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Discovery of Potent and Selective Methylenephosphonic Acid CD73 Inhibitors

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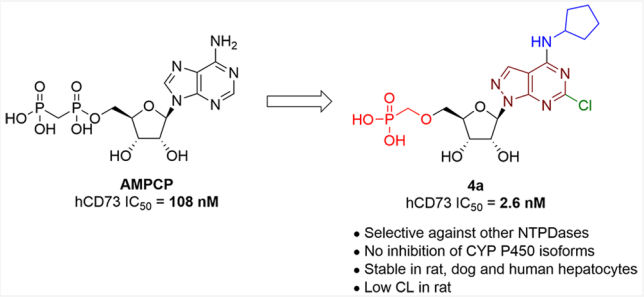
ABSTRACT: Solid tumors are often associated with high levels of extracellular ATP. Ectonucleotidases catalyze the sequential hydrolysis of ATP to adenosine, which potently suppresses T-cell and NK-cell functions via the adenosine receptors (A_{2a} and A_{2b}). The ectonucleotidase CD73 catalyzes the conversion of AMP to adenosine. Thus, increased CD73 enzymatic activity in the tumor microenvironment is a potential mechanism for tumor immune evasion and has been associated with poor prognosis in the clinic. CD73 inhibition is anticipated to restore immune function by skirting this major mechanism of adenosine generation. We have developed a series of potent and selective methylenephosphonic acid CD73 inhibitors via a structure-based design. Key binding interactions of the known inhibitor adenosine-5′-(α,β-methylene)diphosphate (AMPCP) with hCD73 provided the foundation for our early designs. The structure−activity relationship study guided by this structure-based design led to the discovery of **4a**, which exhibits excellent potency against CD73, exquisite selectivity against related ectonucleotidases, and a favorable pharmacokinetic profile.



INTRODUCTION

5′-Ribonucleotide phosphohydrolase, also known as cluster of differentiation 73 (CD73), is a Zn-binding GPI-anchored extracellular protein that functions as a noncovalent homodimer with two structural domains connected by a small hinge region.[1] CD73 has high affinity for adenosine monophosphate (AMP) and catalyzes the conversion of AMP to adenosine.[2] Numerous studies have demonstrated that CD73 is upregulated by hypoxia within the tumor microenvironment.[3] High concentrations of CD73 within the tumor microenvironment contribute substantially to elevated levels of adenosine, which results in immune suppression by activating the adenosine receptors (A_{2a} and A_{2b}) on infiltrating lymphocytes.

CD73 represents a novel therapeutic target for cancer immunology, and several efforts have been undertaken to leverage the role it plays in the development of anticancer therapeutics.[4] For example, anti-CD73 antibody therapy has been shown to inhibit tumor growth and metastasis in animal models.[5] Müller and co-workers have reported derivatives of α-β-methylene-ADP (AMPCP), one of the earlier metabolically stable, potent, and selective CD73 inhibitors known.[6] Similarly, in industrial settings, several efforts have been directed toward the discovery of CD73 inhibitors in recent years.[7,8] Despite these efforts, no CD73 inhibitors are currently approved for clinical use. Therefore, the development of new CD73 inhibitors continues to be vitally important.

RESULTS AND DISCUSSIONS

We started our investigation based on the known CD73 inhibitor AMPCP. The crystal structure of AMPCP bound to human CD73 (hCD73) was disclosed by Sträter et al. in 2012 (PDB: 4H2I).[9] Our initial lead scaffold was designed by investigation of key binding interactions of this known inhibitor with hCD73, i.e., coordination of the phosphono-methyl-phosphonic acid with di-zinc, hydrogen-bonding interaction between *syn*-diol of ribose with Asp-506 and Asn-390 and the hydrophobic π−π stacking between adenine and Phe-500 and Phe-417 shown in Scheme 1.

Early structure−activity relationship (SAR) studies on APMCP were reported by Müller et al., where substitution at the C6 and C8 positions and the methylene bridging the two

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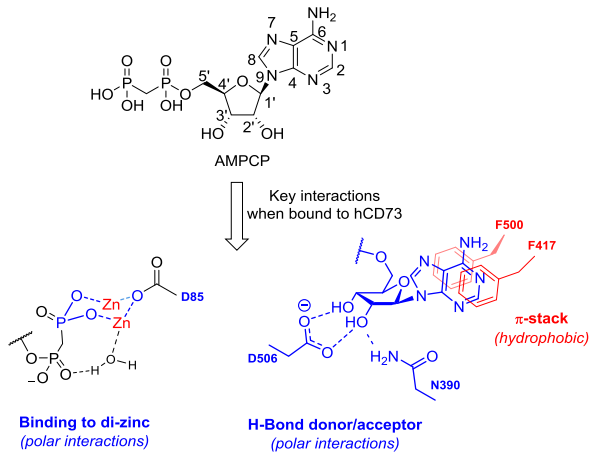
Scheme 1. Key Interactions of AMPCP with hCD73



phosphonic acid units of AMPCP were investigated.[6] Their study found that substitution of the C6 amine with a benzyl group led to analog PSB-12379 (K_i = 2.2 nM) with substantially improved potency relative to AMPCP (K_i = 88.4 nM). While the results were encouraging, their reported SAR was limited to the aforementioned modifications.

During the course of our early investigations, in a parallel program also targeting CD73 using bisphosphonic acids, it was discovered that introduction of a chlorine atom at the C2 position of PSB-12379 led to a 14-fold improvement in potency (IC_{50} = 0.4 nM for **1**, Scheme 2).[7a−c] Similar beneficial effects of C2 chlorine substitution have been reported subsequentially by Müller et al.[10] Our early discovery led us to focus our SAR on **1**. In general, bisphosphonates are poorly absorbed from the gastrointestinal tract; thus, they suffer from poor oral bioavailability. To alleviate the potential liability arising from bisphosphonic acid, we embarked on this simultaneous SAR study by replacing the bisphosphonic acid moiety of **1**.

Our initial effort to replace the bisphosphonic acid moiety in **1** with malonic acid led to a significant drop in potency (**1a**, IC_{50} = 1 μM), presumably due to the loss of interaction with di-zinc (Table 1). When this malonic acid was replaced with phosphonoacetic acid **1b**, an improvement in potency was observed (IC_{50} = 110 nM). However, the ability of the phosphonic acid moiety to engage in the binding interaction with di-zinc was realized to a greater extent when the steric congestion around it was removed. Thus, replacing the phosphonoacetic acid with a methylenephosphonic acid (**1c**) led to adequate interaction with di-zinc and further improvement in potency (IC_{50} = 58 nM). The position of the phosphonic acid also played a key role in the observed potency. For example, if the phosphonic acid moiety was extended by an ethylene (**1d**) or a propylene (**1e**) linker, complete loss of potency was observed. This SAR interrogation resulted in successful replacement of the bisphosphonic acid **1** with methylenephosphonic acid **1c**. Additionally, methylenephosphonic acid analogs offered us the prospect for developing prodrugs to improve oral bioavailability because contrary to earlier experience with bisphosphonic acids, successful development of prodrugs for monophosphonic acids have been reported.[11]

To improve the potency of the methylenephosphonic acid analog **1c**, we carried out a systematic SAR analysis of the adenine core, which began with modification at the C6 position (Table 2).[12] A wide variety of benzylamines and phenethylamines were tolerated at the C6 position. Substitution of the phenyl ring in the phenethylamine moiety with electron-donating or electron-withdrawing groups did not significantly affect the potency (**2a−2c**). Similar observations were made with substitution of the benzylamine (**1c** vs **2f**). In contrast, the stereochemistry at the benzylic position appeared to be important for the observed potency. For example, (*S*)-(−)-α-methylbenzylamine as a C6 substituent (**2d**) exhibited higher potency than its enantiomer (**2e**) (IC_{50} = 65 nM for **2d** vs IC_{50} = 156 nM for **2e**). Heteroaromatic amine substituents like 2-picolylamine **2h**, *N*-methylpyrazole-4-methylamine **2i**, and 4-methyltriazole-3-methylamine **2j** showed decreased potency. C6-amine substituents containing an aliphatic group (**2k**) or carbocyclic groups (**2l−2o**) were also investigated. The most potent analog **2n** was obtained when cyclopentylamine was introduced at the C6 position (IC_{50} = 26 nM). Compounds containing a cyclic secondary amine at the C6 position, such as pyrrolidine (**2p**) or morpholine (**2r**), were found to be less potent. Similarly, C6 amines that contain a heterocyclic substituent as in the case of (*R*)-3-aminotetrahydrofuran (**2q**) did not improve the potency (Table 2).

To better understand the binding interactions of methylenephosphonic acid analog **2n** with hCD73, we obtained an X-ray cocrystal structure of **2n** bound to hCD73 (PDB: 6YE1). The X-ray structure revealed that the methylene phosphonate interacts extensively with the di-zinc, as was observed in the case of AMPCP. Similarly, other polar interactions of the ribose moiety and hydrophobic interactions of the adenine base are also maintained (Figure 1D). From the overlaid cocrystal structures of AMPCP and **2n**, it can be seen that the terminal phosphonates superimpose well but the ribose and the adenine rings of **2n** are shifted 1−1.5 Å relative to AMPCP (Figure 1A,B). This shift of the adenine base toward the di-zinc center indicates that when **2n** is bound to CD73, the N- and C-terminal domains of CD73 move closer together to accommodate the shorter phosphate linker. Further study of the X-ray cocrystal structure revealed a narrow hydrophobic pocket around the C2 chlorine atom (Figure 1C). This presented us with an opportunity to evaluate C2 modifications to improve the binding affinity and boost the potency.

Replacing the C2 chlorine with a phenyl group (**3a**) led to a decrease in potency, presumably due to the conformational

Scheme 2. SAR Study of AMPCP-Based CD73 Inhibitors

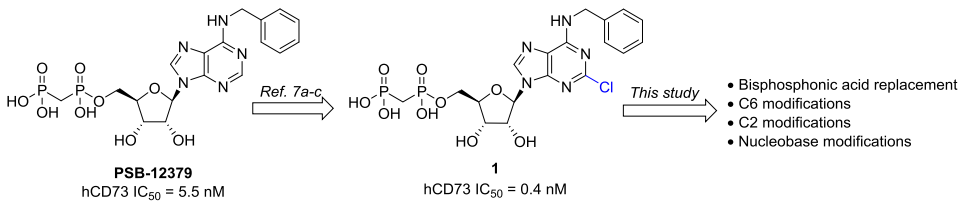


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Table 1. Replacement of Bisphosphonic Acid Residue

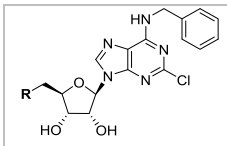
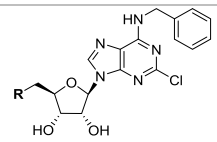
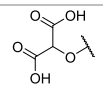
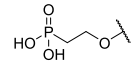
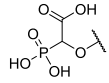
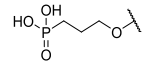
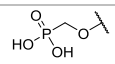
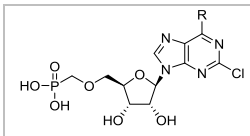
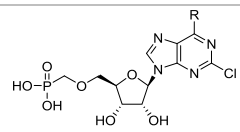
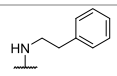
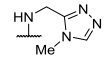
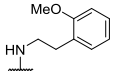
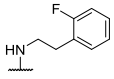
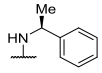
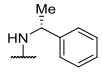
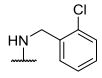
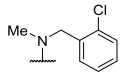
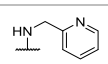
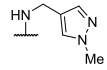
	R	hCD73 IC_{50} (nM)		R	hCD73 IC_{50} (nM)
1a		1,000	**1d**		>10,000
1b		110	**1e**		>10,000
1c		58			

Table 2. Modification of C6 Position

	R	hCD73 IC_{50} (nM)		R	hCD73 IC_{50} (nM)
2a		105	**2j**		3,200
2b		173	**2k**		192
2c		121	**2l**		588
2d		65	**2m**		83
2e		156	**2n**		26
2f		46	**2o**		124
2g		46	**2p**		45
2h		382	**2q**		84
2i		201	**2r**		148

constraints induced by the biaryl system, which might not be accommodated in the nucleotide binding site of the enzyme. As expected, this effect was particularly severe for the *o*-tolyl group (**3b**), which showed a > 130-fold loss in potency (IC_{50} = 3.5 μM for **3b** vs IC_{50} = 26 nM for **2n**). The conformational strain imposed by the biaryl rings could be relieved by a methylene-ether bridge (**3c** and **3d**). In the case of **3d**, a boost in potency was observed when the C2 chlorine was replaced with a phenoxy methyl ether (IC_{50} = 13 nM). Unsurprisingly, when the C2 chlorine was replaced with pyrrolidine-3-methyl

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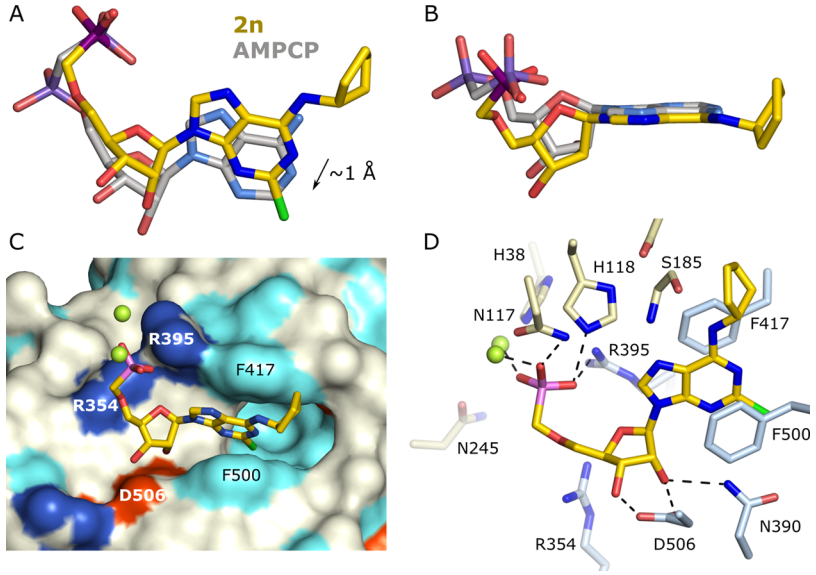
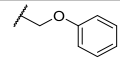
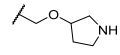


Figure 1. (A) Superposition of AMPCP (PDB: 4H2I) in gray with **2n** (PDB: 6YE1) in yellow. (B) Lateral view of superposition of AMPCP and **2n**. (C) X-ray structure of **2n** bound to CD73, represented by a water-accessible surface in gray for selected residues within 10 Å of the ligand, reveals a narrow hydrophobic pocket around the C2 chlorine. (D) Molecular interactions of **2n** in the cocrystal structure with hCD73.

Table 3. Modification of C2 Position

	R	hCD73 IC_{50} (nM)		R	hCD73 IC_{50} (nM)
3a		245	**3d**		13
3b		3,500	**3e**		>10,000
3c		118			

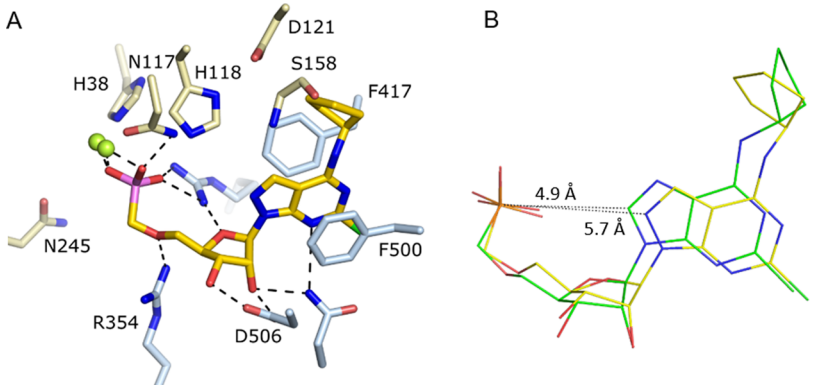


Figure 2. (A) X-ray structure of **4a** cocrystallized with hCD73 (PDB: 6YE2) showing molecular interactions. (B) Overlay of X-ray crystals of **2n** (PDB: 6YE1) in green and **4a** in yellow.

ether, complete loss of activity was observed (**3e**, IC_{50} > 10 μM, Table 3) presumably because of the negative interaction between the polar pyrrolidine and the hydrophobic pocket around the C2 position. Taking into account the potency, synthetic tractability, and physiochemical properties of the C2-modified analogs, we concluded that the C2-chlorine substituent (**2n**) was optimal.

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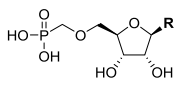
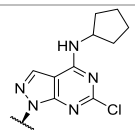
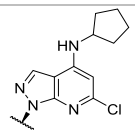
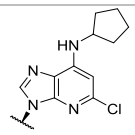
Next, we focused on the modification of the adenine base by changing its electron distribution to modulate hydrophobic interactions arising from the $\pi-\pi$ stacking of Phe-500 and Phe-417 (Figure 2D). When adenine base **2n** (IC_{50} = 26 nM) was replaced with pyrazolopyrimidine **4a**, a 10-fold improvement in potency (IC_{50} = 2.6 nM) was observed (Table 3). Similarly, introduction of pyrazolopyridine (**4b**) led to a fivefold improvement in potency (IC_{50} = 4.5 nM). However, replacing the adenine base with imidazolopyridine (**4c**) did not improve potency (IC_{50} = 33 nM) (Table 4).

Table 4. Modification of the Purine Base

	R	hCD73 IC_{50} (nM)
		
4a		2.6
4b		4.5
4c		33

It is worth noting that heterocyclic core modifications **4a** and **4b**, which incorporate a nitrogen atom at the 8-position, both led to a significant boost in potency. To further understand how these modifications influence the protein-binding interactions that improve potency, we obtained an X-ray cocrystal of **4a** with hCD73 (PDB: 6YE2). Figure 2A shows key ligand interactions. In comparison to the adenine base in **2n**, the position of the pyrazolopyrimidine base in **4a** shifted 0.8 Å away from the N-terminal domain of CD73. This shift can be visualized clearly from the crystal structure overlay of **2n** with **4a** (Figure 2B). These two compounds differ only in the position of the nitrogen atom at the 7- and 8-positions, and the close alignment of the protein atoms of both CD73 domains indicates an almost identical relative domain orientation. Repositioning of the nitrogen atom affects electron distribution of the nucleobase, which presumably influences the hydrophobic stacking interactions of **4a**, resulting in the observed shift. The shifting of the pyrazolopyrimidine away from the phosphonate residue also indicates that the C2 chlorine is being extended deeper into the narrow hydrophobic pocket compared to **2n**. The potentially stronger interaction established due to this conformational orientation might have contributed toward the improvement in potency.

Compound **4a** was characterized further against related targets and evaluated for off-target selectivity (Table 5). It was found to exhibit excellent potency against soluble hCD73 (IC_{50} = 0.86 nM) and soluble mouse CD73 (mCD73; IC_{50} = 3.0 nM), CHO cells overexpressing hCD73 (IC_{50} = 2.6 nM) or

Table 5. Potency and Selectivity of 4a

potency		selectivity	
target	IC_{50} (nM)	target	IC_{50} (nM)
hCD73-CHO	2.6	CD39	>10 000
hCD73 (soluble)	0.86	$A_{2a}R$	>10 000
mCD73-CHO	13	hNTPDase-2	>10 000
mCD73 (soluble)	3.0	hNTPDase-3	>10 000
SKOV-3	0.55	hNTPDase-8	>10 000

mCD73 (IC_{50} = 13 nM), and the human ovary cancer cell line SKOV-3 (IC_{50} = 0.55 nM). It exhibits excellent selectivity (IC_{50} > 10 000 nM) against CD39, $A_{2a}R$, and NTPDase2, 3, and 8. Furthermore, compound **4a** was also found to be stable when cultured with human, dog, rat, or mouse hepatocytes (see the SI) and exhibited low clearance in rats (CL = 0.13 L/h/kg).

During our earlier investigation of C6 substituents on the adenine series (*vide supra*), we found that introduction of α-(*S*)-methylbenzylamine at the C6 position (**2d**) led to decreased clearance, albeit with some loss in potency compared to the corresponding cyclopentylamine (**2n**) (Table 6). Based on this observation, we decided to re-evaluate C6 substitutions on the most potent analogs, **4a** in the pyrazolopyrimidine series and **4b** in the pyrazolopyridine series, to further decrease clearance in rats. The observed trend in potency and clearance of the pyrazolopyrimidine series was similar to that of the adenine series. When cyclopentylamine in **4a** was replaced with α-(*S*)-methylbenzylamine (**5a**), it resulted in an 11-fold loss in potency but a 39-fold decrease in systemic clearance. To improve potency while maintaining low clearance, fluoro-substituted α-(*S*)-methylbenzylamines were explored. The loss in potency due to introduction of α-(*S*)-methylbenzylamine was substantially reclaimed with 2-fluoro- and 4-fluoro-α-(*S*)-methylbenzylamines **5b** (IC_{50} = 7.6 nM) and **5d** (IC_{50} = 12 nM), respectively. Contrary to this, 3-fluoro-α-(*S*)-methylbenzylamine (**5c**) had a deleterious effect on potency (IC_{50} = 39 nM). A similar trend in potencies was also reflected in the pyrazolopyridine series when the C6 cyclopentylamine of **4b** was replaced with the above-mentioned fluoro-benzyl amines (**5e**–**5h**). The positive effect of fluorine incorporation on rat pharmacokinetic (PK) was demonstrated with **5d** in the pyrazolopyrimidine series and with **5h** in the pyrozolopyridine series. Both **5d** and **5h** exhibited remarkably low clearance and improved half-life (CL = 0.020 and 0.018 L/h/kg; $T_{1/2}$ = 4.5 and 4.0 h, respectively).

Chemistry. The bisphosphonic acid replacement analogs (**1a**–**e**) were synthesized according to Scheme 3a. Introduction of the malonate and phosphonoacetate at the C5′ hydroxyl group of commercially available 2,6-dichloro-9-[2,3-*O*-(1-methylethylidene)-β-D-ribofuranosyl]-9*H*-purine **6** was carried out via rhodium catalysis using dimethyl 2-diazomalonate and ethyl 2-diazo-2-(diethoxyphosphoryl)acetate in refluxing toluene to obtain **7** and **8**, respectively.[7d] The benzylamine was then introduced at the C6 position via a S_NAr reaction to obtain **9** and **10**, respectively. Hydrolysis of the ethyl phosphate and removal of the acetonide were carried out with TMS-Br and a successive aqueous quench. Saponification of dimethylmalonate **11** and phosphonoethylacetate **12** with LiOH gave **1a** and **1b**, respectively. Monophosphonic acid derivatives (**1c**–**e**) were synthesized using a four-step sequence (steps e–h) shown in Scheme 3a. The first step in the synthesis involves a S_NAr reaction between commercially

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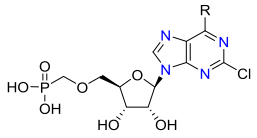
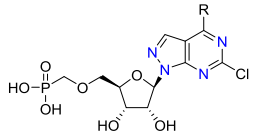
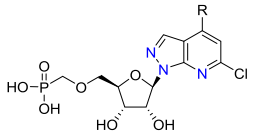
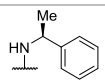
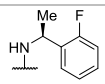
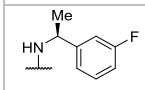
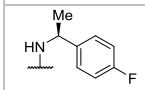
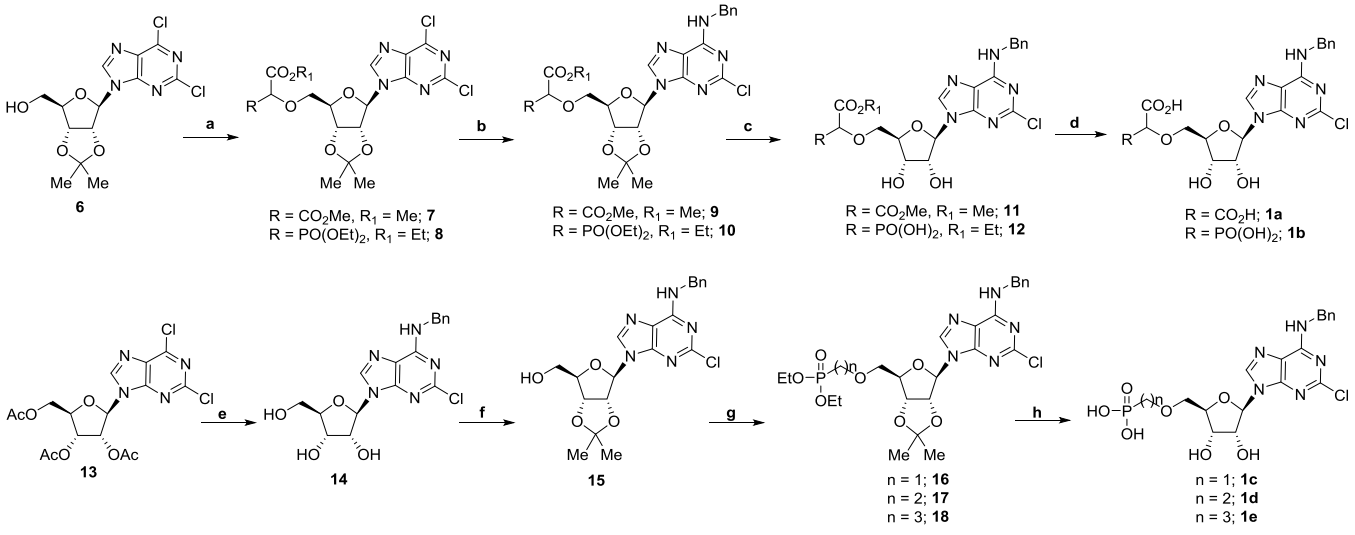
Table 6. Further Improvements in Rat PK

												
R	Compd	IC_{50}[a] (nM)	CL[b] (L/h/k)	$T_{1/2}$[b] (h)	Compd	IC_{50}[a] (nM)	CL[b] (L/h/kg)	$T_{1/2}$[b] (h)	Compd	IC_{50}[a] (nM)	CL[b] (L/h/kg)	$T_{1/2}$[b] (h)
	**2n**	26	0.37	0.97	**4a**	2.6	0.13	1.2	**4b**	4.5	N/D	
	**2d**	65	0.14	1.2	**5a**	29	0.031	2.4	**5e**	19	N/D	
	--				**5b**	7.6	N/D		**5f**	5.9	N/D	
	--				**5c**	39	N/D		**5g**	31	N/D	
	--				**5d**	12	0.020	4.5	**5h**	6.9	0.018	4.0

[a]IC_{50} measured in hCD73-CHO cells. [b]PK experiments were performed on male SD rats at IV dose of 0.25 or 0.5 mg/kg using 31.6% dimethylacetamide (DMA) + 36.8% ethanol + 31.6% poly(ethylene glycol) (PEG) as the vehicle; N/D, not determined.

Scheme 3a. Replacement of Bisphosphonic Acid[a]



[a]Reagents and conditions: (a) $Rh_2(OAc)_4$, dimethyl 2-diazomalonate for **7** or ethyl 2-diazo-2-(diethoxyphosphoryl)acetate for **8**, toluene, reflux, yield = 60% for **7** and 64% for **8**; (b) benzylamine, Et_3N, EtOH, room temperature (rt), yield = quantitative; (c) TMS-Br, CH_3CN, rt and then H_2O, yield = 38% for **11** and 23% for **12**; (d) LiOH, tetrahydrofuran (THF)/H_2O, rt, yield = 89% for **1a**, 15% for **1b**; (e) benzylamine, Et_3N, EtOH, rt and then 7 N NH_3 in MeOH, yield = 55%; (f) 2,2-dimethoxypropane, *p*-TSA, acetone, rt; (g) diethyl *p*-toluenesulfonyloxy methylphosphonate for **1c** or diethyl 2-bromoethylphosphonate for **1d** or diethyl 2-bromopropylphosphonate for **1e**, NaH, DMF, 0 °C–rt; (h) TMS-Br, CH_3CN, rt and then H_2O, yield = 20% for **1c**, 6% for **1d**, 23% for **1e** (three steps).

available 2,6-dichloropurine riboside triacetate **13** and benzylamine in ethanol in the presence of triethylamine. The crude reaction mixture was then subjected to global acetate deprotection using methanolic ammonia to obtain **14**. Next, the 2,3-*syn*-diol was protected as acetonide **15**. At this stage, alkyl phosphonates were introduced at the C5′ hydroxyl of the

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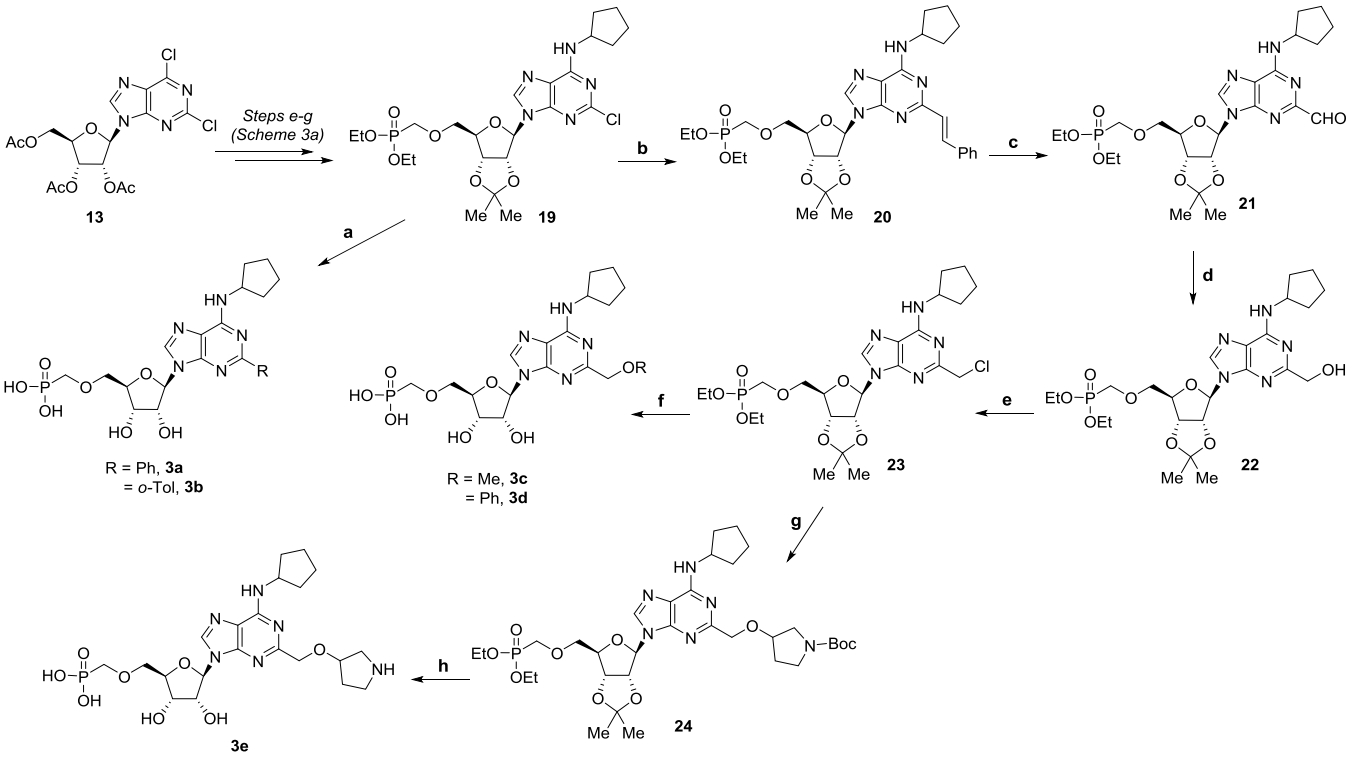
Scheme 3b. Synthesis of C2-Modified Analogs[a]



[a]Reagents and conditions: (a) two steps: (1) phenylboronic acid or tolylboronic acid, $Pd(PPh_3)_4$, Na_3CO_3, THF/H_2O, reflux; (2) TMS-Br, CH_3CN, rt and then H_2O, yields = 20% for **3a** and 10% for **3b**; (b) cinnamylboronic acid, $Pd(PPh_3)_4$, Na_3CO_3, THF/H_2O, reflux; (c) $K_2OsO_4{\cdot}2H_2O$, $NaIO_4$, 2,6-lutidine, THF, H_2O, rt; (d) $NaBH(OAc)_3$, CH_2Cl_2, rt, yield = 60% (three steps); (e) $SOCl_2$, pyridine, CH_3CN, 0 °C, yield = 40%; (f) two steps: (1) K_2CO_3, MeOH or phenol, rt–40 °C; (2) TMS-Br, CH_3CN, rt and then H_2O, yields = 13% for **3c** and 37% for **3d**; (g) *N*-Boc-3-pyrrolidinol, NaH, DMF; (h) TMS-Br, CH_3CN, rt and then H_2O, yield = 15% (two steps).

ribose unit using diethyl *p*-toluenesulfonyloxy methylphosphonate, diethyl 2-bromoethylphosphonate, and diethyl 2-bromopropylphosphonate in the presence of NaH in *N*,*N*-dimethylformamide (DMF) to obtain phosphonates **16**, **17**, and **18**, respectively. As before, hydrolysis of ethylphosphonates and concomitant acetonide removal were achieved with TBS-Br and a successive aqueous quench to yield **1c–e**. The above-mentioned four-step sequence (Scheme 3a, steps e–h) was also used for the synthesis of all C6-modified analogs (**2a–2r**) shown in Table 2. Similar synthesis of 5′-methylphosphonate nucleosides has been reported previously.[13]

Variation of the C2 position was carried out according to Scheme 3b. Following Steps e–g in Scheme 3a, commercially available 2,6-dichloropurine riboside triacetate **13** was converted to **19**, which was used for various C2 modifications. Introduction of a phenyl or *o*-tolyl substituent (**3a** and **3b**, respectively) was achieved via Suzuki coupling of **19** with the corresponding arylboronic acids followed by hydrolysis of the ethylphosphonate using TMS-Br and a successive aqueous quench. For the synthesis of the methylene-ether substituents, intermediate **19** was first coupled with cinnamylboronic acid to obtain **20**. Oxidative cleavage of **20** furnished aldehyde **21**, which was further reduced to carbinol **22**.[14] The carbinol **22** was then treated with thionyl chloride to obtain **23**, which was used for the synthesis of various methylene-ether analogs. Methoxymethyl ether **3c** and phenoxymethylether **3d** were synthesized via S_N2 displacement of the chlorine atom of **23** by methanol and phenol, respectively, in the presence of potassium carbonate followed by hydrolysis of the phosphonate ester. For the synthesis of compound **3e**, commercially available *N*-Boc-3-pyrrolidinol was used in the presence of sodium hydride in *N*,*N*-dimethylformamide. Removal of the Boc group and the hydrolysis of the ethylphosphonate were carried out in one-pot using TMS-Br.

Synthesis of analogs with modifications to the central core was carried out according to Scheme 3c. *N*-Glycosylation of commercially available central cores pyrazolopyrmidine **25** and pyrazolopyridine **26** with ribose tetraacetate were carried out using bis(trimethylsilyl)amine (HMDS) and trimethylsilyl trifluoromethanesulfonate (TMSOTf) to obtain **27** and **28**, respectively. Similar to the synthesis of **1c**, cyclopentylamine was introduced at the C6 position of pyrazolopyrimidine **27** at room temperature in methanol in the presence of triethylamine. Successive removal of the acetate groups with methanolic ammonia gave compounds **29**. *Syn*-diol of **29** was then protected as acetonide **30**. The methylphosphate moiety was then introduced at C5′ hydroxyl to obtain **31**. Finally, global deprotection of **31** gave **4a**. This four-step sequence (steps b–e) was also used for the synthesis of analogs **5a–d**. An alternative approach was adopted for the synthesis of the pyrazolopyridine-derived analog **4b**, in which cyclopentylamine was introduced at the C6 position at the end of the sequence. The glycosylated product **28** was first converted to acetonide **32**, followed by introduction of the phosphate residue at C5′ to obtain **33**. Contrary to the pyrazolopyrimidine **27**, installation of C6 amines on pyrazolopyridine **33** required elevated temperature (refluxing ethanol). As before, hydrolysis of the ethylphosphonate and removal of acetonide

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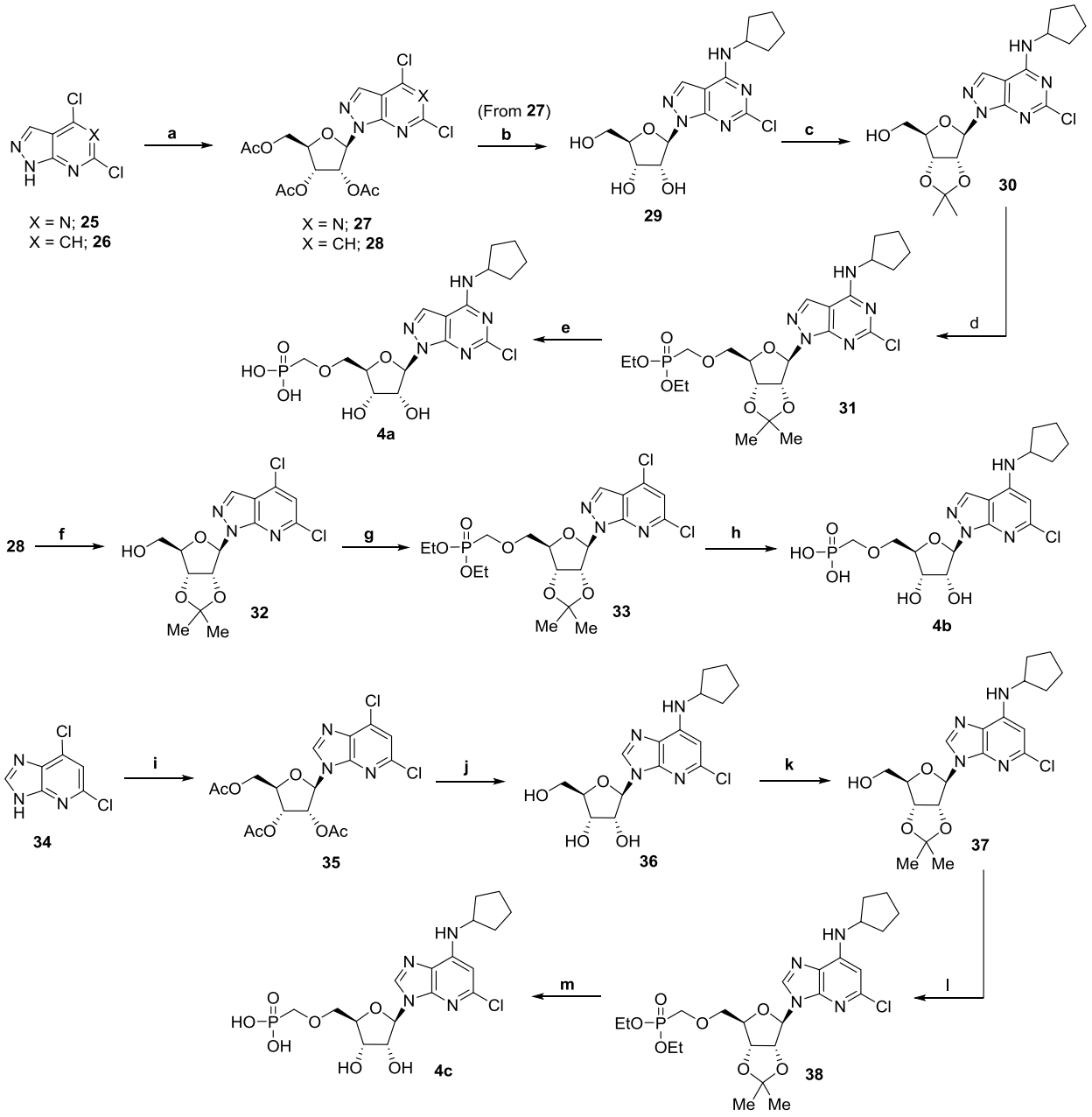
Scheme 3c. Synthesis of Analogs with Modified Purine Base[a]



[a]Reagents and conditions: (a) HMDS, $(NH_4)_2SO_4$, reflux and then β-D-ribofuranose 1,2,3,5-tetraacetate, TMSOTf, CH_3CN, rt, yield = 25% for **27** and 32% for **28**; (b) cyclopentylamine, Et_3N, MeOH, rt and then 7 N NH_3 in MeOH; (c) 2,2-dimethoxypropane, *p*-TSA, acetone, rt; (d) NaHMDS, diethyl *p*-toluenesulfonyloxy methylphosphonate THF, 0 °C−rt; yield = 73% (three steps); (e) TMS-Br, CH_3CN, rt and then MeOH/HCl, yield = 93%; (f) two steps: (1) 7 N NH_3 in MeOH, rt; (2) 2,2-dimethoxypropane, *p*-TSA, acetone, rt, yield = 80%; (g) diethyl *p*-toluenesulfonyloxy methylphosphonate, LiHMDS, THF, −78 °C−rt, yield = 59%; (h) two steps: (1) cyclopentylamine, Et_3N, EtOH, reflux; (2) TMS-Br, CH_3CN, rt and then H_2O, yield = 48%; (i) BSA, CH_3CN, reflux then β-D-ribofuranose 1,2,3,5-tetraacetate, TMSOTf, CH_3CN, reflux; (j) cyclopentylamine, dioxane, reflux, yield = 40%, two steps; (k) 2,2-dimethoxypropane, *p*-TSA, acetone, rt, yield = 78%; (l) NaHMDS, diethyl *p*-toluenesulfonyloxy methylphosphonate THF, 0 °C−rt; yield = 62%; (m) TMS-Br, CH_3CN, rt and then H_2O, yield = 15%.

were carried out in one-pot using TMS-Br to obtain analogs **4b**. Analogs **5e−h** were synthesized in a similar fashion to **4b**. Glycosylation of imidazolopyridine **34** was achieved using *N,O*-bis(trimethylsilyl)acetamide (BSA) and TMSOTf. Similar to pyrazolopyridine core **33**, installation of the C6 cyclopentylamine on imidazolopyridine **35** was achieved at elevated temperature using refluxing dioxane to obtain **36**. Analogous to the synthesis of **4a**, compound **36** was then subjected to steps k−m to furnish **4c**.

CONCLUSIONS

A series of methylenephosphonic acid analogs were synthesized in a systematic SAR study of CD73 inhibitors. Our structure-based design relied on information from the X-ray cocrystal structures of hCD73 with the known inhibitor AMPCP as well as our early lead methylenephosphonic acid **2n**. We have demonstrated that the bisphosphonic acid moiety can be successfully replaced with a methylenephosphonic acid on nucleoside-derived CD73 inhibitors. With additional

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modifications of the adenine base, improved potency and selectivity can be achieved. Our structure-guided approach led to systematic investigation of the C2 position to take advantage of a hydrophobic pocket and nucleobase modification to influence $\pi-\pi$ stacking to boost potency. Similarly, we demonstrated that introduction of 4-fluoro-α-(*S*)-methylbenzylamine at C6 led to a remarkable improvement in rat PK properties. In combination with the results from our additional SAR studies on CD73 inhibitors in a related program,[7c] currently in human clinical trials, this study describes progress toward understanding the molecular requirements for nucleotide-like small-molecule CD73 inhibitors and the discovery of several promising candidates against this promising therapeutic target.

EXPERIMENTAL SECTION

General Chemistry. All reactions were performed using a Teflon-coated magnetic stir bar at the indicated temperature and were conducted under an inert atmosphere when stated. All chemicals were used as received. Reactions were monitored by TLC (silica gel 60 with fluorescence F254, visualized with a short-wave/long-wave UV lamp) and/or liquid chromatography−mass spectrometry (LCMS, Agilent 1100 series LCMS with UV detection at 254 nm using a binary solvent system [0.1% trifluoroacetic acid (TFA) in MeCN/0.1% TFA in H_2O] using either of the following columns: Agilent Eclipse Plus C18 [3.5 μm, 4.6 mm i.d. × 100 mm] or Aeris Widepore C4 [3.6 μm, 2.1 mm i.d. × 50 mm]). Flash chromatography was conducted on silica gel using an automated system (CombiFlash RF+ manufactured by Teledyne ISCO), with detection wavelengths of 254 and 280 nm. Reverse-phase preparative high-performance liquid chromatography (HPLC) was conducted on an Agilent 1260 Infinity series HPLC. Samples were eluted using a binary solvent system (0.1% TFA in MeCN/0.1% TFA in H_2O) with gradient elution on a Gemini C18 110 Å column (21.2 mm i.d. × 250 mm) with detection at 254 nm. Final compounds obtained through preparative HPLC were concentrated through lyophilization. All reported yields are isolated yields. All assayed compounds were purified to ≥95% purity as determined by LCMS (Agilent 1100 series LCMS with UV detection at 254 nm using a binary solvent system [0.1% TFA in MeCN/0.1% TFA in H_2O] using either of the following columns: Agilent Eclipse Plus C18 column [3.5 μm, 4.6 mm i.d. × 100 mm] or Aeris Widepore C4 column [3.6 μm, 2.1 mm i.d. × 50 mm]). ^{1}H NMR spectra were recorded on a Varian 400 MHz NMR spectrometer equipped with an Oxford AS400 magnet. Chemical shifts (δ) are reported as parts per million (ppm) relative to residual undeuterated solvent as an internal reference. The abbreviations s, br. s, d, t, q, dd, dt, ddd, and m stand for singlet, broad singlet, doublet, triplet, quartet, doublet of doublets, doublet of triplets, doublet of doublet of doublets, and multiplet, respectively.

*2-{[(2R,3S,4R,5R)-5-[6-(Benzylamino)-2-chloro-9H-purin-9-yl]-3,4-dihydroxyoxolan-2-yl]methoxy}propanedioic Acid (**1a**).* A mixture of 2,6-dichloro-9-[2,3-*O*-(1-methylethylidene)-β-D-ribofuranosyl]-9*H*-purine **6** (1.05 g, 2.9 mmol), dimethyl 2-diazomalonate (648 mg, 4.1 mmol), and $Rh_2(OAc)_4$ (128 mg, 0.29 mmol) in toluene (50 mL) was stirred at 100 °C overnight. After cooling to rt, the solvent was evaporated from the reaction mixture and the crude material was purified by column chromatography to obtain **7** as a yellow oil in 60% yield (850 mg). ESI MS $[M + H]^+$ for $C_{18}H_{20}Cl_2N_4O_0$, calcd 491.1, found 490.8.

A mixture of **7** (420 mg, 0.86 mmol), benzylamine (98 μL, 0.9 mmol), and triethylamine (125 μL, 0.9 mmol) in EtOH (5 mL) was stirred at rt overnight. The solvent was evaporated from the reaction mixture, and the crude product **9** was used in the next step without purification. ESI MS $[M + H]^+$ for $C_{25}H_{29}ClN_5O_8$, calcd 562.2, found 562.2.

The crude product **9** was dissolved in anhydrous CH_3CN (10 mL). TMS-Br (1.0 mL) was added, and the reaction was stirred at rt overnight. Then, it was quenched with H_2O (2 mL) and stirred at rt for 4 h or until LCMS analysis showed complete cleavage of the acetonide protecting group. The reaction mixture was evaporated and purified by reverse-phase HPLC (C18 column, 0−30% gradient of acetonitrile and water with 0.1% TFA) to give the product **11** as a white solid in 38% yield (210 mg) over two steps: ESI MS $[M + H]^+$ for $C_{22}H_{24}ClN_5O_8$, calcd 522.1, found 522.2.

Compound **11** (170 mg, 0.27 mmol) was dissolved in 4 mL of a 1:1 mixture of THF and H_2O. To this mixture was added $LiOH·H_2O$ (46 mg, 1.1 mmol) and was stirred overnight at rt and then neutralized with AcOH. The solvent was evaporated from the reaction mixture, and the crude material was purified by reverse-phase HPLC (C18 column, 0−30% gradient of acetonitrile and water with 0.1% TFA) to give the product **1a** as a white solid in 89% yield (145 mg) over two steps: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.92 (t, J = 6.2 Hz, 1H), 8.57 (s, 1H), 7.40−7.17 (m, 5H), 5.87 (d, J = 6.3 Hz, 1H), 4.65 (t, J = 5.5 Hz, 1H), 4.59 (d, J = 9.4 Hz, 2H), 4.17 (dd, J = 4.8, 2.7 Hz, 1H), 4.10 (q, J = 3.0 Hz, 1H), 3.76 (dd, J = 10.4, 3.1 Hz, 1H), 3.65 (dd, J = 10.6, 3.3 Hz, 1H). ESI MS $[M + H]^+$ for $C_{20}H_{20}ClN_5O_8$, calcd 494.1, found 494.1.

*2-{[(2R,3S,4R,5R)-5-[6-(Benzylamino)-2-chloro-9H-purin-9-yl]-3,4-dihydroxyoxolan-2-yl]methoxy}-2-phosphonoacetic Acid (**1b**).* Compound **1b** was synthesized in a similar fashion to example **1a** as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.93 (dt, J = 12.6, 6.4 Hz, 1H), 8.61 (d, J = 7.2 Hz, 1H), 7.43−7.17 (m, 5H), 5.86 (d, J = 6.5 Hz, 1H), 4.74−4.53 (m, 2H), 4.19 (dd, J = 18.5, 4.2 Hz, 2H), 4.08 (q, J = 3.2 Hz, 1H), 3.85−3.60 (m, 2H). ESI MS $[M + H]^+$ for $C_{19}H_{21}ClN_5O_9P$, calcd 530.1, found 530.1.

*({[(2R,3S,4R,5R)-5-[6-(Benzylamino)-2-chloro-9H-purin-9-yl]-3,4-dihydroxyoxolan-2-yl]methoxy}methyl) Phosphonic Acid (**1c**).* A mixture of 2,6-dichloro-9-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)purine **6** (29 g, 65 mmol), benzylamine (28.4 mL, 260 mmol), and triethylamine (9 mL, 165 mmol) in EtOH (200 mL) was stirred at rt for overnight. Then, 7 M NH_3 in MeOH (50 mL) was added to the above reaction and was stirred at rt for 24 h. The solvent was evaporated, and the mixture was reconstituted in 400 mL of EtOAc and 300 mL of H_2O. Layers were separated, and the aqueous layer was extracted with EtOAc (2 × 300 mL). The combined organic layer was dried over Na_2SO_4, concentrated, and purified by silica gel chromatography give **14** as a white solid in 55% yield (14 g). ESI MS $[M + H]^+$ for $C_{17}H_{19}ClN_5O_4$, calcd 392.1, found 392.1.

Compound **14** (5.6 g, 14.3 mmol) was dissolved in acetone (100 mL) and 2,2-dimethoxypropane (30 mL). To this mixture was added *p*-TsOH·H_2O (3.4 g, 17.9 mmol). The reaction mixture was stirred at rt overnight, diluted with brine (100 mL), and carefully quenched with saturated $NaHCO_3$ (200 mL). After extraction with EtOAc (2 × 200 mL), combined organics were dried over $MgSO_4$ and evaporated to give the crude product **15** (6.1 g), which was used in the next step without purification. ESI MS $[M + H]^+$ for $C_{20}H_{22}ClN_5O_4$, calcd 432.1, found 432.1.

A sample of the crude product **15** (431.5 mg, 1 mmol) was dissolved in anhydrous DMF (5 mL) and cooled to 0 °C; then, 60% NaH (60 mg, 1.5 mmol) was added and the reaction mixture was stirred at 0 °C for 1 h. Diethyl *p*-toluenesulfonyloxy methylphosphonate (386 mg, 1.2 mmol, 1.2 equiv) was added, and the reaction was slowly warmed up to rt and stirred overnight. H_2O (20 mL) was added to dilute the reaction mixture and extracted with MTBE (2 × 10 mL). The combined organics were dried over $MgSO_4$, filtered, and evaporated to give crude product **16**, which was used in the next step without purification. ESI MS $[M + H]^+$ for $C_{25}H_{33}ClN_5O_7P$, calcd 582.2, found 582.1.

All of the crude product **16** was dissolved in anhydrous CH_3CN (5 mL), TMS-Br (0.5 mL) was added, and the reaction was stirred at rt overnight. It was quenched with H_2O (1 mL) and stirred at rt for 4 h or until LCMS analysis showed complete cleavage of the acetonide protecting group. The reaction mixture was evaporated and purified by reverse-phase HPLC (C18 column, 0−30% gradient of acetonitrile and water with 0.1% TFA) to give the product **1c** as a white solid in 20% yield (123 mg) over three steps: %: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.93 (t, J = 6.2 Hz, 1H), 8.44 (s, 1H), 7.37−7.22 (m, 5H), 5.85 (d, J = 6.0 Hz, 1H), 4.68−4.61 (m, 2H), 4.54 (t, J = 5.5 Hz,

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1H), 4.15−4.09 (m, 1H), 4.05 (q, J = 3.8 Hz, 1H), 3.77−3.65 (m, 2H), 3.61 (d, J = 8.9 Hz, 2H). ESI MS $[M + H]^+$ for $C_{18}H_{22}ClN_5O_7P$, calcd 486.1, found 486.2.

*(2-{[(2R,3S,4R,5R)-5-[6-(Benzylamino)-2-chloro-9H-purin-9-yl]-3,4-dihydroxyoxolan-2-yl]methoxy}ethyl)phosphonic Acid (**1d**).* Compound **1d** was synthesized in a similar fashion to example **1c** as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.46 (d, J = 29.8 Hz, 1H), 7.31 (m, 6H), 5.87 (d, J = 5.8 Hz, 1H), 5.71−5.41 (m, 1H), 4.97 (d, J = 12.7 Hz, 1H), 4.65−3.25 (m, 7H), 2.13−1.84 (m, 2H). ESI MS $[M + H]^+$ for $C_{19}H_{23}ClN_5O_7P$, calcd 500.1, found 500.1.

*(3-{[(2R,3S,4R,5R)-5-[6-(Benzylamino)-2-chloro-9H-purin-9-yl]-3,4-dihydroxyoxolan-2-yl]methoxy}propyl)phophonic Acid (**1e**).* Compound **1e** was synthesized in a similar fashion to example **1c** as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.39 (d, J = 32.3 Hz, 1H), 7.34−7.05 (m, 5H), 5.80 (d, J = 6.1 Hz, 1H), 5.48 (q, J = 16.6 Hz, 1H), 5.02−4.71 (m, 1H), 4.55−4.36 (m, 1H), 4.18−3.93 (m, 2H), 3.88 (q, J = 3.8 Hz, 1H), 3.70−3.22 (m, 3H), 1.88−1.39 (m, 4H). ESI MS $[M + H]^+$ for $C_{20}H_{25}ClN_5O_7P$, calcd 514.1, found 514.1.

*({[(2R,3S,4R,5R)-5-{2-Chloro-6-[(2-phenylethyl)amino]-9H-purin-9-yl}-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)phosphonic Acid (**2a**).* Compound **2a** was synthesized in a similar fashion to example **1c** as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.50−8.33 (m, 2H), 7.33−7.17 (m, 5H), 5.84 (d, J = 6.1 Hz, 1H), 4.54 (t, J = 5.5 Hz, 1H), 4.12 (dd, J = 4.9, 3.2 Hz, 1H), 4.05 (q, J = 3.7 Hz, 1H), 3.79−3.57 (m, 6H), 2.92 (t, J = 7.4 Hz, 2H). ESI MS $[M + H]^+$ for $C_{19}H_{24}ClN_5O_7P$, calcd 500.1, found 500.2.

*({[(2R,3S,4R,5R)-5-(2-Chloro-6-{[2-(2-methoxyphenyl)ethyl]-amino}-9H-purin-9-yl)-3,4-dihydroxyoxolan-2-yl]metoxy}methyl)-phosphonic Acid (**2b**).* Compound **2b** was synthesized in a similar fashion to example **1c** as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.46−8.33 (m, 2H), 7.22−7.11 (m, 2H), 6.96 (d, J = 8.2 Hz, 1H), 6.84 (t, J = 7.4 Hz, 1H), 5.84 (d, J = 6.1 Hz, 1H), 4.57−4.51 (m, 1H), 4.12 (dd, J = 4.9, 3.2 Hz, 1H), 4.05 (q, J = 3.7 Hz, 1H), 3.82−3.56 (m, 9H), 2.89 (t, J = 7.3 Hz, 2H). ESI MS $[M + H]^+$ for $C_{20}H_{26}ClN_5O_8P$, calcd 530.1, found 530.2.

*({[(2R,3S,4R,5R)-5-(2-Chloro-6-{[2-(2-fluorophenyl)ethyl]amino}-9H-purin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)-phosphonic Acid (**2c**).* Compound **2c** was synthesized in a similar fashion to example **1c** as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.54−8.39 (m, 2H), 7.33−7.07 (m, 5H), 5.84 (d, J = 6.1 Hz, 1H), 4.57−4.52 (m, 1H), 4.12 (dd, J = 4.9, 3.2 Hz, 1H), 4.05 (q, J = 3.8 Hz, 1H), 3.77−3.57 (m, 6H), 2.96 (t, J = 7.2 Hz, 2H). ESI MS $[M + H]^+$ for $C_{19}H_{23}ClFN_5O_7P$, calcd 518.1, found 518.2.

*({[(2R,3S,4R,5R)-5-(2-Chloro-6-{[(1S)-1-phenylethyl]amino}-9H-purin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)phosphonic Acid (**2d**).* Compound **2d** was synthesized in a similar fashion to example **1c** as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.89 (d, J = 8.4 Hz, 1H), 8.45 (s, 1H), 7.43 (d, J = 7.5 Hz, 2H), 7.31 (t, J = 7.6 Hz, 2H), 7.21 (t, J = 7.3 Hz, 1H), 5.84 (d, J = 6.0 Hz, 1H), 5.41 (p, J = 7.6 Hz, 1H), 4.51 (t, J = 5.4 Hz, 1H), 4.15−4.08 (m, 1H), 4.07−4.01 (m, 1H), 3.78−3.65 (m, 2H), 3.61 (d, J = 9.0 Hz, 2H), 1.54 (d, J = 7.0 Hz, 3H). ESI MS $[M + H]^+$ for $C_{19}H_{24}ClN_5O_7P$, calcd 500.1, found 500.2.

*({[(2R,3S,4R,5R)-5-(2-Chloro-6-{[(1R)-1-phenylethyl]amino}-9H-purin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)phosphonic Acid (**2e**).* Compound **2e** was synthesized in a similar fashion to example **1c** as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.89 (d, J = 8.4 Hz, 1H), 8.46 (s, 1H), 7.44 (d, J = 7.4 Hz, 2H), 7.31 (t, J = 7.6 Hz, 2H), 7.21 (t, J = 7.2 Hz, 1H), 5.84 (d, J = 6.1 Hz, 1H), 5.41 (p, J = 7.6 Hz, 1H), 4.54 (t, J = 5.5 Hz, 1H), 4.15−4.07 (m, 1H), 4.07−4.01 (m, 1H), 3.77−3.65 (m, 2H), 3.61 (dd, J = 8.9, 1.8 Hz, 2H), 1.54 (d, J = 6.9 Hz, 3H). ESI MS $[M + H]^+$ for $C_{19}H_{24}ClN_5O_7P$, calcd 500.1, found 500.2.

*({[(2R,3S,4R,5R)-5-(2-Chloro-6-{[(2-chlorophenyl)methyl]amino}-9H-purin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)-phosphonic Acid (**2f**).* Compound **2f** was synthesized in a similar fashion to example **1c** as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.97−8.90 (m, 1H), 8.49 (s, 1H), 7.50−7.43 (m, 1H), 7.33−7.25 (m, 3H), 5.87 (d, J = 6.0 Hz, 1H), 4.75−4.67 (m, 2H), 4.56 (t, J = 5.5 Hz, 1H), 4.16−4.02 (m, 2H), 3.78−3.65 (m, 2H), 3.62 (d, J = 8.9 Hz, 2H). ESI MS $[M + H]^+$ for $C_{18}H_{21}Cl_2N_5O_7P$, calcd 520.1, found 520.1.

*({[(2R,3S,4R,5R)-5-(2-Chloro-6-{[(2-chlorophenyl)methyl]-(methyl)amino}-9H-purin-9-yl)-3,4-dihydroxyoxolan-2-yl]-methoxy}methyl)phosphonic Acid (**2g**).* Compound **2g** was synthesized in a similar fashion to example **1c** as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.56−8.33 (m, 1H), 7.54−7.48 (m, 1H), 7.36−7.27 (m, 2H), 7.23−7.08 (m, 1H), 5.88 (d, J = 6.1 Hz, 1H), 5.63 (s, 1H), 5.00 (s, 1H), 4.54 (s, 1H), 4.15−4.01 (m, 2H), 3.78−3.55 (m, 5H), 3.17 (s, 2H). ESI MS $[M + H]^+$ for $C_{19}H_{23}Cl_2N_5O_7P$, calcd 534.1, found 534.1

*({[(2R,3S,4R,5R)-5-{2-Chloro-6-[(pyridin-2-ylmethyl)amino]-9H-purin-9-yl}-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)phosphonic Acid (**2h**).* Compound **2h** was synthesized in a similar fashion to example **1c** as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.05 (t, J = 6.2 Hz, 1H), 8.82−8.78 (m, 1H), 8.72−8.68 (m, 1H), 8.49 (s, 1H), 8.26 (d, J = 8.1 Hz, 1H), 7.83−7.76 (m, 1H), 5.86 (d, J = 6.1 Hz, 1H), 4.78 (d, J = 6.0 Hz, 2H), 4.54 (dd, J = 6.1, 4.9 Hz, 1H), 4.12 (dd, J = 4.9, 3.2 Hz, 1H), 4.05 (q, J = 3.7 Hz, 1H), 3.77−3.66 (m, 2H), 3.61 (dd, J = 8.9, 1.5 Hz, 2H). ESI MS $[M + H]^+$ for $C_{17}H_{21}ClN_6O_7P$, calcd 487.1, found 487.1.

*({[(2R,3S,4R,5R)-5-(2-Chloro-6-{[(1-methyl-1H-pyrazol-4-yl)-methyl]amino}-9H-purin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy}-methyl)phosphonic Acid (**2i**).* Compound **2i** was synthesized in a similar fashion to example **1c** as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.72 (t, J = 6.1 Hz, 1H), 8.43 (s, 1H), 7.58 (s, 1H), 7.38 (s, 1H), 5.84 (d, J = 6.0 Hz, 1H), 4.54 (t, J = 5.5 Hz, 1H), 4.48−4.43 (m, 2H), 4.12 (dd, J = 4.9, 3.3 Hz, 1H), 4.05 (q, J = 3.7 Hz, 1H), 3.76 (s, 3H), 3.75−3.65 (m, 2H), 3.62 (d, J = 8.9 Hz, 2H). ESI MS $[M + H]^+$ for $C_{16}H_{22}ClN_7O_7P$, calcd 490.1, found 490.2.

*({[(2R,3S,4R,5R)-5-(2-Chloro-6-{[(4-methyl-4H-1,2,4-triazol-3-yl)-methyl]amino}-9H-purin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy}-methyl)phosphonic Acid (**2j**).* Compound **2j** was synthesized in a similar fashion to example **1c** as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.12−8.99 (m, 2H), 8.51 (s, 1H), 5.86 (d, J = 6.1 Hz, 1H), 4.87 (s, 2H), 4.54 (t, J = 5.5 Hz, 1H), 4.12 (dd, J = 4.9, 3.2 Hz, 1H), 4.06 (q, J = 3.7 Hz, 1H), 3.86 (s, 3H), 3.77−3.65 (m, 2H), 3.61 (dd, J = 8.9, 1.6 Hz, 2H). ESI MS $[M + H]^+$ for $C_{15}H_{21}ClN_8O_7P$, calcd 491.1, found 491.2.

*({[(2R,3S,4R,5R)-5-{2-Chloro-6-[(propan-2-yl)amino]-9H-purin-9-yl}-3,4-dihydroxyoxolan-2-yl]methoxy}methyl) Phosphonic Acid (**2k**).* Compound **2k** was synthesized in a similar fashion to example **1c** as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.67 (s, 1H), 8.21−8.09 (m, 1H), 5.79 (d, J = 7.1 Hz, 1H), 4.23 (dd, J = 4.8, 1.9 Hz, 1H), 4.02 (s, 1H), 3.66−3.50 (m, 3H), 3.42−3.19 (m, 3H), 1.21 (dd, J = 6.5, 2.1 Hz, 6H). ESI MS $[M + H]^+$ for $C_{14}H_{22}ClN_5O_7P$, calcd 438.1, found 438.1.

*({[(2R,3S,4R,5R)-5-[2-Chloro-6-(cyclopropylamino)-9H-purin-9-yl]-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)phosphonic Acid (**2l**).* Compound **2l** was synthesized in a similar fashion to example **1c** as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.53 (s, 1H), 8.42 (s, 1H), 5.85 (d, J = 6.0 Hz, 1H), 4.54 (t, J = 5.5 Hz, 1H), 4.12 (dd, J = 4.9, 3.2 Hz, 1H), 4.05 (q, J = 3.7 Hz, 1H), 3.77−3.65 (m, 2H), 3.61 (dd, J = 8.9, 1.4 Hz, 2H), 2.97 (s, 1H), 0.84−0.56 (m, 4H). ESI MS $[M + H]^+$ for $C_{14}H_{20}ClN_5O_7P$, calcd 436.1, found 436.1.

*({[(2R,3S,4R,5R)-5-[2-Chloro-6-(cyclobutylamino)-9H-purin-9-yl]-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)phosphonic Acid (**2m**).* Compound **2m** was synthesized in a similar fashion to example **1c** as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.67 (d, J = 7.7 Hz, 1H), 8.43 (s, 1H), 5.84 (d, J = 5.9 Hz, 1H), 4.61 (q, J = 8.1 Hz, 1H), 4.56−4.50 (m, 1H), 4.15−4.09 (m, 1H), 4.05 (q, J = 3.7 Hz, 1H), 3.78−3.65 (m, 2H), 3.61 (d, J = 8.8 Hz, 2H), 2.35−2.00 (m, 4H), 1.74−1.61 (m, 2H). ESI MS $[M + H]^+$ for $C_{15}H_{22}ClN_5O_7P$, calcd 450.1, found 450.1.

*({[(2R,3S,4R,5R)-5-[2-Chloro-6-(cyclopentylamino)-9H-purin-9-yl]-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)phosphonic Acid (**2n**).* Compound **2n** was synthesized in a similar fashion to example **1c** as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.41 (s, 1H), 8.39−8.26 (m, 1H), 5.84 (d, J = 5.9 Hz, 1H), 4.53 (t, J = 5.5 Hz, 1H), 4.48−4.35 (m, 1H), 4.12 (dd, J = 4.9, 3.3 Hz, 1H), 4.05 (q, J = 3.8 Hz, 1H), 3.79−3.65 (m, 2H), 3.62 (d, J = 8.9 Hz, 2H), 2.05−1.85 (m,

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2H), 1.78−1.44 (m, 6H). ESI MS $[M + H]^+$ for $C_{16}H_{24}ClN_5O_7P$, calcd 464.1, found 464.2.

*({[(2R,3S,4R,5R)-5-{2-Chloro-6-[(cyclopentylmethyl)amino]-9H-purin-9-yl}-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)phosphonic Acid (**2o**).* Compound **2o** was synthesized in a similar fashion to example **1c** as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.45 (t, J = 2.4 Hz, 1H), 8.41 (s, 1H), 5.84 (d, J = 6.0 Hz, 1H), 4.61−4.47 (m, 1H), 4.12 (t, J = 4.1 Hz, 1H), 4.04 (d, J = 3.7 Hz, 1H), 3.85−3.51 (m, 5H), 3.36 (m, 1H), 2.38−2.13 (m, 1H), 1.77−1.39 (m, 6H), 1.34−1.12 (m, 2H). ESI MS $[M + H]$ for $C_{17}H_{25}ClN_5O_7P$, calcd 478.1, found 478.2.

*({[(2R,3S,4R,5R)-5-[2-Chloro-6-(pyrrolidin-1-yl)-9H-purin-9-yl]-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)phosphonic Acid (**2p**).* Compound **2p** was synthesized in a similar fashion to example **1c** as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.41 (s, 1H), 5.86 (d, J = 6.1 Hz, 1H), 4.53 (dd, J = 6.2, 4.9 Hz, 1H), 4.11 (dd, J = 4.9, 3.2 Hz, 1H), 4.09−4.00 (m, 3H), 3.76−3.65 (m, 2H), 3.65−3.56 (m, 4H), 2.05−1.86 (m, 4H). ESI MS $[M + H]^+$ for $C_{15}H_{22}ClN_5O_7P$, calcd 450.1, found 450.2.

*({[(2R,3S,4R,5R)-5-(2-Chloro-6-{[(3R)-oxolan-3-yl]amino}-9H-purin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)phosphonic Acid (**2q**).* Compound **2q** was synthesized in a similar fashion to example **1c** as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.63−8.51 (m, 1H), 8.45 (s, 1H), 5.86 (d, J = 6.0 Hz, 1H), 4.63 (s, 1H), 4.57−4.51 (m, 1H), 4.12 (dd, J = 4.9, 3.3 Hz, 1H), 4.05 (q, J = 3.7 Hz, 1H), 3.96−3.83 (m, 2H), 3.79−3.66 (m, 3H), 3.62 (d, J = 8.9 Hz, 3H), 2.29−1.83 (m, 2H). ESI MS $[M + H]^+$ for $C_{15}H_{22}ClN_5O_8P$, calcd 466.1, found 466.1.

*({[(2R,3S,4R,5R)-5-[2-Chloro-6-(morpholin-4-yl)-9H-purin-9-yl]-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)phosphonic Acid (**2r**).* Compound **2r** was synthesized in a similar fashion to example **1c** as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.77 (s, 1H), 5.82 (d, J = 7.5 Hz, 1H), 5.14 (dd, J = 7.5, 4.7 Hz, 1H), 4.27−4.21 (m, 1H), 4.05−4.00 (m, 1H), 3.78−3.66 (m, 4H), 3.61−3.48 (m, 2H), 3.35 (dd, J = 12.1, 7.6 Hz, 1H), 3.27−3.15 (m, 1H). ESI MS $[M + H]^+$ for $C_{15}H_{22}ClN_5O_8P$, calcd 466.1, found 466.1.

*({[(2R,3S,4R,5R)-5-[6-(Cyclopentylamino)-2-phenyl-9H-purin-9-yl]-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)phosphonic Acid (**3a**).* A mixture of 2,6-dichloro-9-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)purine **13** (13.5 g, 30 mmol), cyclopentylamine (3.2 mL, 33 mmol), and triethylamine (4.6 mL, 33 mmol) in MeOH (60 mL) was stirred at rt overnight. Then, 7 M NH_3 in MeOH (20 mL) was added to the above reaction and stirred at rt for 1 day. The reaction mixture was evaporated, the crude product was redissolved in acetone (100 mL), and 2,2-dimethoxypropane (40 mL) and p-TsOH·H_2O (7.1 g, 37.5 mmol) were added. The reaction mixture was stirred at rt overnight, diluted with brine (100 mL), and carefully quenched with saturated $NaHCO_3$ (200 mL). After extraction with EtOAc (2 × 200 mL), combined organics were dried over $MgSO_4$, filtered, and evaporated to give crude acetonide (12.2 g), which was used in the next step without purification. A sample of the crude product (410 mg, 1 mmol) was dissolved in anhydrous DMF (5 mL) and cooled to 0 °C; then, 60% NaH (60 mg, 1.5 mmol) was added and the reaction mixture was stirred at 0 °C for 1 h. Diethyl *p*-toluenesulfonyloxy methylphosphonate (386 mg, 1.2 mmol, 1.2 equiv) was added, and the reaction was slowly warmed up to rt and stirred overnight. It was diluted with H_2O (20 mL) and extracted with MTBE (2 × 10 mL); combined organics were dried over $MgSO_4$, filtered, and evaporated to give the crude product **19**, which was used in the next step without purification. ESI MS $[M + H]^+$ for $C_{23}H_{36}ClN_5O_7P$, calcd 560.2, found 560.1.

In a glass vial, compound **19** (250 mg, 0.45 mmol), phenylboronic acid (82 mg, 0.67 mmol), and sodium carbonate (142 mg, 3.75 mmol) were suspended in 3:1 THF/H_2O (3 mL). This mixture was degassed by N_2 sparge for 10 min. Subsequently, $Pd(PPh_3)_4$ (52 mg, 0.045 mmol) was added, and the resulting mixture was degassed for an additional 5 min and then sealed and heated to 80 °C overnight. After cooling to room temperature, the reaction was diluted with EtOAc and washed with water and brine. The organics were dried over $MgSO_4$, filtered, and concentrated under reduced pressure to obtain the crude product. ESI MS $[M + H]^+$ for $C_{29}H_{40}N_5O_7P$, calcd 602.3, found 602.4.

The crude product obtained above was dissolved in anhydrous CH_3CN (2.5 mL), TMS-Br (0.25 mL) was added, and the reaction was stirred at rt overnight. It was quenched with H_2O (0.25 mL) and stirred at rt for 4 h or until LCMS analysis showed complete cleavage of the acetonide protecting group. The reaction mixture was evaporated and purified by reverse-phase HPLC (C18 column, 0−30% gradient of acetonitrile and water with 0.1% TFA) to give the product **3a** as a white solid in 20% yield (46 mg). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.44−8.37 (m, 3H), 7.55−7.41 (m, 4H), 6.03 (d, J = 6.0 Hz, 1H), 4.73 (t, J = 5.6 Hz, 1H), 4.27−4.17 (m, 1H), 4.08 (app. q, J = 4.0 Hz, 1H), 3.89−3.74 (m, 1H), 3.70 (dd, J = 10.6, 4.8 Hz, 1H), 3.61 (d, J = 8.8 Hz, 2H), 3.52 (dd, J = 8.5, 3.6 Hz, 1H), 2.10−1.98 (m, 3H), 1.80−1.54 (m, 5H). ESI MS $[M - H]^-$ for $C_{22}H_{28}N_5O_7P$, calcd 504.2, found 504.3.

*({[(2R,3S,4R,5R)-5-[6-(Cyclopentylamino)-2-(2-methyl phenyl)-9H-purin-9-yl]-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)-phosphonic Acid (**3b**).* Compound **3b** was synthesized in a similar fashion to example **3a** as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.49 (s, 1H), 7.55−7.09 (m, 4H), 5.97 (d, J = 5.8 Hz, 1H), 4.64 (t, J = 5.4 Hz, 1H), 4.32−4.08 (m, 1H), 4.05 (app. q, J = 3.9 Hz, 1H), 3.75 (dd, J = 10.7, 3.6 Hz, 1H), 3.68 (dd, J = 10.7, 4.6 Hz, 1H), 3.60 (dd, J = 8.9, 1.9 Hz, 2H), 1.97 (br. s, 3H), 1.80−1.45 (m, 8H). ESI MS $[M - H]^-$ for $C_{23}H_{30}N_5O_7P$, calcd 518.2, found 518.2.

*({[(2R,3S,4R,5R)-5-[6-(Cyclopentylamino)-2-(methoxymethyl)-9H-purin-9-yl]-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)-phosphonic Acid (**3c**).* In a round-bottom flask, compound **19** (1.5 g, 2.68 mmol), phenylvinylboronic acid (595 mg, 4.02 mmol), and sodium carbonate (845 mg, 8.04 mmol) were suspended in 3:1 THF/H_2O (15 mL). This mixture was degassed by N_2 sparge for 10 min. Subsequently, $Pd(PPh_3)_4$ (310 mg, 0.27 mmol) was added, and the resulting mixture was degassed for an additional 5 min and then heated to reflux overnight. After cooling to rt, the reaction was diluted with EtOAc and washed with water and brine. The organics were dried over $MgSO_4$, filtered, and concentrated under reduced pressure to obtain the crude product **20** (1.89 g), which was used directly in the following step without purification. ESI MS $[M + H]^+$ for $C_{31}H_{42}N_5O_7P$, calcd 628.3, found 628.4.

To a suspension of the crude product **20** (1.62 g), sodium periodate (3.31 g, 15.48 mmol), and 2,6-lutidine (601 μL, 5.16 mmol) in 2:1 THF/H_2O (28 mL) was added potassium osmate dihydrate (24 mg, 0.065 mmol). The resulting thick suspension was stirred overnight at room temperature and then partitioned between EtOAc and water. The organics were washed sequentially with water and brine, dried over $MgSO_4$, and concentrated under reduced pressure to obtain the crude product **7**, which was used directly in the following step without purification. ESI MS $[M + H]^+$ for $C_{24}H_{36}N_5O_8P$, calcd 554.2, found 554.3.

To a solution of the crude compound **21** (471 mg) in dichloroethane (5.7 mL) was added sodium triacetoxyborohydride (216 mg, 1.02 mmol) in a single portion. The reaction was stirred at room temperature overnight and then partitioned between EtOAc and water. The organics were washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The title compound **22** (285 mg, 60% for three steps) was obtained following column chromatography (SiO_2, 0−15% gradient of MeOH and CH_2Cl_2). ESI MS $[M + H]^+$ for $C_{24}H_{38}N_5O_8P$, calcd 556.3, found 556.3.

To a solution of compound **22** (285 mg, 0.51 mmol) in acetonitrile (5 mL) at 0 °C were added pyridine (125 μL, 1.54 mmol) followed by thionyl chloride (56 μL, 0.77 mmol). The reaction was kept at 0 °C for 1 h. Subsequently, saturated $NaHCO_3$ (aq) was added slowly until gas evolution ceased. The reaction was diluted with EtOAc and washed with sat. $NaHCO_3$, water, and brine. The organics were dried over $MgSO_4$ and concentrated under reduced pressure. The title compound **23** (115 mg, 40%) was obtained following column chromatography (SiO_2, 0−10% gradient of MeOH and CH_2Cl_2). ESI MS $[M + H]^+$ for $C_{24}H_{37}ClN_5O_7P$, calcd 574.2, found 574.3.

To a flask charged with compound **23** (115 mg, 0.20 mmol) in methanol (5 ml) was added potassium carbonate (138 mg, 1.0

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mmol). The resulting suspension was stirred overnight at room temperature, diluted with EtOAc, and washed with water and brine. The organics were dried over $MgSO_4$ and concentrated under reduced pressure. The crude mixture (35 mg) so obtained comprised the desired product as well as an inconsequential mixture of methylphosphonates resulting from transesterification. ESI MS $[M + H]^+$ for $C_{25}H_{40}N_5O_8P$, calcd 570.3, found 570.3.

The crude product obtained above (35 mg) was dissolved in anhydrous CH_3CN (2.5 mL), TMS-Br (0.25 mL) was added, and the reaction was stirred at rt for 1.5 h. It was quenched with H_2O (0.25 mL) and stirred at rt for 1 h or until LCMS analysis showed complete cleavage of the acetonide protecting group. The reaction mixture was evaporated and purified by reverse-phase HPLC (C18 column, 0−30% gradient of acetonitrile and water with 0.1% TFA) to give the product **3c** as a white solid in 13% yield (12 mg), two steps: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.48 (br. s, 1H), 5.94 (d, J = 6.2 Hz, 1H), 4.61 (t, J = 5.6 Hz, 1H), 4.42 (s, 3H), 4.15 (dd, J = 4.9, 3.1 Hz, 1H), 4.05 (t, J = 3.8 Hz, 1H), 3.82−3.65 (m, 2H), 3.61 (d, J = 8.9 Hz, 2H), 3.39 (d, J = 1.3 Hz, 3H), 1.97 (br. s, 2H), 1.82−1.65 (m, 2H), 1.58 (br. s, 4H). ESI MS $[M - H]^-$ for $C_{18}H_{28}N_5O_8P$, calcd 472.2, found 472.3.

*({[(2R,3S,4R,5R)-5-[6-(Cyclopentylamino)-2-(phenoxy methyl)-9H-purin-9-yl]-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)-phosphonic Acid (**3d**).* Compound **3d** was synthesized in a similar fashion to example **3c** as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.33 (s, 1H), 7.30−7.12 (m, 3H), 6.93 (d, J = 7.8 Hz, 2H), 6.84 (t, J = 7.3 Hz, 1H), 5.84 (d, J = 5.9 Hz, 1H), 4.99 (s, 3H), 4.53 (dd, J = 6.1, 5.0 Hz, 1H), 4.07 (dd, J = 5.0, 3.4 Hz, 1H), 3.95 (q, J = 3.9 Hz, 1H), 3.65 (dd, J = 10.7, 3.7 Hz, 1H), 3.59−3.49 (m, 3H), 1.81 (s, 3H), 1.63 (br. s, 3H), 1.46 (br. s, 5H). ESI MS $[M + H]^+$ for $C_{23}H_{30}N_5O_8P$, calcd 536.2, found 536.3.

*({[(2R,3S,4R,5R)-5-[6-(Cyclopentylamino)-2-[(pyrrolidin-3-yloxy)-methyl]-9H-purin-9-yl]-3,4-dihydroxyoxolan-2-yl]methoxy}-methyl)phosphonic Acid (**3e**).* To a vial containing 1-boc-3-hydroxypyrrolidine (107 mg, 0.57 mmol) in DMF (1.5 mL) was added sodium hydride (60 wt % oil dispersion, 23 mg, 0.57 mmol). After 2 min, compound **23** (65 mg, 0.11 mmol) in DMF (500 μL) was added and the reaction was stirred overnight at rt. The reaction was quenched with water, diluted with EtOAc, and washed with water and brine. The organics were dried over $MgSO_4$ and concentrated under reduced pressure. The crude product **24** was used directly in the following step without further purification. ESI MS $[M + H]^+$ for $C_{33}H_{53}N_6O_{10}P$, calcd 724.4, found 725.4.

All of the crude product **24** was dissolved in anhydrous CH_3CN (3.0 mL), TMS-Br (0.3 mL) was added, and the reaction was stirred at rt for 1.5 h. It was quenched with H_2O (0.3 mL) and stirred at rt for 4 h or until LCMS analysis showed complete cleavage of the acetonide and BOC protecting groups. The reaction mixture was evaporated and purified by reverse-phase HPLC (C18 column, 0−30% gradient of acetonitrile and water with 0.1% TFA) to give the product **3e** as a white solid in 15% yield (9 mg, two steps): ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.00 (s, 1H), 8.82 (s, 1H), 5.91 (d, J = 6.1 Hz, 1H), 4.62 (t, J = 5.6 Hz, 1H), 4.54−4.41 (m, 4H), 4.16 (t, J = 4.1 Hz, 1H), 4.04 (q, J = 4.0 Hz, 1H), 3.78−3.67 (m, 2H), 3.61 (d, J = 8.8 Hz, 2H), 3.40−3.13 (m, 5H), 2.20−2.09 (m, 1H), 2.04−1.88 (m, 4H), 1.66−1.47 (m, 4H). ESI MS $[M - H]^-$ for $C_{21}H_{33}N_6O_8P$, calcd 527.2, found 527.4.

*({[(2R,3S,4R,5R)-5-[6-Chloro-4-(cyclopentylamino)-1H-pyrazolo-[3,4-d]pyrimidin-1-yl]-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)-phosphonic Acid (**4a**).* The mixture of 4,6-dichloro-1*H*-pyrazolo[3,4-*d*]pyrimidine **25** (47.7 g, 252.4 mmol) and $(NH_4)_2SO_4$ (300 mg) in HMDS (200 mL) was stirred under reflux for 5 h and then concentrated under a vacuum to give a dark-brown oil that was immediately used in the next step. The crude TMS-protected heterocycle was dissolved in anhydrous CH_3CN (500 mL), and 1,2,3,5-tetraacetate-β-D-ribofuranose (88.3 g, 277.6 mmol) was added. The reaction mixture was stirred at rt until all starting materials dissolved, and then, TfO-TMS (68.3 mL, 378.6 mmol) was added dropwise. It was stirred at rt overnight, concentrated under a vacuum to ∼50% of the original volume, and then carefully quenched with saturated $NaHCO_3$ (1 L) and extracted with EtOAc (3 × 500 mL). Combined organics were dried over $MgSO_4$, filtered, and evaporated to give a dark-brown oil that was purified by column chromatography (SiO_2, hexane/EtOAc, 100−40%) to give **27** (yellow oil, 28.5 g, 25%). ESI MS $[M + Na]^+$ for $C_{16}H_{16}Cl_2N_4NaO_7$, calcd 469.0, found 469.0.

Compound **27** (22 g, 49.3 mmol) was dissolved in MeOH (100 mL) and cooled to 0 °C. Cyclopentylamine (5.1 g, 51.8 mmol) and triethylamine (7.2 mL, 51.8 mmol) were added, and the reaction mixture was stirred at 0 °C for 15 min and then at rt for 4 h. Then, 7 M NH_3 in MeOH (60 mL) was added and the reaction was stirred at rt for 1 day. The reaction mixture was evaporated, and the crude product **29** was used in the next step without purification. ESI MS $[M + H]^+$ for $C_{15}H_{21}ClN_5O_4$, calcd 370.1, found 370.2.

The crude product **29** (10.5 g, 28.4 mmol) was dissolved in acetone (50 mL), and 2,2-dimethoxypropane (50 mL) and p-TsOH·H_2O (6.7 g, 35.5 mmol) were added. The reaction mixture was stirred at rt overnight, diluted with brine (100 mL), and carefully quenched with saturated $NaHCO_3$ (100 mL). After extraction with EtOAc (2 × 100 mL), combined organics were dried over $MgSO_4$, filtered, and evaporated to give the crude product **30** (6.2 g), which was used in the next step without purification. ESI MS $[M + H]^+$ for $C_{18}H_{25}ClN_5O_4$, calcd 410.2, found 410.2.

The crude compound **30** (3.2 g, 7.8 mmol) in anhydrous THF (50 mL) was cooled to 0 °C, and 1 M NaHMDS in THF (12.4 mL, 12.4 mmol) was added dropwise. The reaction mixture was stirred at 0 °C for 1 h; then, (diethoxyphosphoryl)methyl trifluoromethanesulfonate (3.4 g, 12.4 mmol) was added. The cooling bath was removed, and the reaction was stirred at rt overnight. It was quenched with a saturated solution of NH_4Cl (50 mL) and diluted with MTBE (100 mL). The organic layer was separated, dried over $MgSO_4$, filtered, and evaporated. The crude product was purified by column chromatography (SiO_2, Hex → Hex:EtOAc, 2:8) to give compound **31** as a white foamy solid (3.2 g, 73%). ESI MS $[M + H]^+$ for $C_{23}H_{36}ClN_5O_7P$, calcd 560.2, found 560.3.

Product **31** (3.2 g, 5.7 mmol) was dissolved in anhydrous CH_3CN (30 mL), TMS-Br (1.6 mL) was added, and the reaction was stirred at rt overnight. The solvent was evaporated to obtain a white foamy solid. To a portion of the white solid (250 mg) were added 4 mL of MeOH and 2 mL of 1 N HCl. The reaction mixture was stirred for 8 h at rt, after which the solvent was evaporated and purified by reverse-phase HPLC (C18 column, 0−30% gradient of acetonitrile and water with 0.1% TFA) to give the product **4a** as a white solid in 93% yield (267 mg): ^{1}H NMR (400 MHz, DMSO-d_6) 8.71 (d, J = 7.2 Hz, 1H), 8.25 (s, 1H), 6.00 (d, J = 3.9 Hz, 1H), 4.51−4.37 (m, 2H), 4.19 (t, J = 5.2 Hz, 1H), 4.07−3.98 (m, 1H), 3.71 (dd, J = 10.7, 4.0 Hz, 1H), 3.59−3.48 (m, 3H), 2.06−1.93 (m, 3H), 1.79−1.47 (m, 5H). ESI MS $[M + H]^+$ for $C_{16}H_{24}ClN_5O_7P$, calcd 464.1, found 464.2.

*({[(2R,3S,4R,5R)-5-(6-Chloro-4-{[(1S)-1-phenylethyl]amino}-1H-pyrazolo[3,4-d]pyrimidin-1-yl)-3,4-dihydroxyoxolan-2-yl]-methoxy}methyl)phosphonic Acid (**5a**).* Compound **5a** was synthesized in a similar fashion to example **4a** as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.49 (s, 1H), 7.55−7.09 (m, 4H), 5.97 (d, J = 5.8 Hz, 1H), 4.64 (t, J = 5.4 Hz, 1H), 4.32−4.08 (m, 1H), 4.05 (app. q, J = 3.9 Hz, 1H), 3.75 (dd, J = 10.7, 3.6 Hz, 1H), 3.68 (dd, J = 10.7, 4.6 Hz, 1H), 3.60 (dd, J = 8.9, 1.9 Hz, 2H), 1.97 (br. s, 3H), 1.80−1.45 (m, 8H). ESI MS $[M - H]^-$ for $C_{19}H_{23}ClN_5O_7P$, calcd 498.1, found 498.3.

*({[(2R,3S,4R,5R)-5-(6-Chloro-4-{[(1S)-1-(2-fluorophenyl)ethyl]-amino}-1H-pyrazolo[3,4-d]pyrimidin-1-yl)-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)phosphonic Acid (**5b**).* Compound **5b** was synthesized in a similar fashion to example **4a** as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.21 (t, J = 6.6 Hz, 1H), 8.32 (dt, J = 4.7, 1.0 Hz, 1H), 7.43 (t, J = 7.8 Hz, 1H), 7.36−7.23 (m, 1H), 7.22−7.12 (m, 2H), 6.03−5.92 (m, 1H), 5.58 (q, J = 7.0 Hz, 1H), 4.44 (t, J = 4.5 Hz, 1H), 4.16 (d, J = 5.6 Hz, 1H), 4.05−3.93 (m, 1H), 3.69 (dd, J = 10.8, 4.0 Hz, 1H), 3.52 (t, J = 8.2 Hz, 2H), 1.53 (d, J = 6.9 Hz, 3H). ESI MS $[M + H]^+$ for $C_{19}H_{22}ClFN_5O_7P$, calcd 518.1, found 518.1.

({[(2R,3S,4R,5R)-5-(6-Chloro-4-{[(1S)-1-(3-fluorophenyl)ethyl]-amino}-1H-pyrazolo[3,4-d]pyrimidin-1-yl)-3,4-dihydroxyoxolan-2-

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*yl]methoxy}methyl)phosphonic Acid (**5c**).* Compound **5c** was synthesized in a similar fashion to example **4a** as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.16 (d, J = 7.8 Hz, 1H), 8.35−8.21 (m, 1H), 7.50−7.28 (m, 1H), 7.22 (d, J = 8.7 Hz, 2H), 7.07 (t, J = 8.7 Hz, 1H), 5.99 (d, J = 4.1 Hz, 1H), 5.41 (t, J = 7.2 Hz, 1H), 4.44 (t, J = 4.4 Hz, 1H), 4.17 (t, J = 5.1 Hz, 1H), 3.99 (s, 1H), 3.69 (dd, J = 10.7, 3.9 Hz, 1H), 3.51 (d, J = 8.7 Hz, 2H), 1.53 (d, J = 6.8 Hz, 3H). ESI MS [M + H]$^+$ for $C_{19}H_{22}ClFN_5O_7P$, calcd 518.1, found 518.1.

*({[(2R,3S,4R,5R)-5-(6-Chloro-4-{[(1S)-1-(4-fluorophenyl)ethyl]-amino}-1H-pyrazolo[3,4-d]pyrimidin-1-yl)-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)phosphonic Acid (**5d**).* Compound **5d** was synthesized in a similar fashion to example **4a** as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.14 (d, J = 7.8 Hz, 1H), 8.29 (s, 1H), 7.42 (t, J = 6.8 Hz, 2H), 7.22−7.05 (m, 2H), 5.98 (d, J = 4.1 Hz, 1H), 5.48−5.31 (m, 1H), 4.44 (t, J = 4.6 Hz, 1H), 4.17 (t, J = 5.1 Hz, 1H), 3.99 (d, J = 5.9 Hz, 1H), 3.69 (dd, J = 10.9, 4.0 Hz, 1H), 3.51 (d, J = 8.6 Hz, 2H), 1.57−1.48 (m, 3H). ESI MS [M + H]$^+$ for $C_{19}H_{22}ClFN_5O_7P$, calcd 518.1, found 518.1

*({[(2R,3S,4R,5R)-5-[6-Chloro-4-(cyclopentylamino)-1H-pyrazolo-[3,4-b]pyridin-1-yl]-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)-phosphonic Acid (**4b**).* A mixture of 4,6-dichloro-1*H*-pyrazolo[3,4-*b*]pyridine **26** (9.5 g, 50.5 mmol) and $(NH_4)_2SO_4$ (100 mg) in HMDS (50 mL) was stirred under reflux for 5 h and then concentrated under a vacuum to give a dark-brown oil that was immediately used in the next step. The crude TMS-protected heterocycle was dissolved in anhydrous CH_3CN (100 mL), and 1,2,3,5-tetraacetate-β-D-ribofuranose (17.6 g, 55.5 mmol) was added. The reaction mixture was stirred at rt until all starting materials dissolved; then, TfO-TMS (13.7 mL, 75.7 mmol) was added dropwise. It was stirred at rt overnight, concentrated under a vacuum to ∼50% of the original volume, and then carefully quenched with saturated $NaHCO_3$ (300 mL) and extracted with EtOAc (3 × 100 mL). Combined organics were dried over $MgSO_4$, filtered, and evaporated to give a dark-brown oil that was purified by column chromatography (SiO_2, hexane/EtOAc, 100−40%) to give **28** (yellow oil, 7.2 g, 32%). ESI MS [M + Na]$^+$ for $C_{17}H_{17}Cl_2N_3NaO_7$, calcd 468.0, found 468.0.

Compound **28** (3.1 g, 6.9 mmol) was dissolved in MeOH (10 mL) and cooled to 0 °C. Then, 7 M NH_3 in MeOH (5 mL) was added and the reaction was stirred at rt for 1 day. The reaction mixture was evaporated, the crude material was redissolved in acetone (10 mL), and 2,2-dimethoxypropane (10 mL) and p-TsOH·H_2O (1.63 g, 8.6 mmol) were added. The reaction mixture was stirred at rt overnight, diluted with brine (20 mL), and carefully quenched with saturated $NaHCO_3$ (30 mL). After extraction with EtOAc (2 × 50 mL), combined organics were dried over $MgSO_4$, filtered, and evaporated. The crude material was purified by column chromatography (SiO_2, hexane/EtOAc, 1:1) to give **32** (white solid, 2.0 g, 80%, overall). ESI MS [M + H]$^+$ for $C_{14}H_{16}Cl_2N_3O_4$, calcd 360.1, found 360.1.

A solution of compound **32** (2.7 g, 7.5 mmol) in anhydrous THF (20 mL) was cooled to 0 °C, and 1 M LiHMDS in THF (9.0 mL, 9.0 mmol) was added dropwise. The reaction mixture was stirred at −78 °C for 1 h; then, (diethoxyphosphoryl)methyl trifluoromethanesulfonate (2.4 g, 9.0 mmol) was added. The cooling bath was removed, and the reaction was stirred at rt overnight. It was quenched with a saturated solution of NH_4Cl (6.0 mL) and diluted with MTBE (20 mL). The organic layer was separated, dried over $MgSO_4$, filtered, and evaporated. The crude product was purified by column chromatography (SiO_2, Hex → Hex:EtOAc, 2:8) to give compound **33** as a white foamy solid (2.0 g, 59%). ESI MS [M + H]$^+$ for $C_{19}H_{27}Cl_2N_3O_7P$, calcd 510.1, found 510.0.

In a glass vial, compound **33** (400 mg, 0.78 mmol) was dissolved in EtOH (1.0 mL). To this solution were added cyclopentylamine (115 μL, 1.17 mmol) and triethylamine (163 μL, 1.17 mmol). The reaction mixture was heated to reflux in the sealed vial for 16 h. Volatiles were removed under reduced pressure, and the crude mass was redissolved in anhydrous CH_3CN (5.0 mL), followed by addition of TMS-Br (0.5 mL). The reaction mixture was stirred at rt overnight before quenching with H_2O (1 mL) and stirring for an additional 4 h at rt or until LCMS analysis showed complete cleavage of the acetonide protecting group. The reaction mixture was evaporated and purified by reverse-phase HPLC (C18 column, 0−30% gradient of acetonitrile and water with 0.1% TFA) to give the product **4b** as a white solid (200 mg; 48%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.26 (s, 1H), 7.65 (d, J = 6.7 Hz, 1H), 6.22 (s, 1H), 6.06 (d, J = 3.9 Hz, 1H), 4.47 (t, J = 4.5 Hz, 1H), 4.17 (t, J = 5.2 Hz, 1H), 4.02−3.92 (m, 2H), 3.69 (dd, J = 10.9, 3.9 Hz, 1H), 3.50 (d, J = 8.6 Hz, 3H), 2.06−1.88 (m, 2H), 1.75−1.47 (m, 6H). ESI MS [M + H]$^+$ for $C_{17}H_{25}ClN_4O_7P$, calcd 463.1, found 463.2.

*({[(2R,3S,4R,5R)-5-(6-Chloro-4-{[(1S)-1-phenylethyl]amino}-1H-pyrazolo[3,4-b]pyridin-1-yl)-3,4-dihydroxyoxolan-2-yl]methoxy}-methyl)phosphonic Acid (**5e**).* Compound **5e** was synthesized in a similar fashion to example **4b** as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.37 (s, 1H), 8.19 (d, J = 7.2 Hz, 1H), 7.39 (d, J = 8.1 Hz, 2H), 7.32 (t, J = 7.6 Hz, 2H), 7.24−7.18 (m, 1H), 6.04 (d, J = 4.1 Hz, 1H), 6.02−5.92 (m, 1H), 4.85 (s, 1H), 4.46 (t, J = 4.6 Hz, 1H), 4.17 (t, J = 5.4 Hz, 1H), 3.96 (t, J = 5.5 Hz, 1H), 3.67 (td, J = 13.5, 12.1, 7.3 Hz, 1H), 3.51 (d, J = 8.3 Hz, 2H), 1.52 (d, J = 6.7 Hz, 3H). ESI MS [M + H]$^+$ for $C_{20}H_{24}ClN_4O_7P$, calcd 499.9, found 499.8.

*({[(2R,3S,4R,5R)-5-(6-Chloro-4-{[(1S)-1-(2-fluorophenyl)ethyl]-amino}-1H-pyrazolo[3,4-b]pyridin-1-yl)-3,4-dihydroxyoxolan-2-yl]-methoxy}methyl)phosphonic Acid (**5f**).* Compound **5f** was synthesized in a similar fashion to example **4b** as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.37 (s, 1H), 8.23 (d, J = 7.1 Hz, 1H), 7.44−7.34 (m, 1H), 7.34−7.26 (m, 1H), 7.26−7.09 (m, 2H), 6.05 (d, J = 4.0 Hz, 1H), 6.00−5.73 (m, 1H), 5.04 (s, 1H), 4.53−4.40 (m, 1H), 4.17 (t, J = 5.2 Hz, 1H), 3.98 (dt, J = 6.4, 4.5 Hz, 1H), 3.69 (dd, J = 10.8, 4.0 Hz, 1H), 3.51 (t, J = 7.4 Hz, 3H), 1.56 (d, J = 6.7 Hz, 3H). ESI MS [M + H]$^+$ for $C_{20}H_{23}ClFN_4O_7P$, calcd 517.1, found 517.1.

*({[(2R,3S,4R,5R)-5-(6-Chloro-4-{[(1S)-1-(3-fluorophenyl)ethyl]-amino}-1H-pyrazolo[3,4-b]pyridin-1-yl)-3,4-dihydroxyoxolan-2-yl]-methoxy}methyl)phosphonic Acid (**5g**).* Compound **5g** was synthesized in a similar fashion to example **4b** as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.35 (s, 1H), 8.18 (d, J = 7.3 Hz, 1H), 7.42−7.31 (m, 1H), 7.31−7.18 (m, 2H), 7.10−6.99 (m, 1H), 6.04 (d, J = 4.0 Hz, 1H), 4.91 (s, 1H), 4.51−4.41 (m, 1H), 4.17 (t, J = 5.3 Hz, 1H), 4.04−3.90 (m, 1H), 3.69 (dd, J = 10.8, 4.0 Hz, 1H), 3.51 (d, J = 8.3 Hz, 3H), 1.57−1.46 (m, 3H). ESI MS [M + H]$^+$ for $C_{20}H_{23}ClFN_4O_7P$, calcd 517.1, found 517.1.

*({[(2R,3S,4R,5R)-5-(6-Chloro-4-{[(1S)-1-(4-fluorophenyl)ethyl]-amino}-1H-pyrazolo[3,4-b]pyridin-1-yl)-3,4-dihydroxyoxolan-2-yl]-methoxy}methyl)phosphonic Acid (**5h**).* Compound **5h** was synthesized in a similar fashion to example **4b** as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.35 (s, 1H), 8.17 (d, J = 7.2 Hz, 1H), 7.43 (ddd, J = 8.8, 5.5, 1.5 Hz, 2H), 7.14 (td, J = 8.8, 1.4 Hz, 2H), 6.04 (d, J = 4.1 Hz, 2H), 4.89 (s, 1H), 4.52−4.40 (m, 1H), 4.17 (t, J = 5.2 Hz, 1H), 3.97 (dt, J = 6.3, 4.4 Hz, 1H), 3.68 (dd, J = 10.8, 4.0 Hz, 1H), 3.60−3.38 (m, 3H), 1.50 (d, J = 6.5 Hz, 3H). ESI MS [M + H]$^+$ for $C_{20}H_{23}ClFN_4O_7P$, calcd 517.1, found 517.1.

*({[(2R,3S,4R,5R)-5-[5-Chloro-7-(cyclopentylamino)-3H-imidazo-[4,5-b]pyridin-3-yl]-3,4-dihydroxyoxolan-2-yl]methoxy}methyl)-phosphonic Acid (**4c**).* To a solution of 5,7-dichloroimidazo[4,5-*b*]pyridine **34** (376 mg, 2 mmol) in MeCN (14 mL) at rt was added *N*,*O*-bis(trimethylsilyl)acetamide (0.523 mL, 2.14 mmol) dropwise, and the reaction mixture was heated to 85 °C for 1 h. The mixture was cooled to rt, and a solution of β-D-ribofuranose 1,2,3,5-tetraacetate (726 mg, 2.28 mmol) in MeCN (7 mL) and trimethylsilyl trifluoromethanesulfonate (0.471 mL, 2.60 mmol) were added sequentially dropwise. The reaction mixture was heated to 85 °C for 4 h. After cooling to rt, aqueous saturated sodium bicarbonate (50 mL) was added, subsequently extracted three times with EtOAc (100 mL), dried over sodium sulfate, and concentrated. The crude material **35** thus obtained was redissolved in dioxane (2 mL), and cyclopentylamine (0.987 mL, 10 mmol) was added. The mixture was heated to 100 °C for 16 h. The reaction mixture was then directly loaded onto silica gel and purified by silica gel chromatography (0−10% MeOH in DCM) to afford the desired product **36** as a brown solid (298 mg, 40%, two steps). ESI MS [M + H]$^+$ for $C_{16}H_{22}ClN_4O_4$, calcd 369.1, found 369.1.

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A solution of the compound **36** (298 mg; 0.808 mmol) and *p*-toluenesulfonic acid monohydrate (154 mg, 0.808 mmol) in 2,2-dimethoxypropane (1.6 mL) and acetone (1.6 mL) was stirred at rt for 20 h. Triethylamine (0.5 mL) was added, the solvent was removed, and the residue was purified by silica gel chromatography (50−100% EtOAc in hexanes) to afford the desired product **37** as a white solid (258 mg; 78%). ESI MS $[M + H]^+$ for $C_{19}H_{26}ClN_4O_4$, calcd 409.2, found 409.2.

Compound **37** (126 mg, 0.308 mmol) in anhydrous THF (1.5 mL) was cooled to 0 °C, and 1 M NaHMDS in THF (0.43 mL, 0.43 mmol) was added dropwise. The reaction mixture was stirred at 0 °C for 1 h; then, (diethoxyphosphoryl)methyl trifluoromethanesulfonate (139 mg, 0.47 mmol) was added. The cooling bath was removed, and the reaction was stirred at rt overnight. It was quenched with a saturated solution of NH_4Cl (1.0 mL) and diluted with MTBE (100 mL). The organic layer was separated, dried over $MgSO_4$, filtered, and evaporated. The crude product was purified by column chromatography to obtain **38** as a white solid (107 mg; 62%). ESI MS $[M + H]^+$ for $C_{24}H_{37}ClN_4O_7P$, calcd 559.9, found 559.9.

Compound **38** (107 mg, 0.19 mmol) was dissolved in anhydrous CH_3CN (0.46 mL), TMS-Br (0.25 mL) was added, and the reaction was stirred at rt overnight. It was quenched with H_2O (1 mL) and stirred at rt for 4 h or until LCMS analysis showed complete cleavage of the acetonide protecting group. The reaction mixture was evaporated and purified by reverse-phase HPLC (C18 column, 0−30% gradient of acetonitrile and water with 0.1% TFA) to give the product **4c** as a white solid (14 mg; 15%). ^1H NMR (400 MHz, DMSO-d_6) δ 8.47 (s, 1H), 7.12 (d, J = 7.4 Hz, 1H), 6.41 (s, 1H), 5.90 (d, J = 5.8 Hz, 1H), 4.68−4.45 (m, 1H), 4.16−4.10 (m, 1H), 4.03 (q, J = 3.9 Hz, 1H), 3.78−3.65 (m, 2H), 3.61 (d, J = 8.9 Hz, 2H), 2.05−1.89 (m, 2H), 1.77−1.63 (m, 2H), 1.63−1.47 (m, 4H). ESI MS $[M - H]^-$ for $C_{17}H_{23}ClN_4O_7P$, calcd 461.8, found 461.2.

Generation and Expansion of the Human CD73 Stable CHO Cell Line. Stable cell lines were generated by Lake Pharma (Belmont, CA) using a standard protocol to transform CHO cells with a pcDNA3.1(+) vector carrying the human NT5E (CD73) gene. Antibiotic selection was performed in CD OptiCHO cell media containing 5 μg/mL Puromycin and 200 μg/mL Hygromycin B. Pools of surviving CHO-CD73 were collected and frozen in 7.5% dimethyl sulfoxide (DMSO) in CD OptiCHO cell media. Cryopreserved cells were defrosted in a water bath at 37 °C by agitating the vial until the cells were completely thawed. Cells were then transferred to a 15 mL Falcon tube prior to centrifuging at 225*g* for 5 min to pellet the cells. The cell pellet was resuspended in a fresh warm CD OptiCHO Growth Medium supplemented with 2 mM Glutamax and transferred to a T175 flask. After two days and on reaching ∼80% confluence (∼20 million cells/flask), cells were split 1:3 into three fresh T175 flasks. After a further three days, cells were transferred to 15 mL Falcon tubes and centrifuged at 250*g* for 5 min to pellet. Cells were resuspended at a density of 3 million cells/mL in CellBanker2 cryopreservation media and aliquoted into cryogenic vials. Cell aliquots were stored at −80 °C until needed.

CD73 CHO Cellular Assay. On the day of the experiment, one vial of CHO-CD73 cells was thawed and cells were resuspended in 10 mL of assay buffer consisting of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 4.2 mM $NaHCO_3$, and 0.1% glucose. A 16-point, 1:3 master serial dilution of the test compound was prepared in 100% DMSO to span a final concentration range of 100−0 μM or 0.1−0 μM. Twenty microliters of assay buffer was added to each well of a 96-well round-bottomed polypropylene plate followed by addition of 10 or 1 μL of the test compound at 10-fold or 100-fold final concentration. Fifty or thirty microliters of cells was added to each well, and the plate was incubated for a further 60 min at 37 °C prior to addition of 20 μL of 125 μM AMP or 49 μL of 51 μM AMP in an assay buffer. Final assay conditions comprised 1500 cells per well in 10 or 2% DMSO and 25 μM AMP substrate. After a further 60 min of incubation at 37 °C and 5% CO_2, the plate was centrifuged at 225*g* for 5 min. Eighty microliters of supernatant was transferred to a 96-well Spectra Plate, the wells of which had been preloaded with 20 μL of the PiColorLock Gold colorimetric assay reagent (Cat. No. 30 300 30, Thermo Fisher). The inorganic phosphate reaction product was assessed as a function of color developed by reaction with the PiColorLock Gold reagent and quantitated using an Envision 2102 Multilabel Reader fitted with a 620 nm filter. The CD73 enzymatic activity was evaluated as a correlate of phosphate product levels. Percentage maximum activity in each test well was calculated based on DMSO (maximum activity) and wells not containing cells (baseline activity). The IC_{50} values of the test compounds were determined from the compound's dose−response curve fitted using a standard four-parameter fit equation.

CD39 CHO Cellular Assay. CD39 stable cell lines were generated by Lake Pharma (Belmont, CA) using the same protocol as the generation of CD73 stable cells. The CD39 CHO cellular assay was carried out using the same protocol as the CD73 CHO cellular assay but with modification to cell number, substrate concentration, and compound preincubation time. Final assay conditions comprised 500 cells per well in 2% DMSO and 15 μM ATP substrate with 4 h of compound preincubation.

Preparation of Chinese Hamster Ovary (CHO) K1 Cells Transiently Transfected with NTPDase2, NTPDase3, or NTPDase8. One day prior to the assay, 5 $\times 10^6$ CHO-K1 cells (ATCC, #CCL-61) were seeded in 11 mL of cell media (Hams F-12, (Fisher, 11765047), supplemented with 10% FBS (Gibco, Cat. No. A3160502), and 1% GlutaMax (Invitrogen, Cat. No. 35050-061)) in T75 flasks (Corning, 430641U). Then, 10 μL of 100 ng/μL NTPDase2, NTPDase3, or NTPDase8 plasmid DNA was added to 2 mL of serum-free Opti-MEM media (Fisher, 31985062). The diluted plasmid was combined with 2 mL of serum-free Opti-MEM media containing 100 μL of the Lipofectamine LTX Reagent (Fisher, #15338030). The final DNA/LTX mixture was incubated for 30 min at ∼25 °C and added to the T75 flask containing CHO-K1 cells. The cells were incubated at 37 °C and 5% CO_2 overnight. The following day, media were removed by gentle aspiration and cells were washed with 10 mL of phosphate-buffered saline (PBS, Gibco, 14190250) twice. The cells were harvested by adding 2 mL of Accutase (Fisher, #00-4555-56) to the flask. The flask was then incubated at 37 °C until the cells detached from the flask when lightly tapped by hand. Next, 8 mL of PBS was added to the flask to dislodge cells and then the cells were transferred to a 15 mL conical centrifuge tube (Falcon, 339650) and centrifuged at 225*g* for 5 min. PBS was removed by aspiration, and the cells were suspended in 5 mL of an assay buffer consisting of 20 mM HEPES, pH 7.4, 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 4.2 mM $NaHCO_3$, and 0.1% glucose. Cell density was determined using ViaCell XR (Becman Coulter). The cells were then diluted to 0.25 $\times$ 10^6 cells/mL.

NTPDase2, NTPDase3, or NTPDase8 Cellular Assays. A 16-point, 1:3 master serial dilution of the test compound was prepared in 100% DMSO to span a final concentration range of 10−0 μM. Then, 18 μL of an assay buffer was added to each well of a 96-well polypropylene plate (Corning, Cat no. 3365) followed by addition of 2 μL of the test compound at 50-fold final concentration. Then, 40 μL of NTPDase2, 3, or 8 cells in an assay buffer was added to each well and the plate was incubated for a further 1 h at 37 °C prior to addition of 40 μL of 62.5 μM (NTPDase2), 40 μL of 37.5 μM (NTPDase3), 40 μL of 75 μM (NTPdase8), and adenosine-5′-triphosphate monohydrate (ATP) (Sigma, Cat. No. A26209-5G) in the assay buffer. Final assay conditions comprised 10 000 cells per well in 2% DMSO and 25/15/30 μM ATP substrate. After a further 50 min of incubation at 37 °C and 5% CO_2, the plate was centrifuged at 225*g* for 5 min. Then, 80 μL of the supernatant was transferred to a 96-well Spectra Plate (Perkin Elmer, Cat. No. 6005640), the wells of which had been preloaded with 20 μL of PiColorLock Gold colorimetric assay reagent (Thermo Fisher, Cat. No. 30 300 30). The inorganic phosphate reaction product was quantitated as a function of color developed by reaction with PiColorLock Gold reagent and determined using an Envision 2102 Multilabel Reader (Perkin Elmer) fitted with a 620 nm filter. NTPDase2, 3, or 8 activity was assessed as a correlate of phosphate product levels. Percentage maximum activity in each of the compound test wells was calculated

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based on DMSO (maximum activity) and no cell control wells (baseline activity). The IC_{50} values of the test compounds were determined using Prism software (Graphpad Software Inc., version 7.02, La Jolla, CA 92037), and the compound's dose−response curve was fitted using a standard four-parameter fit equation.

$A2_aR$ Assay. The compound inhibition constant (IC_{50}) was determined as a function of cellular cAMP levels in NECA-stimulated CHO cells stably transfected with adenosine $2A_a$ receptor. Clonal CHO cells stably transfected with *ADORA2A* and induced with tetracycline were seeded into 384-well OptiPlates at a density of between 2000 cells per well and preincubated with 20-point, 1:2 serial dilution of the compound to span a final concentration range of 10−0 μM. A 30 nM fixed concentration of NECA was prepared and added to the wells to generate a full serial dilution of NECA against each of the five compound concentrations. Cells were incubated for a further 30 min at 37 °C. The reaction was quenched by addition of 5 μL of Ulight-anti-cAMP diluted 1:150 with a conjugate and lysis buffer. Five microliters of Eu-cAMP tracer diluted 1:50 dilution in the conjugate and lysis buffer was then added to each well, and the plate was incubated for an additional 60 min at 37 °C. Cellular cAMP levels were then quantitated as a function of the fluorescence resonance energy transfer (FRET) signal measured using an Envision 2102 Multilabel Reader (Perkin Elmer) fitted with 615 nm excitation and 665 nm emission filters. The IC_{50} potency was performed using Prism software (Graphpad Software Inc., version 7.02, La Jolla, CA 92037) to calculate the compound IC_{50}.

Pharmacokinetic Studies. All animal procedures were conducted under protocols approved by the Arcus Institutional Animal Care and Use Committee (IACUC). Male rats, weighing between 0.27 and 0.30 kg, were purchased from Charles River Laboratories (Hollister, CA) and were acclimated before use. Test articles were dissolved in 31.6% DMA, 36.8% ethanol, and 31.6% PEG and administered intravenously via the femoral vein at 0.25 or 0.5 mg/kg. Blood was sampled via the jugular vein at predose, 5, 15, and 30 min, 1, 2, 4, 6, 8, and 24 h post dose. Blood samples were placed in polypropylene tubes containing K2-EDTA as the anticoagulant and centrifuged at 8000 rpm and 4 °C for 7 min to obtain plasma, which were stored at −80 °C until analysis. Plasma samples were prepared by protein precipitation and analyzed by LCMS/MS (Shimadzu Nexera X2 UHPLC and API4000 mass spectrometer). Pharmacokinetic analysis was performed using Phoenix WinNonlin v6.4.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.0c01835.

Molecular formula strings (CSV)

Hepatocyte stability assay, CYP inhibition assay, and crystallographic methods (PDF)

Accession Codes

Atomic coordinates have been deposited in the Protein Data Bank (PDB: 6YE1 for **2n** and PDB: 6YE2 for **4a**). Structures will be released upon article publication.

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Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

SAR, structure−activity relationship; GPI, glycosylphosphatidylinositol; hCD73, human cluster of differentiation 73; CYP, cytochrome P450; PDB, protein data bank; hNTPDases, human nucleoside triphosphate diphosphohydrolases; POM, pivaloyloxymethyl; POC, isopropyloxycarbonyloxymethyl; HDP, hexadecyloxypropyl; DMA, dimethylacetamide; PEG, poly(ethylene glycol); DMF, *N*,*N*-dimethylformamide; THF, tetrahydrofuran; *p*TsOH, para-toluenesulfonic acid; HMDS, bis(trimethylsilyl)amine; BSA, *N*,*O*-bis(trimethylsilyl)-acetamide; HPLC, high-performance liquid chromatography; LCMS, liquid chromatography−mass spectrometry; ESI, electrospray ionization; rt, room temperature; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography

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