 (MH$^+$); ESI-MS (HR) calcd for $C_{21}H_{24}N_6O_7SI$ (MH$^+$): 631.0472, found: 631.0470.

4.1.3. Diethyl 1,1-difluoro-5-(triisopropylsilyl)pent-4-ynylphosphonate (17)

To a solution of **15** (100 mg, 0.29 mmol) and **16** (110 mg, 0.35 mmol) in benzene (1.95 mL) was added dropwise a solution of hexabutyldistannane (74 μL, 0.15 mmol). After the solution was irradiated by 500 W Hg-lamp (Pyrex filter) for 16 h at the room temperature, the reaction mixture was poured into the ice-water and extracted with AcOEt. The extracts were washed with brine, dried over $MgSO_4$, and evaporated. The resulting residue was chromatographed on silica gel (hexane/AcOEt = 8:1) to give **17** (48 mg, 42%) as a pale yellow oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.28 (dq, 4H, OC*H*$_2$CH$_3$ × 2, $J_{H,H} = J_{H,P}$ = 7.2 Hz), 2.55 (m, 2H, 3-H × 2), 2.35 (m, 2H, 2-H × 2), 1.39 (t, 6H, OCH$_2$C*H*$_3$ × 2, J = 7.2 Hz), 1.03 (m, 21H, Si(CH(CH$_3$)$_2$)$_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 119.89 (dt, $J_{C,P}$ = 215.4 Hz, $J_{C,F}$ = 260.3 Hz), 106.16, 81.41, 64.73 (d, $J_{C,P}$ = 6.6 Hz, 2 carbons), 33.91 (dt, $J_{C,P}$ = 14.8 Hz, $J_{C,F}$ = 20.6 Hz), 18.72 (3 carbons), 16.54 (d, $J_{C,P}$ = 5.5 Hz, 2 carbons), 11.89 (m), 11.33 (6 carbons); ^{19}F NMR (282 MHz, $CDCl_3$) δ −50.43 (dt, 2F, $J_{F,H}$ = 18.8 Hz, $J_{F,P}$ = 107.1 Hz); ^{31}P NMR (122 MHz, $CDCl_3$) δ 7.29 (t, 1P, $J_{P,F}$ = 107.1 Hz); ESI-MS (LR) *m/z* 397 (MH$^+$); ESI-MS (HR) calcd for $C_{18}H_{36}F_2O_3PSi$ (MH$^+$): 397.2139, found: 313.2114.

4.1.4. Diethyl 1,1-difluoropent-4-ynylphosphonate (4b)

To a solution of **17** (40.5 mg, 0.10 mmol) in THF (3.5 mL) was added dropwise a 1.0 M THF solution of TBAF (204 μL, 0.20 mmol) at 0 °C. After the stirring was continued for 5 min at the same temperature, the reaction mixture was poured into water and extracted with AcOEt. The organic extracts were washed with brine, dried over $MgSO_4$, and evaporated. The resulting residue was chromatographed on silica gel (hexane/AcOEt = 6:1) to give **4b** (16.4 mg, 67%) as a pale yellow oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.28 (dq, 4H, OCH_2CH$_3$ × 2, $J_{H,H} = J_{H,P} = 7.3$ Hz), 2.50 (dt, 2H, 3-H × 2, $J_{3,5} = 2.6$ Hz, $J_{3,2} = 7.5$ Hz), 2.35 (m, 2H, 2-H × 2), 1.98 (t, 1H, 5-H, $J_{5,3} = 2.6$ Hz), 1.39 (t, 6H, OCH$_2$CH_3 × 2, $J = 7.3$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 119.67 (dt, $J_{C,P} = 215.7$ Hz, $J_{C,F} = 260.5$ Hz), 81.94, 69.10, 64.59 (d, $J_{C,P} = 7.0$ Hz, 2 carbons), 33.37 (dt, $J_{C,P} = 15.2$ Hz, $J_{C,F} = 20.9$ Hz), 16.38 (d, $J_{C,P} = 5.4$ Hz, 2 carbons), 10.88 (m); ^{19}F NMR (282 MHz, $CDCl_3$) δ -50.61 (dt, 2F, $J_{F,H} = 18.6$ Hz, $J_{F,P} = 106.9$ Hz); ^{31}P NMR (122 MHz, $CDCl_3$) δ 6.97 (t, 1P, $J_{P,F} = 106.9$ Hz); ESI-MS (LR) *m/z* 241 (MH^+); ESI-MS (HR) calcd for $C_9H_{16}F_2O_3P$ (MH^+): 241.0805, found: 241.0810.

4.1.5. 9-[5′,5′-Difluoro-5′-(diethylphosphono)pent-1′-ynyl]-2*N*-(*N*,*N*-dimethylaminomethylidene)-7*N*-(2-nitrobenzene) sulfonyl-1*N*-(pivaloyloxy)methyl-9-deazaguanine (18b)

To a solution of **4b** (144 mg, 600 μmol) in MeCN (2.5 mL) was added Et_3N (139 μL, 1.00 mmol), $PdCl_2(PPh_3)_2$ (7.0 mg, 10.0 μmol) and CuI (1.0 mg, 5.00 μmol) at room temperature. After being stirred for 1 h at the same temperature, to the mixture was added **1** (315 mg, 500 μmol). After being stirred for 15 h at 60 °C, the mixture was evaporated. The resulting residue was chromatographed on silica gel (hexane/AcOEt = 1:1) to give **18b** (244 mg, 66%) as a pale yellow solid: UV λ_{max} (MeOH) 320 nm, 216 nm; mp 192–194 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.59 (s, 1H, C*H*NMe$_2$), 8.34 (dd, 1H, CH of Ns, $J = 1.4$ Hz, 7.9 Hz), 8.06 (dd, 1H, CH of Ns, $J = 1.3$ Hz, 7.9 Hz), 8.01–7.92 (m, 3H, CH of Ns × 2, 8-H), 6.02 (s, 2H, CH_2OC(O)^{t}Bu), 4.23 (dq, 4H, OCH_2CH$_3$ × 2, $J_{H,H} = J_{H,P} = 7.1$ Hz), 3.18, 2.99 (each s, 6H, CHN(CH_3)$_2$), 2.74 (t, 2H, 3′-H × 2, $J_{3',4'} = 7.6$ Hz), 2.40 (m, 2H, 4′-H × 2), 1.30 (t, 6H, OCH$_2$CH_3 × 2, $J = 7.1$ Hz), 1.02 (s, 9H, ^{t}Bu); ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.46, 157.86, 156.65, 152.11, 149.73, 147.32, 136.44, 134.26, 133.02, 132.82, 129.88, 125.28, 119.96 (dt, $J_{C,P} = 214.0$ Hz, $J_{C,F} = 260.3$ Hz), 112.25, 103.42, 92.42, 70.74, 65.48, 64.37 (d, $J_{C,P} = 6.4$ Hz, 2 carbons), 40.84, 38.22, 34.75, 32.59 (dt, $J_{C,P} = 14.9$ Hz, $J_{C,F} = 20.3$ Hz), 26.64 (3 carbons), 16.22 (d, $J_{C,P} = 4.8$ Hz, 2 carbons), 11.58 (m); ^{19}F NMR (376 MHz, DMSO-d_6) δ −50.74 (dt, 2F, $J_{F,H} = 19.5$ Hz, $J_{F,P} = 104.0$ Hz); ^{31}P NMR (162 MHz, DMSO-d_6) δ 7.44 (t, 1P, $J_{P,F} = 104.0$ Hz); ESI-MS (LR) *m/z* 743 (MH^+); ESI-MS (HR) calcd for $C_{30}H_{38}F_2N_6O_{10}PS$ (MH^+): 743.2075, found: 743.2028.

4.1.6. 9-[5′,5′-Difluoro-5′-(diethylphosphono)pent-1′-ynyl]-2*N*-(*N*,*N*-dimethylaminomethylidene)-1*N*-(pivaloyloxy)methyl-9-deazaguanine (19b)

To a solution of **18b** (152 mg, 172 μmol) in DMF (5.8 mL) was added PhSH (21.0 μL, 206 μmol), K_2CO_3 (71.0 mg, 516 μmol.) at room temperature. After being stirred for 1.5 h at the same temperature, the reaction mixture was diluted with $CHCl_3$ and washed with brine, dried over $MgSO_4$, and evaporated. The resulting residue was chromatographed on silica gel ($CHCl_3$) to give **19b** (86.5 mg, 90%) as a yellow solids: mp 161–166 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.09 (s, 1H, NH), 8.51 (s, 1H, C*H*NMe$_2$), 7.46 (d, 1H, 8-H, $J = 3.2$ Hz), 6.18 (s, 2H, CH_2OC(O)^{t}Bu), 4.22 (m, 4H, OCH_2CH$_3$ × 2), 3.15, 2.97 (each s, 6H, CHN(CH_3)$_2$), 2.67 (t, 2H, 3′-H × 2, $J_{3',4'} = 7.7$ Hz), 2.34 (m, 2H, 4′-H × 2), 1.29 (t, 6H, OCH$_2$CH_3 × 2, $J = 7.1$ Hz), 1.08 (s, 9H, ^{t}Bu); ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.65, 156.79, 153.98, 153.49, 144.40, 130.30, 119.93 (dt, $J_{C,P} = 216.2$ Hz, $J_{C,F} = 261.8$ Hz), 113.29, 97.80, 88.35, 73.82, 65.39, 64.35 (d, $J_{C,P} = 6.7$ Hz, 2 carbons), 40.46, 38.24, 34.45, 33.09 (dt, $J_{C,P} = 14.4$ Hz, $J_{C,F} = 20.4$ Hz), 26.70 (3 carbons), 16.23 (d, $J_{C,P} = 5.0$ Hz, 2 carbons), 11.59 (m); ^{19}F NMR (376 MHz, DMSO-d_6) δ −50.82 (dt, 2F, $J_{F,H} = 20.0$ Hz, $J_{F,P} = 105.0$ Hz); ^{31}P NMR (162 MHz, DMSO-d_6) δ 7.50 (t, 1P, $J_{P,F} = 105.0$ Hz); ESI-MS (LR) *m/z* 558 (MH^+); ESI-MS (HR) calcd for $C_{24}H_{34}F_2N_5O_6P$ (MH^+): 558.2292, found: 558.2271.

4.1.7. 9-[5′,5′-Difluoro-5′-(diethylphosphono)pentyl]-2*N*-(*N*,*N*-dimethylaminomethylidene)-1*N*-(pivaloyloxy)methyl-9-deazaguanine (22b)

A solution of **19b** (1.00 g, 1.79 mmol) in MeOH/$CHCl_3$ (1:1) (180 mL) was hydrogenated over 10% Pd–C (1.0 g) for 20 min at room temperature under an atmospheric pressure. The catalyst was removed through Celite, and the filtrate was concentrated in vacuo. The resulting residue was chromatographed on silica gel (3% MeOH in $CHCl_3$) to give **22b** (1.01 g, ca. 100%) as a white solid: UV λ_{max} (MeOH) 303 nm, 265 nm; mp 111–118 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.24 (s, 1H, NH), 8.56 (s, 1H, C*H*NMe$_2$), 7.29 (s, 1H, 8-H), 6.11 (s, 2H, CH_2OC(O)^{t}Bu), 4.17 (m, 4H, OCH_2CH$_3$ × 2), 3.23, 3.04 (each s, 6H, CHN(CH_3)$_2$), 2.65 (m, 2H, 1′-H × 2), 2.05 (m, 2H, 4′-H × 2), 1.65 (m, 2H, 2′-H × 2), 1.53 (m, 2H, 3′-H × 2), 1.26 (t, 6H, OCH$_2$CH_3 × 2, $J = 7.1$ Hz), 1.10 (s, 9H, ^{t}Bu); ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.46, 158.81, 153.70, 152.17, 128.00, 120.99 (dt, $J_{C,P} = 213.6$ Hz, $J_{C,F} = 259.6$ Hz), 112.68, 112.15, 65.39, 64.06 (d, $J_{C,P} = 7.0$ Hz, 2 carbons), 41.16, 38.30, 34.96, 33.12 (dt, $J_{C,P} = 14.6$ Hz, $J_{C,F} = 20.3$ Hz), 29.54, 26.64 (3 carbons), 22.94, 19.87 (m), 16.23 (d, $J_{C,P} = 5.2$ Hz, 2 carbons); ^{19}F NMR (376 MHz, DMSO-d_6) δ −49.41 (dt, 2F, $J_{F,H} = 20.4$ Hz, $J_{F,P} = 107.1$ Hz); ^{31}P NMR (162 MHz, DMSO-d_6) δ 8.10 (t, 1P, $J_{P,F} = 107.1$ Hz); ESI-MS (LR) *m/z* 562 (MH^+); ESI-MS (HR) calcd for $C_{24}H_{39}F_2N_5O_6P$ (MH^+): 562.2605, found: 562.2585.

4.1.8. 9-[5′,5′-Difluoro-5′-(diethylphosphono)pentyl]-2*N*-(*N*,*N*-dimethylaminomethylidene)-9-deazaguanine (23b)

To a solution of **22b** (924 mg, 1.65 mmol) in MeOH (83 mL) was added NaOMe (446 mg, 8.25 mmol). After being stirred for 22 h at room temperature, the reaction mixture was quenched with 1 M HCl solution at 0 °C and evaporated. The resulting residue was chromatographed on silica gel (3% MeOH in $CHCl_3$) to give **23b** (604 mg, 82%) as a white solid: mp 110–113 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.28 (s, 1H, 1-NH), 10.88 (s, 1H, 7-NH), 8.46 (s, 1H, C*H*NMe$_2$), 6.99 (d, 1H, 8-H, $J = 2.7$ Hz), 4.16 (m, 4H, OCH_2CH$_3$ × 2), 3.10, 2.98 (each s, 6H, CHN(CH_3)$_2$), 2.54 (t, 2H, 1′-H × 2, $J_{1',2'} = 7.4$ Hz), 2.03 (m, 2H, 4′-H × 2), 1.65 (tt, 2H, 2′-H × 2, $J_{2',1'} = J_{2',3'} = 7.4$ Hz), 1.51 (m, 2H, 3′-H × 2), 1.25 (t, 6H, OCH$_2$CH_3 × 2, $J = 7.1$ Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ 156.66, 155.19, 153.54, 144.00, 124.73, 121.08 (dt, $J_{C,P} = 213.6$ Hz, $J_{C,F} = 258.5$ Hz), 114.94, 64.05 (d, $J_{C,P} = 6.8$ Hz, 2 carbons), 40.29, 34.41, 33.16 (dt, $J_{C,P} = 14.2$ Hz, $J_{C,F} = 20.5$ Hz), 29.34, 23.07, 20.04 (m), 16.22 (d, $J_{C,P} = 5.0$ Hz, 2 carbons); ^{19}F NMR (376 MHz, DMSO-d_6) δ −49.37 (dt, 2F, $J_{F,H} = 20.5$ Hz, $J_{F,P} = 106.8$ Hz); ^{31}P NMR (162 MHz, DMSO-d_6) δ 8.16 (t, 1P, $J_{P,F} = 106.8$ Hz); ESI-MS (LR) *m/z* 448 (MH^+); ESI-MS (HR) calcd for $C_{18}H_{29}F_2N_5O_4P$ (MH^+): 448.1925, found: 448.1927.

4.1.9. 9-[5′,5′-Difluoro-5′-(diethylphosphono)pentyl]-9-deazaguanine (DFPP-DG; 25)

A solution of **23b** (342 mg, 764 μmol) in concd HCl solution (12 mL) was refluxed for 20 h. The reaction mixture was diluted with water and washed with AcOEt. The water layer was concentrated and co-evaporated with EtOH. The resulting precipitate was suspended with water/MeOH (1:1) and collected by filtration to give a solid. The solid was heated for 24 h at 40 °C in vacuo to give **25** (238 mg, 93%) as a pale brown solid: mp >287 °C (colored); UV λ_{max} (0.1 N HCl) 230 nm (ε = 17,000), 274 nm (ε = 12,700), UV

λ_{max} (pH 7.0 hepes buffer) 231 nm (ε = 17,500), 273 nm (ε = 10,100), UV λ_{max} (0.1 N NaOH) 231 nm (ε = 18,400), 267 nm (ε = 6800), 285 nm (ε = 6100); ^{1}H NMR (400 MHz, D_2O, NaOD) δ 7.15 (s, 1H, 8-H), 2.62 (t, 2H, 1′-H × 2, $J_{1',2'}$ = 7.0 Hz), 2.01 (m, 2H, 4′-H × 2), 1.69–1.62 (m, 4H, 2′-H × 2, 3′-H × 2); ^{13}C NMR (100 MHz, D_2O, NaOD) δ 167.25, 162.22, 148.56, 128.03 (dt, $J_{C,P}$ = 184.9 Hz, $J_{C,F}$ = 257.3 Hz), 127.71, 117.39, 116.50, 36.48 (dt, $J_{C,P}$ = 12.3 Hz, $J_{C,F}$ = 21.5 Hz), 32.44, 25.92, 23.11 (m); ^{19}F NMR (376 MHz, D_2O, NaOD) δ −47.96 (dt, 2F, $J_{F,H}$ = 21.4 Hz, $J_{F,P}$ = 87.7 Hz); ^{31}P NMR (162 MHz, D_2O, NaOD) δ 7.33 (t, 1P, $J_{P,F}$ = 87.7 Hz); ESI-MS (LR) *m/z* 337 (MH^+); ESI-MS (HR) calcd for $C_{11}H_{16}F_2N_4O_4P$ (MH^+): 337.0877, found: 337.0856.

4.2. Biological studies

4.2.1. Enzymatic procedures, inhibition types and inhibition constants

Kinetic studies, if not otherwise indicated, were conducted at 25 °C in 50 mM hepes/NaOH buffer pH 7.0 in the presence of 1 mM phosphate buffer for the determination of the inhibition constants and in the presence of 50 mM phosphate buffer for determination of the enzyme specific activity.

One unit of PNP is defined as the amount of enzyme that causes phosphorolysis of 1 μmol of Inosine (Ino) to hypoxanthine and ribose-1-phosphate per min under standard conditions i.e. at 25 °C with 0.5 mM Ino and 50 mM sodium phosphate buffer pH 7.0. The phosphorolysis of Ino was therefore measured to determine enzyme specific activity. A standard coupled xanthine oxidase procedure[28] was used with observation wavelength λ_{obs} = 300 nm and molar extinction coefficient difference $\Delta\varepsilon$ 300 nm = 9600 M^{-1} cm^{-1}.[22a]

PNP is known for its non-hyperbolic kinetics and deviations form the classical Michaelis–Menten kinetics depend on the nucleoside substrate and concentration of the co-substrate, phosphate.[22a] Therefore, inhibition type and inhibition constants were determined, if not otherwise indicated, using 7-methylguanosine as the variable substrate since it was shown that for this substrate the classical Michaelis–Menten[29] equation is sufficient for data analysis.[22a]

The phosphorolysis of 7-methylguanosine was examined spectrophotometrically by a direct method.[30] Observation wavelength, λ_{obs} = 260 nm corresponds to the maximal difference between the extinction coefficients of the nucleoside substrate, m^7Guo, and the respective purine base, 7-methylguanine: $\Delta\varepsilon$ = 4 600 M^{-1} cm^{-1} at 260 nm at pH 7.0 for the mixture of cationic and zwitterionic forms of m^7Guo.[21a,30]

The reaction mixture for the direct method and for the coupled method had 1 mL volume in a 10-mm path-length cuvette and was kept at 25 °C. It contained 50 mM Hepes pH 7.0, both substrates of the phosphorolytic reaction (phosphate buffer of the same pH as the main buffer, and a nucleoside, m^7Guo or Ino). In the case of Ino phosphorolysis, xanthine oxidase was also present (about 0.1 U/mL), while in the case of inhibition studies an inhibitor was also included in the reaction mixture. If not otherwise indicated, the reaction was started by the addition of PNP (calf or human).

Standard initial rate procedures were employed in all kinetic studies In the case of inhibition studies for each combination of the initial substrate concentration c_o and the inhibitor concentration [I], the rates were determined at least two times. The initial velocities, v_o, were measured directly from the computer controlling the spectrophotometer. A linear regression program was used for the determination of the slopes, with their standard errors, of absorbance versus time.

Data were initially presented in a form of Michaelis–Menten, Lineveawer–Burk, and Dixon plots to visualize kinetic data obtained in the particular experiment and to visually inspect the type of inhibition observed, but not to calculate kinetic constants. Initial rates were analyzed and kinetic constants were obtained with the use of the weighted least-squares non-linear regression using the program Leonora developed by Cornish-Bowden.[23] Models of competitive, uncompetitive, and mixed-type inhibition[29] were compared by analysis of variance. Inhibition constants were determined by fitting the appropriate equation (for competitive, uncompetitive and mixed-type inhibition models) to the whole data set obtained for the inhibitor, that is, for all triplets: v_o, c_o, [I], where c_o is initial substrate concentration, v_o is initial velocity and [I] is inhibitor concentration.

IC_{50}s were also calculated from the experimental data by fitting the exponential decay equation to the initial velocities, v_o versus inhibitor concentration [I] obtained for fixed concentration of substrates: 25 μM of 7-methylguanosine and 1 mM of phosphate.

The time dependence of inhibition was studied by incubation of calf-spleen PNP (1–4 nM subunits), **DFPP-DG** and its analogues (1–50 nM), and one substrate—1 mM phosphate—in hepes buffer pH 7.0 at 25 °C in a total volume of 1.2 mL. After a given time interval, t (0.5–20 min), 0.03 mL of the second substrate, m^7Guo (2000 μM), was mixed with 0.97 mL of the incubated solution. The final concentration of m^7Guo was therefore 60 μM, with all other concentration changed by only 3%, so may be treated as equal to the initial values. The initial velocities observed after various incubation times for each inhibitor concentration, $v_o(t, [I])$, were measured, that is, the rate of phosphorolysis (7-methylguanine formation) using the spectrophotometric assay as described above.

For each inhibitor concentration, the velocity at equilibrium, that is, at infinite time, $v_o(\infty, [I])$ (later referred to as $v_s[I]$—steady-state velocity observed in the presence of inhibitor at [I] concentration, was determined. This was done by fitting the one-phase exponential decay to each set of velocities observed with various [I], $v_o(t, [I])$:

$$v_o(t;[I]) = A\exp(-kt) + v_s[I] \quad (1)$$

In separate experiments k_c was determined as the initial velocity obtained at time t = 0 (no incubation) with a saturating concentration of m^7Guo (120 μM) and in the absence of inhibitor, that is, $v_o(0; [0]) = k_c$. It was also found that 120 min incubation has no influence on enzyme activity, hence one may assume that $v_o(0; [0]) = v_o(120\ min; [0])$. The Michaelis constant was determined as previously described,[22a] and the value obtained K_m = 17 μM, was used in the following calculations.

The inhibition constant at equilibrium, K_i^{eq}, was finally determined as reported previously for immucillins[24], from the equation:

$$V_s[I]/k_c = [S]/(K_m(1 + [I]/K_i^{eq}) + [S]) \quad (2)$$

where [S] is the concentration of m^7Guo (60 μM), and K_m and k_c are constants.

4.2.2. Extinction coefficients and pK_a determination

Extinction coefficients and pK_a were determined spectrophotometrically. Britton–Robisnon universal buffers[31] were used to get buffers covering the broad pH range 1.8–12. In addition, spectra at pH 1.0 and 13.0 (in 0.1 N HCl and in 0.1 N NaOH) were recorded.

Two milligrams of an inhibitor (**DFPP-DG** or one of its analogues) were dissolved in 1–10 mL DMSO (depending on solubility of the compound examined) to get stock solutions of several mM concentrations. From the stock solution samples, desired pH was prepared in a two-step dilution. In the first, step 0.1–0.3 mL of the stock was added to 50 mL of water, and in the next step, 0.9 mL of the solution in water was mixed with 0.1 mL of the desired buffer. The spectra of these solutions were recorded in a range of 220–360 nm and used to determine extinction coefficients and pK_a. pK_a was determined by fitting the sigmoidal dose–

response curve to the absorbance versus pH, that is, assuming titration of one ionizable proton:

$$A\,(\mathrm{pH}) = A_o + \Delta A\,(1 + 10^{\mathrm{p}K_a - \mathrm{pH}})$$

where A_o is absorbance of one ionic form and ΔA is the difference of absorbance of two ionic forms (ΔA could be positive or negative, depending of the observation wavelength). Such a fitting was done separately for several (3–5) observation wavelengths. The mean values were then calculated for a particular compound and those are given in Section 2.

DMSO concentrations in solutions used for spectral and inhibitory experiments were typically up to 0.2% and never exceeded 0.6%.

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Supplementary data

Supplementary data (additional experimental details: synthetic procedures and physical data for all new compounds not described in the experimental section, and materials and instrumentation for the biological studies) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.01.062.

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