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Synthesis and biological evaluation of 9-deazaguanine derivatives connected by a linker to difluoromethylene phosphonic acid as multi-substrate analogue inhibitors of PNP

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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 adjusted by length of the linker achieved by the Sonogashira-coupling reaction between a 9-deaza-9-iodoguanine derivative and ω-alkynyldifluoromethylene phosphonates as a key reaction.**DFPP-DG** is a very potent PNP inhibitor with apparent inhibition constants (in the presence of 1 mM phosphate) of 4.4 and 8.1 nM versus calf spleen and human erythrocyte PNPs, respectively. One of its analogues, ***homo*-DFPP-DG**, with longer chain linking phosphonate and 9-deazaguanine is even more potent versus human enzyme, with an apparent inhibition constant of 5.3 nM (in the presence of 1 mM phosphate).

Purine nucleoside phosphorylase (PNP, E.C.2.4.2.1) is a ubiquitous enzyme of purine metabolism that functions in the salvage pathway, thus enabling cells to utilize purine bases recovered from metabolized purine ribo- and deoxy-ribonucleosides to synthesize purine nucleotides.[1] In mammals, homotrimeric PNP catalyzes the reversible phosphorolytic cleavage of the glycosidic bond of 6-keto purine nucleosides. PNP is crucial for the integrity of the immune system, since PNP deficiency in humans leads to defective T-cell response.[1] Therefore, inhibitors of PNP may be useful in the treatment of various autoimmune diseases, other T-cell proliferative disorders, and T-cell cancers.[1]

Since PNP accomplishes the reversible phosphorylation of purine nucleosides via a ternary complex of the 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 for PNP. Therefore, a number of metabolically stable acyclic nucleotides containing a purine and phosphate-like moiety connected by a linker have been synthesized.[2] 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.[3]

During our previous studies directed toward the design and synthesis of a multi-substrate analogue inhibitor of trimeric PNP based on the use of difluoromethylene-phosphonic acid as a phosphate mimic, we have examined structural modification of the linker and base moieties of **DFPP-G**.[4] In these studies, we have identified several conformationally constrained analogues of **DFPP-G** that show better inhibition motifs than those observed for **DFPP-G**.[4,5]

In an effort to logically extend on our previous results, we have recently succeeded in crystallizing a binary complex with **DFPP-G** and calf spleen PNP (Fig. 1).[6] High-resolution X-ray differentiation data confirmed for the first time that **DFPP-G** acts as a multi-substrate

Keywords: Purine nucleoside phosphorylase; Multi-substrate analogue inhibitors; 9-Deazaguanine; Difluoromethylene phosphonic acids; Sonogashira reactions.
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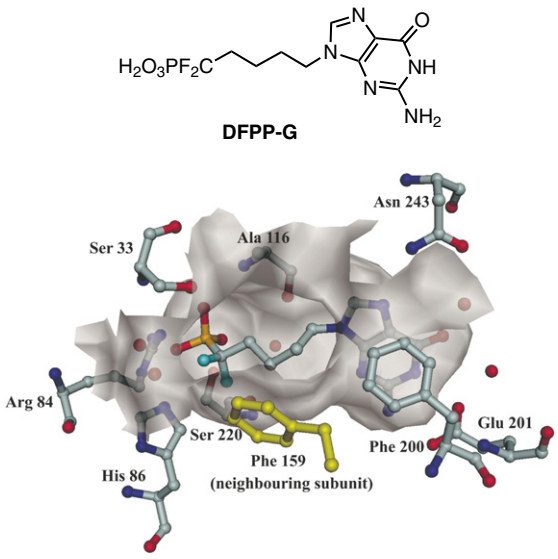


Figure 1. Structure of **DFPP-G** and its interactions with calf spleen PNP in the binary complex (figure from Ref. 6).

analogue inhibitor as it binds to both nucleoside- and phosphate-binding sites. In addition, the putative hydrogen bonds identified in the base-binding site indicate that the contact of guanine O^6 with Asn243 $O^{\delta 1}$ is not a direct contact but is mediated by a water molecule (Figs. 1 and 2). It is apparent that the bridging water molecule is entropically disfavored, and exclusion of the water molecule from the complex may induce tighter binding to PNP.

On the basis of these findings and hypothesis, we designed a new candidate, 9-(5′,5′-difluoro-5′-phosphonopentyl)-9-deazaguanine (**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 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 Asn243$O^{\delta 1}$, while the Asn243$N^{\delta 1}$ 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-arylmethyl-9-deazaguanine

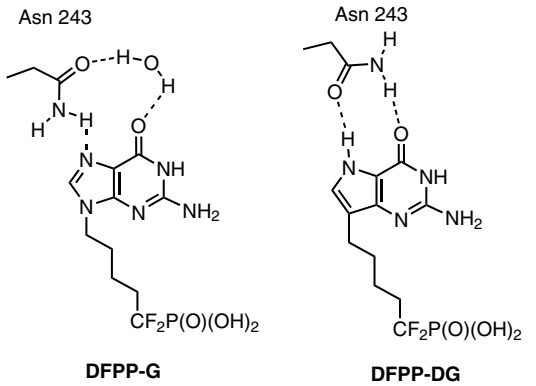


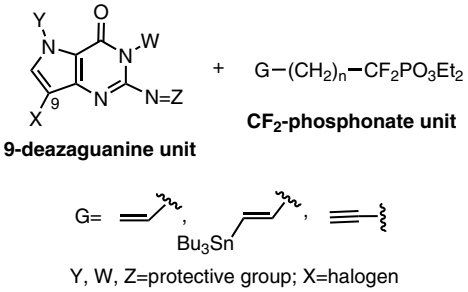
Figure 2. Binding patterns of **DFPP-G** and expected binding patterns of **DFPP-DG** with the base-binding site of PNP.

derivatives.[1,7] 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.[6,8] In this paper, we now describe the synthesis and biological evaluation of **DFPP-DG** and its related analogues as a multi-substrate analogue inhibitor of PNP.

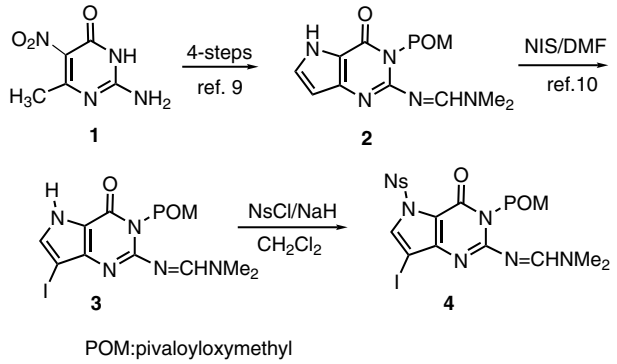
To synthesize analogous compounds of **DFPP-DG**, a strategy which has high diversity for adjusting the length of the linker is desirable. In this context, a cross-coupling reaction between a 9-deazaguanine unit and a CF_2-phosphonate unit appropriately functionalized at the ω-position is one of the reactions to be chosen. This strategy would make a variety of analogues of **DFPP-DG** upon adjusting the number of carbon atoms between '$CF_2PO_3Et_2$' moiety and ω-functional group (G) within the CF_2-phosphonate unit (Scheme 1).

Keeping this strategy in mind, we first examined synthesis of the 9-deazaguanine unit. Inspections of literature revealed that suitably protected 9-deazaguanine derivative **2** has been previously prepared from readily accessible pyrimidinone **1**,[9] and compound **2** was transformed to 9-iodo-9-deazaguanine derivative **3** by NIS-mediated selective iodination at the 9-position.[10] Then, according to the literature procedure, we were able to obtain multi-grams of **3** without any difficulty. To protect the acidic proton at the 7-position, **3** was nosylated by conventional method to give **4** in a quantitative yield (Scheme 2).

Multi-grams of suitably protected 9-iodo-9-deazaguanine **4** in hand, we next focused on the synthesis of



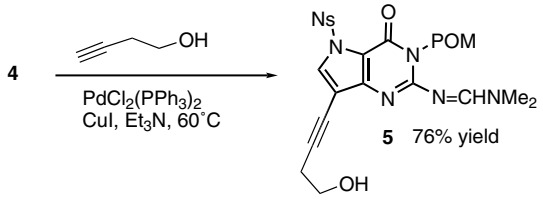
Scheme 1. Cross-coupling strategy for synthesis of **DFPP-DG** analogues.



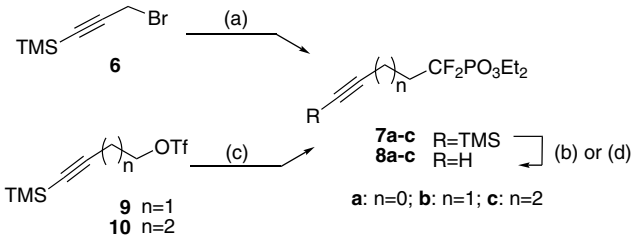
Scheme 2. Preparation of 9-iodo-9-deazaguanine derivatives.

CF_2-phosphonate units. Preliminary studies of the coupling reaction of **4** 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,[11] respectively, revealed that only the Sonogashira reaction with 3-butyn-1-ol gave the corresponding coupling-product **5** in good yield, while the Still and Heck reactions gave no desired products (Scheme 3).

Therefore, we decided to examine the cross-coupling reaction of **4** with ω-alkynyldifluoromethylenephsophonate units under Sonogashira conditions for the synthesis of **DFPP-DG** analogues. A series of requisite ω-alkynyldifluoromethylene phosphonate units having 3–5 carbon atom linker were prepared as shown in Scheme 4.



Scheme 3. Sonogashira reaction of **4** with 3-butyn-1-ol.

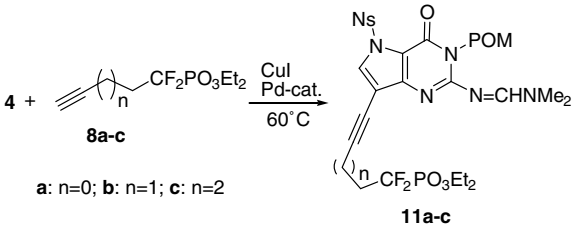


Scheme 4. Synthesis of ω-alkynyldifluoromethylenephosphonate units. Reagents and conditions: (a) $BrZnCF_2PO_3Et_2$, CuBr, DMF, rt; (b) TBAF, THF, AcOH; (c) $LiCF_2PO_3Et_2$, THF, −78 °C; (d) TBAF, THF.

Treatment of TMS-protected bromopropyne **6** with $BrZnCF_2PO_3Et_2$ in the presence of CuBr in DMF according to our previously reported protocols[12,13] gave **7a** in a 70% yield. Tetrabutylammonium fluoride (TBAF)-induced protodesilylation of **7a** in THF in the presence of AcOH gave ω-alkenyldifluoromethylene phosphonate unit **8a** (C3-phosphonate unit) in a 97% yield.[14] To synthesize C4-phosphonate unit **8b** and C5-phosphonate unit **8c**, alkylation of $LiCF_2PO_3Et_2$ with readily available triflates **9** and **10** was applied according to the method described by Berkowitz.[13,15] While the reaction with **10** under the conditions proceeded smoothly to give **7c** in an 89% yield, the reaction with **9** was problematic due to competitive formation of large amounts of enyne products by β-elimination of HF under the conditions. However, **7b** could be isolated in a very low yield (8%). The compounds **7b** and **c** were treated with TBAF in THF to give the requisite C4-phosphonate unit **8b** and C5-phosphonate unit **8c** in 75% and 90% yields, respectively.

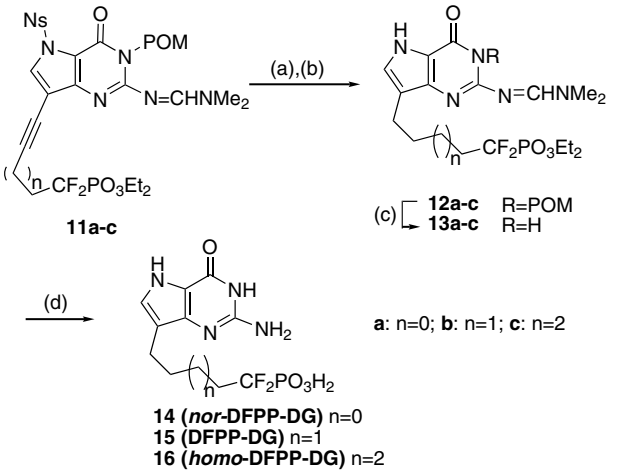
The Sonogashira-coupling reaction between the 9-deazaguanine unit **4** and the phosphonate units (**8a–c**) was first examined with C5-phosphonate unit **8c** to verify proper reaction conditions, since **8c** was readily available (Table 1). When the Sonogashira reaction of **4** was carried out with a slight excess of **8c** 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,[16] desired coupling product **11 c**[17] was obtained in a 58% yield along with a large amount of recovered **4** (36%) (run 1). Yield of **11c** was not increased upon using the Pd(0) catalysis under the same conditions (run 2). In an effort to increase the yield, modified conditions using

Table 1. Sonogashira reaction of **4** with phosphonate units (P-units)[a]



Run	P-unit	Pd-cat.[b]	Et_3N (equiv)	Solvent	**11**	
					Yield, %[c]	
1	**8c**	$PdCl_2(PPh_3)_2$	>60	None	**11c**	58 (36)
2	**8c**	$Pd(PPh_3)_4$	>60	None	**11c**	22 (78)
3	**8c**	$PdCl_2(PPh_3)_2$	2	THF	**11c**	15 (69)
4	**8c**	$PdCl_2(PPh_3)_2$	2	DMF	**11c**	41 (23)
5	**8c**	$PdCl_2(PPh_3)_2$	2	MeCN	**11c**	69 (5)
6	**8a**	$PdCl_2(PPh_3)_2$	2	MeCN	**11a**	39 (22)
7[d]	**8a**	$PdCl_2(PPh_3)_2$	1.2	MeCN	**11a**	59 (41)
8	**8b**	$PdCl_2(PPh_3)_2$	2	MeCN	**11b**	66 (8)

[a] All reactions were carried out in the presence of CuI (1 mol%) at 60 °C for 15 h unless otherwise stated.
[b] Two mol% of Pd-catalysis was utilized.
[c] Yields in parentheses refer to recovered **4**.
[d] Reaction time was 1 h.



Scheme 5. Synthesis of **DFPP-DG** analogues. Reagents: (a) thiophenol, K_2CO_3, DMF; (b) H_2, Pd-C, MeOH–$CHCl_3$; (c) NaOMe, MeOH; (d) concd HCl.

representative solvents composed by 2 equivalents of Et_3N were examined (runs 3–6 and 8). This survey identified MeCN was a good solvent to induce a good yield (69%) of **11c** and minimize the recovered yield of **4** (run 5). Under these conditions, phosphonate units **8a** and **b** were coupled with **4** to give **11a** and **b** in 39% and 66% yields, respectively (runs 6 and 8). The yield of **11a** was significantly improved to 59%, when the coupling reaction was carried out in the presence of a slight excess of Et_3N for 1 h (run 7). It should be noted that **11a** did not form upon conducting the coupling in a large excess of the amine without the solvent, due to rapid decomposition of **11a** under these conditions.

The compounds **11a**–**c** thus obtained were readily transformed to **DFPP-DG** and its homo- and nor-analogues (Scheme 5). Thiophenol-mediated removal of the nosyl protecting group for **11b**, followed by hydrogenation, gave **12b** in a 90% yield. The compound **12b** was treated with NaOMe in MeOH to give **13b** in an 82% yield. Removal of the ethyl protecting group and the dimethylaminomethylene group for **13b** was achieved in concd HCl at reflux (17 h) to give **DFPP-DG** (**15**) as white solids in a 91% yield. The compounds **11a** and **c** were, respectively, transformed to ***nor*-DFPP-DG** (**14**) and ***homo*-DFPP-DG**(**16**) by the same procedure as a series of **DFPP-DG** in good overall yield.

Inhibitory activities of **DFPP-DG** and its analogues with calf spleen and human erythrocyte PNPs were measured as previously described for other PNP inhibitors[5,18] and evaluated in comparison with that of **DFPP-G**. The results are summarized in Table 2.

DFPP-DG is about a two-fold more potent inhibitor of calf spleen PNP than its counterpart with guanine base, **DFPP-G**. The inhibition constant, as expected for the multisubstrate analogue inhibitor competing with phosphate for the phosphate-binding site, decreases from (4.4 ± 0.6) nM at 1 mM phosphate concentration to (1.0 ± 0.2) nM at 0.025 mM phosphate concentration (vs calf spleen and human erythrocyte enzymes, see Table 2). By contrast, there are almost no differences in inhibition parameters versus human erythrocyte PNP in the case of **DFPP-G** and **DFPP-DG**. The shorter chain in ***nor*-DFPP-DG** results in about nine-fold weaker binding versus human PNP. However, the longer chain in ***homo*-DFPP-DG** which allows more favorable interactions with base-, pentose-, and phosphate-binding sites yields IC_{50} two-fold lower than observed in the case of **DFPP-DG**, 9.5 nM as compared with 20.4 nM (at 1 mM phosphate concentration, see Table 2). Also apparent inhibition constant for ***homo*-DFPP-DG** is lower than that observed for the **DFPP-DG**, (5.3 ± 0.4) and (8.1 ± 0.6) nM.

Table 2. Inhibitory properties versus calf spleen and human erythrocyte PNPs determined, if not otherwise indicated, in the presence of 1 mM phosphate[a]

Compound	IC_{50} (nM) calf PNP[b]	IC_{50} (nM) human PNP[b]	K_i^{app} [nM] calf PNP	K_i^{app} (nM) human PNP
DFPP-G	18.7	20.2	6.9 ± 0.7[c]	10.8 ± 0.7
DFPP-DG	10.2	20.4	4.4 ± 0.6	8.1 ± 0.6
***nor*-DFPP-DG**	—	173	—	—
***homo*-DFPP-DG**	—	9.5	5.7 ± 0.6	5.3 ± 0.4
DFPP-G[d]	—	—	2.7 ± 0.2	—
DFPP-DG[d]	—	—	1.0 ± 0.2	1.0 ± 0.2

[a] All reactions were carried out in 50 mM Hepes buffer, pH 7.0, at 25 °C, with 7-methylguanosine as a variable substrate, and, if not otherwise indicated, in the presence of 1 mM phosphate.
[b] With 25 μM 7-methylguanosine.
[c] Data from Ref. 5.
[d] With 0.025 mM phosphate.

DFPP-G and some of its analogues, with cyclic linkers between the guanine and difluoromethylene phosphonic acid groups, 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.[19] It is therefore very likely that the effect of the newly synthesized compounds on the proliferation of autoimmune lymphocytes (from patients affected by Hashimoto's thyroiditis and other autoimmune diseases) will be even more significant. Such studies are now underway.

Acknowledgments

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17. All new compounds gave satisfactory spectroscopic and analytical data. Compound **11a**: mp 132–137 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.86 (1H, s), 9.34 (1H, dd, J = 9.6, 1.7 Hz). 9.29 (1H, dd, J = 9.4, 1.1 Hz), 9.18–9.07 (3H, m), 6.76 (2H, s), 4.61 (4H, dq, $J_{HH} = J_{HP}$ = 8.6 Hz), 3.66 (2H, td, J_{HF} = 19.9 Hz, J_{HP} = 7.2 Hz), 3.32, 3.09 (6H, each s), 1.04 (6H, t, J = 8.6 Hz), 0.72 (9H, s); ^{31}P NMR (162 MHz, DMSO-d_6) δ 6.89 (1P, t, J_{PF} = 101.5 Hz); ^{19}F NMR (376 MHz, DMSO-d_6) δ −47.28 (2F, dt, J_{FP} = 101.5 Hz, J_{FH} = 19.9 Hz), ESI-MS *m/z* 729.1926 (calcd for $C_{29}H_{35}F_2N_6O_{10}PS$: 729.1919). Compound **11b**: mp 192–194 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.59 (1H, s), 8.34 (1H, dd, J = 7.9, 1.4 Hz), 8.98 (1H, dd, J = 7.9, 1.3 Hz), 8.01–7.92 (3H, m), 6.02 (2H, s), 4.23 (4H, dq, $J_{HH} = J_{HP}$ = 7.1 Hz), 3.18, 2.99 (6H, each s), 2.74 (2H, t, J = 7.6 Hz), 2.40 (2H, m), 1.30 (6H, t, J = 7.1 Hz), 1.02 (9H, s); ^{31}P NMR (162 MHz, DMSO-d_6) δ 7.44 (1P, t, J_{PF} = 104.0 Hz); ^{19}F NMR (376 MHz, DMSO-d_6) δ −50.74 (2F, dt, J_{FP} = 104.0 Hz, J_{FH} = 19.5 Hz); ESI-MS *m/z* 743.2028 (calcd for $C_{30}H_{37}F_2N_6O_{10}PS$: 743.2075). Compound **11c**: mp 115–121 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.56 (1H, s), 8.32 (1H, dd, J = 7.9, 1.6 Hz), 8.10 (1H, dd, J = 7.7, 1.4 Hz), 8.00–7.91 (3H, m), 6.02 (2H, s), 4.19 (4H, dq, $J_{HH} = J_{HP}$ = 7.2 Hz), 3.17, 2.99 (6H, each s), 2.59 (2H, t, J = 6.8 Hz), 2.27 (2H, m), 1.80 (2H, m), 1.27 (6H, t, J = 7.2 Hz), 1.02 (9H, s); ^{31}P NMR (162 MHz, DMSO-d_6) δ 7.90 (1P, t, J_{PF} = 106.4 Hz); ^{19}F NMR (376 MHz, DMSO-d_6) δ −49.25 (2F, dt, J_{FP} = 106.4 Hz,J_{FH} = 20.4 Hz); ESI-MS *m/z* 757.2220 (calcd for $C_{31}H_{39}F_2N_6O_{10}PS$: 757.2232). ***nor*-DFPP-DG**: mp >300 °C; ^{1}H NMR (400 MHz, D_2O, NaOD) δ 7.17 (1H, s), 2.65 (2H, t, J = 7.2 Hz), 2.10 (2H, m), 1.87 (2H, m); ^{31}P NMR (162 MHz, D_2O, NaOD) δ 7.24 (1P, t, J_{PF} = 87.7 Hz); ^{19}F NMR (376 MHz, D_2O, NaOD) −47.80 (2F, dt, J_{FP} = 87.7 Hz, J_{FH} = 21.4 Hz). ESI-MS *m/z* 323.0717 (calcd for $C_{10}H_{13}F_2N_4O_4P$: 323.0720); UV 0.1 N HCl 229 (23,000), 274 (13,600), pH 7.0 Hepes buffer 230 (23,100), 273 (10,500), 0.1 N NaOH 230 (24,600) 265 (7100), 287 (6200). **DFPP-DG**: mp >287 °C; ^{1}H NMR (400 MHz, D_2O, NaOD) δ 7.15 (1H, s), 2.62 (2H, t, J = 7.0 Hz), 2.01 (2H, m), 1.69–1.62 (4H, m); ^{31}P NMR (162 MHz, D_2O, NaOD) δ 7.33 (1P, t, J_{PF} = 87.7 Hz); ^{19}F NMR (376 MHz, D_2O, NaOD) δ −47.96 (2F, dt, J_{FP} = 87.7 Hz, J_{FH} = 21.4 Hz); ESI-MS *m/z* 337.0856 (calcd for $C_{11}H_{15}F_2N_4O_4P$: 337.0877); UV 0.1 N HCl 230 (17,000), 274 (12,700), pH 7.0 Hepes buffer 231 (17,500), 273 (10,100), 0.1 N NaOH 231 (18,400), 267 (6800), 285 (6100); pK_{a1} = 5.1, pK_{a2} = 10.6 (from spectrophotometric titrations). ***homo*-DFPP-DG**: mp >300 °C; ^{1}H NMR (400 MHz, D_2O, NaOD) δ 7.10 (1H, s), 2.56 (2H, t, J = 7.4 Hz), 1.98 (2H, m), 1.64–1.55 (4H, m), 1.40 (2H, m); ^{31}P NMR (162 MHz, D_2O, NaOD) δ 7.35 (1P, t, J_{PF} = 88.0 Hz); ^{19}F NMR (376 MHz, D_2O, NaOD) δ −47.80 (2F, dt, J_{FP} = 88.0 Hz, J_{FH} = 21.4 Hz). ESI-MS *m/z* 351.1047 (calcd for $C_{12}H_{17}F_2N_4O_4P$: 351.1033); UV, due to poor solubility extinction coefficients for this analogue were not determined; for calculation of the inhibitor concentration, extinction coefficients obtained for **DFPP-DG** were used.
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