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Synthesis and Evaluation of Multisubstrate Analogue Inhibitors of Purine Nucleoside Phosphorylases

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Abstract—1,1-Difluoro-2-(tetrahydro-3-furanyl)ethylphosphonic acids (±)-*cis*-**4a** and (±)-*trans*-**4a** possessing a (purine-9-yl)methyl functionality at the ring as well as their homologues (±)-*cis*-**4b** and (±)-*trans*-**4b** were synthesized and tested as 'multi-substrate analogue' inhibitors for purine nucleoside phosphorylases. Radical cyclization of allylic α,α-difluorophosphonates **8a,b** was applied to construct the α,α-difluorophosphonate-functionalized oxacycles **9a,b**. The IC_{50} values of the nucleotide analogues (±)-*cis*-**4a** and (±)-*cis*-**4b** were 88 and 38 nM, respectively, for human erythrocyte PNP-catalyzed phosphorylation of inosine in the presence of 100 mM orthophosphate. The stereochemistry of the inhibitors was found to affect significantly the inhibitory potency. The *trans*-isomers (±)-*trans*-**4a** and (±)-*trans*-**4b** were ca. 4-fold less potent than the corresponding *cis*-isomers. At an intracellular concentration of orthophosphate (1 mM), (±)-*cis*-**4b**, the most potent compound of this series, was shown to have IC_{50} and K_i values of 8.7 and 3.5 nM, respectively.

Introduction

Purine nucleoside phosphorylase (PNP: EC 2.4.2.1) is a ubiquitous enzyme of the purine salvage pathway. It catalyzes the reversible phosphorylation of ribo- and 2′-deoxyribo-nucleosides of guanine and hypoxanthine in higher organisms, as well as of adenine in some prokaryotes (eqn 1).[1] Interest in PNP stems largely from its potential as a drug target. Inhibitors of PNP have been suggested to have therapeutic value in the treatment of T-cell proliferative disease such as T-cell leukemia,[2] since individuals who genetically lack PNP suffer from impairment of their immune system but have normal B-cell function.[3] PNP inhibitors may also prolong the plasma half-lives of some chemotherapeutic agents such as 2′,3′-dideoxyinosine by preventing the PNP-catalyzed inactivation.[4] Consequently, extensive drug-discovery research has been devoted to the design and synthesis of inhibitors of PNP.

$$\beta\text{-purine nucleoside} + \text{orthophosphate} \rightleftarrows \text{purine} + \alpha\text{-D-pentose-1-phosphate} \quad (1)$$

Since PNP accomplishes the reversible phosphorylation of the purine nucleosides via a ternary complex of enzyme, nucleoside, and orthophosphate, compounds that contain in their structure covalently linked elements of both substrates (nucleoside and orthophosphate) are expected to act as a 'multi-substrate analogue' inhibitor for PNP.[5a] Therefore, a number of metabolically stable acyclic nucleotide analogues containing a purine and a phosphate-like moiety have been synthesized.[5]

In the previous paper,[6a–c] we described the synthesis and biological evaluation of a series of novel nucleotide analogues constrained at the χ^2-bond of the acyclic nucleotide analogue **1**, originally developed by Danzin and Halazy as a multi-substrate analogue inhibitor for PNPs.[5g] The study resulted in identification of a novel cyclopropane nucleotide analogue (±)-**3** which showed a better inhibition motif for PNP isolated from *Cellulomonas* sp. rather than from human erythrocytes.[6b] The study also revealed that the hypoxanthine derivative (±)-**3** ($K_i = 8.8$ nM) possesses a higher binding affinity for *Cellulomonas* sp. PNP than the corresponding guanine derivative (±)-**2** ($K_i = 28.2$ nM).[6a,b] However, no significant difference in the inhibition potencies (IC_{50}) of our inhibitors (±)-**2** and (±)-**3** and the Danzin inhibitor **1** was observed for human erythrocyte-derived PNP.

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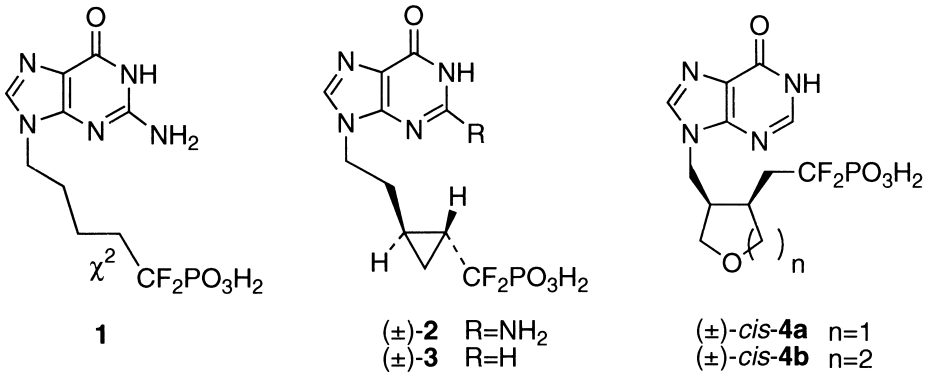


Figure 1. Multi-substrate analogue inhibitors for PNP.

To search for effective motifs to inhibit human PNP, we successively synthesized the novel nucleotide analogue (±)-*cis*-**4a** in which a hypoxanthine and a difluoromethylene phosphonic acid are linked by an alkyl spacer composed of a five-membered oxacycle. The design was based on the hypothesis that the oxacycles incorporated to the alkyl spacer would be interactive with a hydrophobic patch of the ribose-binding pocket in the active site of human PNP in the desired manner.[7,8] We reported that nucleotide analogue (±)-*cis*-**4a** showed good inhibitory potency toward both human erythrocyte PNP and *Cellulomonas* sp. PNP.[8] The *trans*-isomer ((±)-*trans*-**4a**) of (±)-*cis*-**4a** showed modest inhibition profiles for human erythrocyte PNP. Thus, the vicinal sterochemistry of the side-chains of the oxacycle significantly affected the inhibitory potency toward human erythrocyte PNP.[8] These findings prompted us to undertake further biological evaluation of the related nucleotide analogues (±)-*cis*-**4b** and (±)-*trans*-**4b** having the 6-membered oxacycle in the alkyl spacer. In this paper, we describe experimental details for the synthesis and biological evaluation of (±)-*cis*-**4a,b** and (±)-*trans*-**4a,b**.

Results

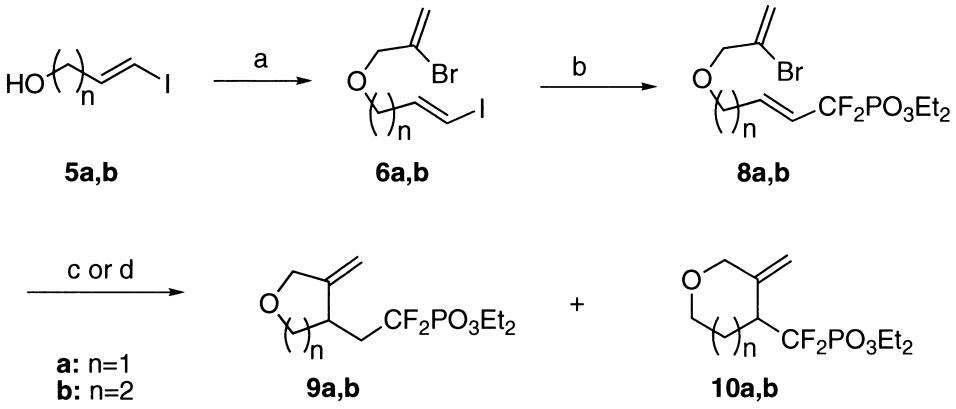
Synthesis of novel nucleotide analogues (±)-*cis*-4a,b and (±)-*trans*-4a,b

Radical cyclization of allylic α,α-difluorophosphonates **7a,b** was applied to construct the suitably functionalized oxacycles **9a,b**, key intermediates for the synthesis of the target nucleotide analogues (±)-*cis*-**4a,b** and (±)-*trans*-**4a,b** (Scheme 1). Treatment of iodoalkenols **5a** and **5b** with 2,3-dibromopropene in aqueous NaOH in the presence of a phase-transfer catalyst (n-$Bu_4N(HSO_4)$) gave the bis-functionalized ethers **6a** and **6b** in 84 and 73% yield. The cross-coupling reaction of **6a** and **6b** with the zinc reagent **7**,[9,10] generated from diethyl bromodifluromethylphosphonate ($BrCF_2PO_3Et_2$) in dimethylacetamide (DMA), in the presence of CuBr under ultrasound irradiation proceeded selectively at the iodo-carbon to give the radical-processors **8a** and **8b** in 87 and 91% yield, respectively. The solvent and sonication were critical for inducing a good yield; the yield of **8a,b** was low (ca. 15%), and a substantial amount of starting material was recovered upon conducting the cross-coupling reaction in dimethylformamide (DMF) without sonication.[10] Radical cyclization of **8a,b** was conducted in the presence of n-Bu_3SnH in refluxing toluene (110 °C) initiated by 2,2′-azobisisobutyronitrile (AIBN). Alternatively, the cyclization was carried out at low temperature (−20 °C) in the presence of n-Bu_3SnH in THF initiated by Et_3B. When **8a** was cyclized by the AIBN-mediated chain-reaction, a mixture of **9a** and **10a** was obtained in 82% yield in a ratio of 88:12. The ratio was significantly improved when the cyclization was conducted by the Et_3B-mediated reaction. This reaction proceeded rapidly (10 min) to give exclusively *5-exo-trig* cyclization product **9a** in 91% yield. In the case of cyclization of the homologous **8b**, both conditions gave the desired 6-*exo-trig* cyclization product **9b** in good to modest yield without formation of **10b**. In this cyclization, the yield for the AIBN-mediated reaction (71%) was much better than that for the Et_3B-mediated reaction (42%).

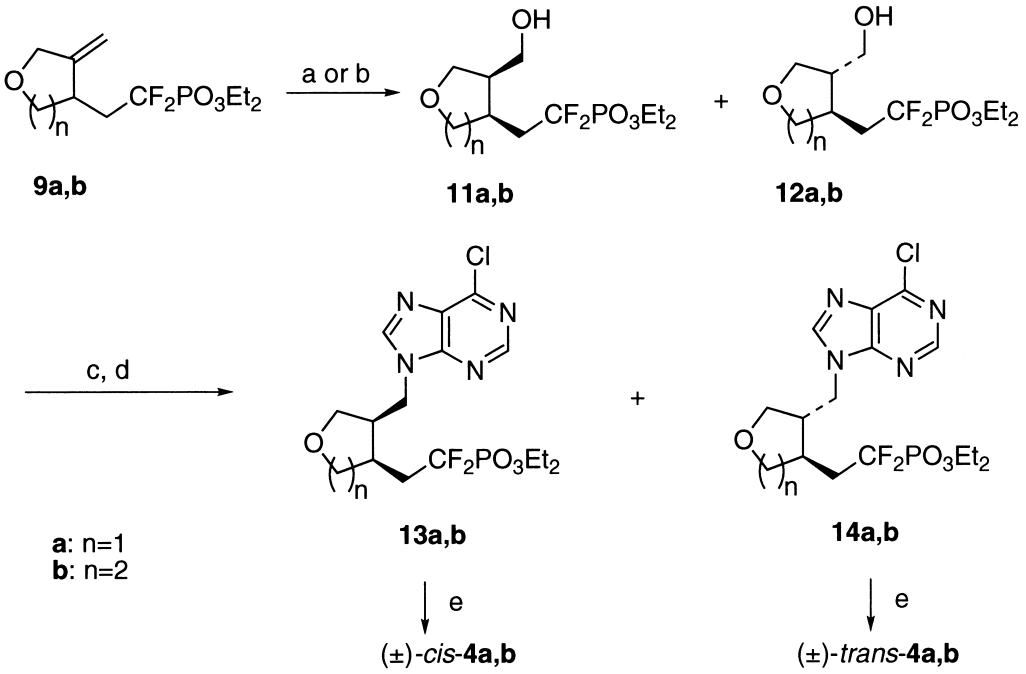
Conversion of **9a,b** to target nucleotide analogues (±)-*cis*-**4a,b** and (±)-*trans*-**4a,b** was accomplished as shown in Schemes 2 and 3. Hydroboration of **9a,b** with a borane–THF complex in THF at 0 °C, followed by oxidative work up (30% H_2O_2, aqueous NaOAc), gave a diastereomeric mixture of alcohols **11a,b** and **12a,b** in favor of the *cis*-stereoisomers (**11a**:**12a** = 64:36, 83% yield; **11b**:**12b** = 72:28, 50% yield). The diastereomeric ratios were slightly improved when the hydroboration was carried out with 9-borabicyclo[3.3.1]nonane (9-BBN) in place of a borane–THF complex (**11a**:**12a** = 73:27, 23% yield; **11b**:**12b** = 84:16, 51% yield).

Practically, the *cis*-enriched alcohols **11a** (*cis*:*trans* = 64:36) and **11b** (*cis*:*trans* = 84:16) were coupled with 6-chloropurine under the Mitsunobu conditions [diethyl azodicarboxylate (DEAD), Ph_3P, THF] to give 6-chloropurine derivatives **13a** and **13b** contaminating the corresponding *trans*-isomers. At this stage, diastereomerically pure *cis*-isomers **13a** and **13b** were isolated through preparative HPLC in 43 and 75% yield, respectively.

The 6-chloropurine derivatives **14a,b** of *trans*-stereochemistry were prepared through epimerization of the hydroxymethyl functional group of **11a,b** (Schemes 2 and 3). Oxidation of *cis*-enriched alcohols **11a** (*cis*:*trans* = 64:27) and **11b** (*cis*:*trans* = 84:16) with Jones reagent, followed by esterification (3% HCl–MeOH),



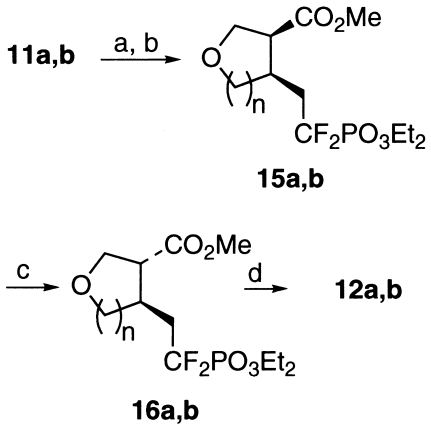
Scheme 1. (a) 2,3-Dibromopropene, aq NaOH, *n*-$Bu_4N(HSO_4)$; (b) $BrZnCF_2PO_3Et_2$ (**7**), CuBr, DMA, ultrasound; (c) *n*-Bu_3SnH, Et_3B, THF, −20 °C; (d) *n*-Bu_3SnH, AIBN, toluene, 110 °C.



Scheme 2. (a) BH_3·THF, THF, 0 °C, then H_2O_2, aq NaOAc; (b) 9-BBN, THF, 0 °C then H_2O_2, aq NaOAc; (c) 6-chloropurine, DEAD, Ph_3P, THF; (d) HPLC-separation, (e)TMSBr, CH_2Cl_2, then H_2O.

gave the corresponding methyl esters **15a** and **15b**. Upon treatment of the methyl esters with Cs_2CO_3 in MeOH at room temperature for 12 h, epimerization took place to give the *trans*-enriched methyl esters **16a** (*cis*:*trans* = 15:85) and **16b** (*cis*:*trans* = 50:50) in 44 and 42% yield. Reduction of the methyl esters with $LiBH_4$ in ether gave the alcohols **12a** and **12b** contaminating the *cis*-isomers, reflecting the diastereomeric ratios of the methyl esters **16a,b** in 74 and 59% yield, respectively. As in the case of preparation of **13a** and **13b**, the alcohols **12a** and **12b** were manipulated to diastereomerically pure 6-chloropurine derivatives **14a** and **14b**, which were respectively isolated in 57 and 34% yield after preparative HPLC separation.

Removal of the ethyl protecting group and hydrolysis of a 6-chloropurine for **13a,b** and **14a,b** were performed by treatment with bromotrimethylsilane (TMSBr) in CH_2Cl_2,



Scheme 3. (a) Jones oxid; (b) 3% HCl, MeOH; (c) Cs_2CO_3, MeOH; (d) $LiBH_4$, ether.

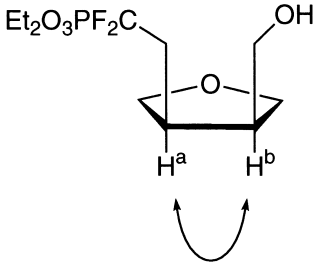
followed by hydrolysis with H_2O in one-pot, to give (±)-*cis*-**4a,b** and (±)-*trans*-**4a,b** as amorphous powders.

The relative stereochemistry of (±)-*cis*-**4a** and(±)-*trans*-**4a** was confirmed at the stage of alcohols **11a** and **12a** by NOESY experiments. Diastereomerically pure alcohols **11a** and **12a** were obtained for the analytical purpose by preparative HPLC separation of the corresponding trityl ethers, followed by deprotection. The representative NOESY correlations (500 MHz, $CDCl_3$) for **11a** and **12a** are depicted in Figure 2. In the NOESY spectrum of **11a** a correlation between the methine protons (H^a and H^b) was observed, whereas there was no correlation from H^b to the α-methylene protons of the hydroxyl group. On the other hand, the NOESY spectrum of **12a** revealed that there were correlations between H^b and the methylene protons α to the hydroxyl group. These results show that the stereochemistry of the major hydroboration product **11a** should be *cis*.

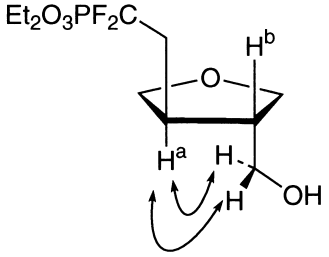
The relative stereochemistry of (±)-*cis*-**4b** and (±)-*trans*-**4b** was ascertained at the stage of 6-chloropurine derivatives **13b** and **14b** through NOESY experiments (500 MHz, $CDCl_3$). The stereochemistry of **13b** was assigned to be *cis* by a diagnostic NOESY correlation between the two methine protons (H^a and H^b) which reside in an axial/equatorial relationship on the tetrahydropyranyl ring, along with the supporting cross-peaks depicted in Figure 3. This assignment was further confirmed by the NOESY experiments with **14b**; no correlation between the two methine protons was observed in the NOESY spectrum.

PNP inhibitory activity of (±)-*cis*-4a,b and (±)-*trans*-4a,b

The inhibitory potencies of (±)-*cis*-**4a,b** and (±)-*trans*-**4a,b** toward human erythrocyte PNP were assessed with

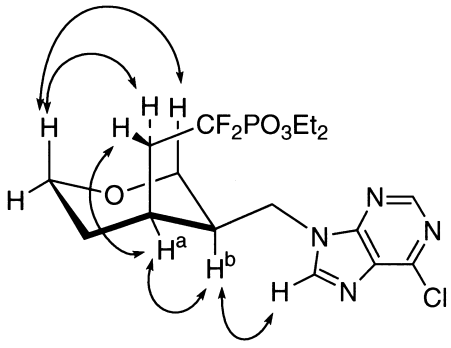


11a



12a

Figure 2. Representative NOESY correlations of **11a** and **12a**.



13b

Figure 3. Representative NOESY correlations of **13b**.

either the IC_{50} or K_i value in the presence of inosine and orthophosphate being fixed at the concentrations of 0.1 and 100 mM, respectively, as described previously.[6,8] The results summarized in Table 1 indicate that *cis*-**4a** with an IC_{50} of 88 nM is about 4-fold more potent than *trans*-**4a** with that of 320 nM. The nucleotide analogue (±)-*cis*-**4a** is apparently more potent than the previously synthesized inhibitor (±)-**3** (IC_{50} = 340 nM) in comparison to their IC_{50} values, while the binding affinities of these nucleotide analogues are approximately the same (K_i = 15–17 nM). Under the same conditions, the reference nucleotide analogue **1** shows modest inhibitory potency (IC_{50} = 380 nM) and binding affinity (K_i = 53.0 nM) toward the PNP. Tetrahydropyranyl derivatives (±)-*cis*-**4b** and (±)-*trans*-**4b** were more potent than the tetrahydrofuranyl derivatives (±)-*cis*-**4a** and (±)-*trans*-**4a**; the IC_{50} values of (±)-*cis*-**4b** and (±)-*trans*-**4b** were determined to be 38 and 106 nM, respectively, under these conditions. The vicinal stereochemistry on the tetrahydropyranyl ring is reflected in the inhibition of human PNP preferable to the *cis*-isomer as in the case of the tetrahydrofuranyl derivatives. It should be noted that the vicinal stereochemistry did not affect the inhibition of PNP derived from *Cellulomonas* sp.; both (±)-*cis*-**4a** (IC_{50} = 35 nM) and (±)-*trans*-**4a** (IC_{50} = 37 nM) were equally strong inhibitors for the PNP. From the assessments on human PNP, the most potent compound in this set was identified to be (±)-*cis*-**4b**.

Table 1. Comparison of inhibition constants of **1**, (±)-**2**, (±)-**3**, (±)-*cis*-**4a,b**, and (±)-*trans*-**4a,b** for human erythrocyte PNP[a]

Compound	IC_{50} (nM)[b]	K_i (nM)[b]
1	380[c]	53.0[c]
(±)-**2**	330[c]	43.4[c]
(±)-**3**	340[c]	17.3[c]
(±)-*cis*-**4a**	88	15.2
(±)-*trans*-**4a**	320	ND[d]
(±)-*cis*-**4b**	38	26.9
(±)-*trans*-**4b**	106	ND[d]

[a]Purchased from Sigma.
[b]Determined in the presence of 0.1 mM inosine in 100 mM potassium phosphate buffer (pH 7.5).
[c]The data were taken from the previous reports.[6b]
[d]Not determined.

Table 2. Effects of Pi concentration on inhibition of **1** and (±)-*cis*-**4b** for human PNP

Compound	IC_{50} (nM)[a]		K_i (nM)[a]	
	1 mM PO_4	100 mM PO_4	1 mM PO_4	100 mM PO_4
1	32.5	380	17.5	53.0
(±)-*cis*-**4b**	8.7	38	3.5	26.9

[a]Determined in the presence of inosine (0.1 mM) at pH 7.5.

Inhibition constants of (±)-*cis*-**4b** for the human PNP was further evaluated at a physiological concentration of orthophosphate (1 mM), because the inhibition of PNPs by phosphates and phosphonic acids is inversely proportional to the concentration of orthophosphate.[5a,11] Table 2 summarizes the IC_{50} and K_i values of (±)-*cis*-**4b** at both 1 mM and 100 mM orthophosphate in comparison of those of the reference acyclic nucleotide analogue **1**. As observed with **1**, inhibition of the PNP by (±)-*cis*-**4b** is proportional to the concentration of orthophosphate, albeit the ratios (4.2–6.4) of the inhibition constants respectively obtained at 1 mM or 100 mM potassium phosphate buffer (pH 7.5) were modest. The IC_{50} and K_i values of (±)-*cis*-**4b** were determined to be 8.7 and 3.5 nM, respectively, under the physiological concentration of orthophosphate. These data suggest that the compound (±)-*cis*-**4b** could occupy both the phosphate and nucleoside binding sites in human erythrocyte PNP as a multisubstrate analogue inhibitor.

In summary, we have demonstrated that the structural modification of **1** with oxacycles and a hypoxanthine nucleobase is a useful strategy for increasing the binding affinity and inhibitory potency to human PNP. Additionally, the present results indicate that the stereochemistry of the compounds *cis*-**4a,b** greatly influences the inhibitory potency for human PNP, but not for *Cellulomonas* sp. PNP, although it remains to be evaluated which enantiomers of these compounds are desirable. Therefore, more detailed studies addressing how the stereoisomers of *cis*-**4a,b** can preferably interact with the active site of human erythrocyte PNP will be required. Identification of the optically active congeners and clarification of their inhibitory mechanism may lead to possible creation of a highly specific inhibitor for human PNP.

Experimental

General

All reactions were carried out under nitrogen atmosphere. The sonicated reactions were carried out under ultrasound irradiation generated by an ultrasonic cleaner (Iuchi VS-100, 100 W, 50 KHz). NMR data were obtained on a Bruker DPX 400 using $CDCl_3$ or CD_3OD as a solvent unless otherwise specified. ^{13}C NMR (100 MHz) and ^{31}P NMR (162 MHz) were taken with broad-band ^1H decoupling. The chemical shift data for each signal on ^1H NMR (400 MHz) are expressed as relative ppm from $CHCl_3$ (δ 7.26) or CH_3OH (δ 3.30). The chemical shifts of ^{13}C are reported relative to $CDCl_3$ (δ 77.0) or CD_3OD (δ 49.0). The chemical shifts of ^{31}P are recorded relative to external 85% H_3PO_4. ^{19}F NMR spectra (376 MHz) were measured using benzotrifluoride (BTF) as an internal reference. IR spectra were recorded on a JASCO FTIR-620 spectrometer. Mass spectra were measured on a Finnigan TSQ-700 or a VG Auto Spec E spectrometer.

(*E*)-3-[(2-Bromoallyl)oxy]-1-iodo-1-propene (6a). To a stirred solution of NaOH (3.48 g, 0.096 mol) and tetrabutylammonium hydrogen sulfate (440 mg, 1.3 mmol) in H_2O (20 mL) was added successively iodoalkenol **5a** (4.0 g, 21.7 mmol) and 2,3-dibromopropene (1.67 mL, 17.4 mmol) under ice-cooling. After being stirred for 12 h at room temperature, the mixture was diluted with water and extracted with ether. The extracts were washed with brine, dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography on silica gel eluted with *n*-hexane:EtOAc = 50:1 to give **6a** (4.41 g, 84%) as an oil. IR (film) 1639, 1612 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.63 (1H, dt, J = 14.5, 5.7 Hz), 6.44 (1H, dt, J = 14.5, 1.1 Hz), 5.95 (1H, bd, J = 1.3 Hz), 5.64 (1H, t, J = 0.7 Hz), 4.10 (2H, s), 3.96 (2H, dd, J = 5.7, 1.4 Hz); ^{13}C NMR ($CDCl_3$) δ 141.3, 128.7, 117.5, 79.3, 73.6, 71.3; EIMS m/z 302 ($M^+ + 1$). HREIMS m/z calcd for C_6H_7BrIO: 300.8725. Found: 300.8734.

(*E*)-4-[(2-Bromoallyl)oxy]-1-iodo-1-butene (6b). This compound was obtained as an oil from **5b** (8.12 g, 41 mmol) and 2,3-dibromopropene (3.15 mL, 32.8 mmol) in an analogous manner to that for preparation of **6a**. Yield: 73%; IR (film) 1640, 1608 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.56 (1H, dt, J = 14.4, 7.2 Hz), 6.15 (1H, dt, J = 14.4, 1.4 Hz), 5.91–5.88 (1H, m), 5.63–5.60 (1H, m), 4.08 (2H, s), 2.51 (2H, t, J = 6.5 Hz), 2.39–2.33 (2H, m); ^{13}C NMR ($CDCl_3$) δ 143.2, 130.0, 118.1, 77.5, 75.0, 68.7, 36.2; EIMS m/z 315 (M^+). HREIMS calcd for $C_7H_{10}BrIO$: 315.8960. Found: 315.8975.

Diethyl {(*E*)-4-[(2-bromoallyl)oxy]-1,1-difluoro-2-butenyl} phosphonate (8a). To a stirred suspension of Zn dust (1.94 g, 29.7 mmol) in dry DMA (30 mL) was slowly added a solution of diethyl bromodifluoromethylphosphonate (7.93 g, 29.7 mmol) in DMA (30 mL). During the addition, an exothermic reaction occurred. The addition was controlled so that the internal temperature was maintained at 50–60 °C. After the addition was completed, the solution was stirred at room temperature for an additional 3 h, and then CuBr (860 mg, 6 mmol) was added in one portion. The mixture was stirred at the same temperature for 30 min. Iodoalkene **6a** (6.00 g, 19.8 mmol) in DMA (30 mL) was added dropwise at room temperature. The mixture was sonicated for 14 h at 25 °C and portioned between water and ether. The biphasic mixture was passed through Celite, and was extracted with Et_2O. The extracts were washed with brine, and dried over $MgSO_4$. The volatile component of the extracts was removed in vacuo, and the residue was chromatographed on silica gel (*n*-hexane:EtOAc = 40:1) to give **8a** (6.23 g, 87%) as an oil: IR (film) 1678, 1640, 1270, 1099 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.38–6.30 (1H, m), 6.06–5.94 (1H, m), 5.92 (1H, dt, J = 1.7, 1.5 Hz), 5.64 (1H, t, J = 0.8 Hz), 4.33–4.23 (4H, m), 4.17–4.13 (2H,

m), 4.13 (2H, dt, $J=1.1$ Hz), 1.38 (6H, t, $J=7.0$ Hz); ^{13}C NMR ($CDCl_3$) δ 135.1 (dt, $J_{CP}=5.7$ Hz, $J_{CF}=10.0$ Hz), 122.0 (dt, $J_{CP}=13.4$ Hz, $J_{CF}=21.8$ Hz), 117.9, 116.8 (dt, $J_{CP}=221.2$ Hz, $J_{CF}=258.8$ Hz), 74.4, 68.5, 64.7 (dt, $J_{CP}=6.7$ Hz), 16.4 (d, $J_{CP}=5.4$ Hz); ^{19}F NMR ($CDCl_3$) δ −46.4 (2F, ddd with small splits, $J_{FP}=112.8$ Hz, $J_{FH}=3.4$ Hz); ^{31}P NMR ($CDCl_3$) δ 5.89 (t, $J_{PF}=113.2$ Hz). EIMS m/z 363 ($M^+ + 1$). Anal. calcd for $C_{11}H_{18}BrF_2O_4P$: C, 34.46; H, 5.01. Found: C, 35.98; H, 5.04.

Diethyl {(*E*)-5-[(2-bromoallyl)oxy]-1,1-difluoro-1-pentenyl} phosphonate (8b). This compound was obtained as an oil from **6b** in an analogous manner to that for preparation of **8a**. Yield: 91%; an oil: IR (film) 1672, 1640, 1271 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 6.38–6.29 (1H, m) 5.91–5.90 (1H, m), 5.85–5.71 (1H, m), 5.69–5.61 (1H, m), 4.33–4.20 (4H, m), 4.10–4.07 (2H, m), 3.57 (2H, t, $J=6.5$ Hz), 2.52–2.43 (2H, m), 1.37 (6H, t, $J=7.1$ Hz); ^{13}C NMR ($CDCl_3$) δ 137.0 (dt, $J_{CP}=5.9$ Hz, $J_{CF}=10.3$ Hz), 129.1, 123.4 (dt, $J_{CP}=13.3$ Hz, $J_{CF}=21.4$ Hz), 117.4, 117.2 (dt, $J_{CP}=219.8$ Hz, $J_{CF}=258.9$ Hz), 74.7, 68.4, 63.4 (d, $J_{CP}=6.7$ Hz), 32.2, 16.1 (d, $J_{CP}=5.3$ Hz); ^{19}F NMR ($CDCl_3$) δ −46.0 (2F, ddd with small splits, $J_{FP}=114.3$ Hz, $J_{FH}=12.8$, 2.8 Hz); ^{31}P NMR ($CDCl_3$) δ 7.00 (t, $J_{PF}=114.7$ Hz). EIMS m/z 377 ($M^+ + 1$). Anal. calcd for $C_{12}H_{20}BrF_2O_4P$: C, 38.30; H, 5.36. Found: C, 38.27; H, 5.36.

Diethyl [1,1-difluoro-2-(4-methylenetetrahydro-3-furanyl) ethyl]phosphonate (9a). To a stirred solution of **8a** (8.79 g, 24 mmol) and *n*-Bu_3SnH (9.7 mL, 36 mmol) in THF (800 mL) was added Et_3B (1 M solution in hexane, 24 mL) at −20 °C. The mixture was stirred at the same temperature for 10 min under aerobic conditions. The reaction was quenched with MeOH (1 mL). The volatile component of the mixture was removed in vacuo. The residue was portioned between petroleum ether and acetonitrile. The extracts by acetonitrile were evaporated and the residue was purified by column chromatography on silica gel (*n*-hexane:EtOAc = 10:1) to give **9a** (6.2 g, 91%) as an oil: IR (film) 1668, 1272, 1024 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 5.02 (1H, dd, $J=4.4$, 2.1 Hz), 4.97 (1H, dd, $J=4.8$, 2.4 Hz), 4.39–4.19 (7H, m), 3.56 (1H, t, $J=8.65$ Hz), 3.15–3.01 (1H, br s), 2.53–2.37 (1H, m), 2.18–2.00 (1H, m), 1.39 (6H, t, $J=7.1$ Hz); ^{13}C NMR ($CDCl_3$) δ 150.3, 120.2 (dt, $J_{CP}=260.2$ Hz, $J_{CF}=260.1$ Hz), 104.2, 73.8, 70.6, 64.8 (d, $J_{CP}=6.8$ Hz), 36.9 (t, $J_{CF}=3.0$ Hz), 36.2 (dt, $J_{CP}=14.8$ Hz, $J_{CF}=20.4$ Hz), 16.2 (d, $J_{CP}=5.2$ Hz); ^{19}F NMR ($CDCl_3$) δ −46.3 (1F, dddd, $J_{FF}=297.1$ Hz, $J_{FP}=107.2$ Hz, $J_{FH}=32.5$, 8.4 Hz), −50.2 (1F, dddd, $J_{FF}=297.1$ Hz, $J_{FP}=107.6$ Hz, $J_{FH}=29.9$, 12.6 Hz); ^{31}P NMR ($CDCl_3$) δ 7.48 (t, $J_{PF}=107.1$ Hz). EIMS m/z 284 (M^+). HREIMS m/z calcd for $C_{11}H_{19}F_2O_4P$: 284.0989. Found: 284.0997.

Diethyl 1,1-difluoro-2-(3-methylidenetetrahydro-2*H*-pyran-4-yl)ethylphosphonate (9b). A solution of *n*-Bu_3SnH (9.7 ml; 36 mmol) in toluene (360 mL) was added to a refluxing solution of **8b** (9.05 g, 24 mmol) and AIBN (6.90 mg, 0.10 mmol) in toluene (440 mL) during 30 min. The mixture was refluxed for an additional 10 h. AIBN (6.90 mg, 0.10 mmol) was added every 2 h. Volatile component of the mixture was removed in vacuo. The residue was portioned between petroleum ether and acetonitrile. The extracts by acetonitrile were evaporated and the residue was purified by column chromatography on silica gel (*n*-hexane:EtOAc = 10:1) to give **9b** (5.08 g, 71%) as an oil: IR (film) 1652, 1271 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 4.87 (1H, s), 4.75 (1H, s), 4.27–4.20 (4H, m), 4.12–4.02 (1H, m), 3.96–3.86 (2H, m), 3.62–3.56 (1H, m), 2.81–2.71 (1H, m), 2.55–2.35 (1H, m), 2.18–1.91 (2H, m), 1.56–1.46 (1H, m), 1.34 (6H, t, $J=7.1$ Hz); ^{13}C NMR ($CDCl_3$) δ 145.9, 121.7 (dt, $J_{CP}=215.9$ Hz, $J_{CF}=260.7$ Hz), 108.7, 72.8, 66.7, 64.6–64.3 (m), 35.4 (dt, $J_{CP}=14.7$ Hz, $J_{CF}=19.9$ Hz), 34.8, 33.7-33.4 (m), 16.4 (d, $J_{CP}=5.3$ Hz); ^{19}F NMR ($CDCl_3$) δ −45.6 (1F, dddd, $J_{FF}=297.0$ Hz, $J_{FP}=108.0$ Hz, $J_{FH}=32.0$, 10.0 Hz); ^{31}P NMR ($CDCl_3$) δ 7.50 (t, $J_{PF}=108.6$ Hz). EIMS m/z 298 (M^+). Anal. calcd for $C_{12}H_{21}F_2O_4P$: C, 48.30; H, 7.10. Found: C, 48.79; H, 7.12.

Diethyl 1,1-difluoro-2-[(3*S,4*S**)-4-(hydroxymethyl)tetrahydro-3-furanyl]ethylphosphonate (11a) and diethyl 1,1-difluoro-2-[(3*S**,4*R**)-4-(hydroxymethyl)tetrahydro-3-furanyl]ethylphosphonate (12a).** To a stirred solution of **8a** (4.57 g, 16.1 mmol) in THF (100 mL) was added a borane–THF complex (1.0 M solution in THF, 16 mL) under ice-cooling. The mixture was stirred at room temperature for 15 min. 30% Hydrogen peroxide (10.9 mL) and 3 M sodium acetate (5.4 mL) were successively added. The mixture was stirred at room temperature for 3 h. The biphasic mixture was extracted with ether. The extracts were washed with brine, dried ($MgSO_4$), and evaporated. The residue was purified by column chromatography on silica gel (*n*-hexane: EtOAc = 3:1) to give a mixture of **11a** and **12a** (4.03 g, 83%) in a ratio of 64:36. The mixture was practically used for the next reaction without further purification. The analytical samples for **11a** and **12a** were obtained as oils through preparative HPLC separation [Inertsil (GL-science), EtOAc, flow rate: 5 mL/min, UV-detector (254 nm)] of the corresponding trityl ethers, followed by deprotection [Amberlyst 15E®, MeOH]: **11a**: IR (film) 3624, 1264, 1164 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 4.33–4.23 (4H, m), 4.03 (1H, dt, $J=8.2$ Hz, 1.1 Hz), 3.91 (1H, dd, $J=8.8$, 6.4 Hz), 3.80 (1H, dd, $J=8.8$, 3.7 Hz), 3.77–3.72 (1H, m), 3.69–3.59 (1H, m), 3.54 (1H, t, $J=8.5$ Hz), 2.82–2.70 (1H, m), 2.55–2.36 (2H, m), 2.23–2.02 (1H, m), 1.80 (1H, broad s), 1.39 (6H, t, $J=7.1$ Hz); ^{19}F NMR ($CDCl_3$) δ −46.9 (1F, dddd, $J_{FF}=296.1$ Hz, $J_{FP}=107.4$ Hz, $J_{FH}=30.6$, 9.9 Hz), −50.4 (1F, dddd, $J_{FF}=296.1$ Hz, $J_{FP}=107.9$ Hz, $J_{FH}=29.1$, 12.1 Hz). ^{31}P NMR ($CDCl_3$) δ 7.50 (t, $J_{PF}=107.3$ Hz). EIMS m/z 303 ($M^+ + 1$). HREIMS m/z calcd for $C_{11}H_{22}F_2O_5P$: 303.1173. Found: 303.1160. **12a**: IR (film) 3444, 1261, 1165 cm^1; ^{1}H NMR ($CDCl_3$) δ 4.32–4.24 (4H, m), 4.10 (1H, t, $J=7.9$ Hz), 3.81 (1H, dd, $J=8.7$, 7.9 Hz), 3.73–3.60 (3H, m), 3.44 (1H, dd, $J=8.9$, 7.2 Hz), 2.49–2.32 (2H, m), 2.22–1.96 (2H, m), 1.58 (1H, s), 1.39 (6H, t, $J=7.1$ Hz); ^{13}C NMR ($CDCl_3$) δ 121.1 (dt, $J_{CP}=215.0$ Hz, $J_{CF}=260.3$ Hz), 74.0, 70.2, 64.6 (d, $J_{CP}=6.7$ Hz), 63.5, 48.0, 37.3 (dt, $J_{CP}=14.8$ Hz, $J_{CF}=20.5$ Hz), 35.4 (d, $J_{CP}=3.9$ Hz), 16.2 (d, $J_{CP}=5.2$ Hz); ^{19}F NMR ($CDCl_3$) δ −46.0 (1F, dddd, $J_{FF}=297.3$ Hz, $J_{FP}=107.3$ Hz, $J_{FH}=26.5$, 10.5 Hz), −48.5 (1F, dddd, $J_{FF}=297.3$ Hz, $J_{FP}=107.3$ Hz, $J_{FH}=25.8$, 15.5 Hz); ^{31}P NMR ($CDCl_3$) δ 7.45

(t, J_{PF} = 107.1 Hz). EIMS *m/z* 303 ($M^+ + 1$). HREIMS *m/z* calcd for $C_{11}H_{22}F_2O_5P$: 303.1173. Found: 303.1187.

Diethyl 1,1-difluoro-2-[(3*R,4*R**)-3-(hydoroxymethyl)tetrahydro-2*H*-pyran-4-yl]ethylphosphonate (11b) and diethyl 1,1-difluoro-2-[(3*S**,4*R**)-3-(hydroxymethyl)tetrahydro-2*H*-pyran-4-yl]ethylphosphonate (12b).** To a stirred solution of **9b** (3.0 g, 10 mmol) in THF (100 mL) was added 9-BBN (40 mL of 0.5 M solution in hexane, 20 mmol) under ice cooling. The mixture was stirred at room temperature for 10 h. 30% Hydrogen peroxide (6.8 mL) and 3 M sodium acetate (6.7 mL) were successively added. The mixture was stirred at room temperature for 30 min. The biphasic mixture was extracted with ether. The extracts were washed with brine, dried ($MgSO_4$), and evaporated. The residue was purified by column chromatography on silica gel (*n*-hexane:EtOAc = 100:1) to give a mixture of **11b** and **12b** (1.6 g, 51%) as an oil in a ratio of 84:16. ^{31}P NMR ($CDCl_3$) δ 7.48 (0.84P, t, J_{PF} = 106.8 Hz) and 7.19 (0.16P, t, J_{PF} = 107.0 Hz); FABMS *m/z* 339 (MNa^+), 317 (MH^+). The mixture was used for the next reaction without further purification.

Transformation of the *cis*-enriched alcohol 11a,b to the *trans*-enriched alcohol 12a,b. The *cis*-enriched alcohol **11a** (4.2 g, 14 mmol, *cis*:*trans* = 64:36) was oxidized with Jones reagent (5.7 mL) in acetone (150 mL) for 12 h at room temperature. Work-up as usual gave the corresponding carboxylic acid, which was treated with 3% HCl–MeOH (80 mL) at room temperature for 6 h. The volatile component mixture was removed in vacuo and the residue was diluted with water. The mixture was extracted with $CHCl_3$ and the extracts were washed with brine, dried ($MgSO_4$), and evaporated to give the *cis*-enriched methyl ester **15a** [3.8 g; ^{31}P NMR ($CDCl_3$) δ 7.57 (0.64P, t, J_{PF} = 107 Hz) and 7.13 (0.36P, t, J_{PF} = 107 Hz); EIMS *m/z* 345 ($M^+ + 1$)] as an oil. This oil was treated with Cs_2CO_3 (2.83 g, 8.7 mmol) in MeOH (25 mL) for 12 h at room temperature. The volatile component of the mixture was evaporated. The residue was diluted with water and extracted with $CHCl_3$. The extracts were washed with brine, dried ($MgSO_4$), and evaporated to give the *trans*-enriched methyl ester **16a** [2.1 g; ^{31}P NMR ($CDCl_3$) δ 7.57 (0.15P, t, J_{PF} = 107 Hz), 7.13 (0.85P, t, J_{PF} = 107 Hz); EIMS *m/z* 345 ($M^+ + 1$); Anal. calcd for $C_{12}H_{21}F_2O_6P$: C, 43.64; H, 6.41. Found: C, 43.60; H, 6.45] as an oil. This oil was dissolved in ether (60 mL) and treated with $LiBH_4$ (199 mg, 9.1 mmol) at room temperature for 12 h. The mixture was diluted with water and extracted with $CHCl_3$. The extracts were dried ($MgSO_4$) and evaporated. The residue was purified by column chromatography (SiO_2; *n*-hexane:EtOAc = 3:1) to give the *trans*-enriched alcohol **12a** [1.4 g (36% for the 4-steps); ^{31}P NMR ($CDCl_3$) δ 7.50 (0.15P, t, J_{PF} = 107.0 Hz) and 7.45 (0.85P, t, J_{PF} = 107.0 Hz); EIMS *m/z* 303 ($M^+ + 1$)]. The mixture was used for the next reaction without further purification. The *trans*-enriched alcohol **12b** [^{31}P NMR ($CDCl_3$) δ 7.48 (0.5P, t, J_{PF} = 106.2 Hz), 7.19 (0.5P, t, J_{PF} = 107.0 Hz); FABMS *m/z* 317 (MH^+)] was obtained in 25% overall yield (4-steps) as an oil via methyl esters **15b** [^{31}P NMR ($CDCl_3$) δ 7.35 (t, J_{PF} = 108.9 Hz)] and **16b** [^{31}P NMR ($CDCl_3$) δ 7.18 (t, J_{PF} = 107.0 Hz)] in an analogous manner to that for preparation of the *trans*-enriched alcohol **12a**.

Diethyl 2-{(3*S,4*S**)-4-[(6-chloro-9*H*-purin-9-yl)methyl]-tetrahydro-3-furanyl}-1,1-difluoroethylphosphonate (13a).** To a stirred solution of the *cis*-enriched alcohol **11a** (2.63 g, 8.71 mmol; *cis*:*trans* = 64:36), Ph_3P (2.97 g, 11.3 mmol), and 6-chloropurine (1.75 g, 11.3 mmol) in THF (100 mL) was added DEAD (5.7 mL of 40% toluene solution, 13.0 mmol) under ice-cooling. The mixture was stirred at room temperature for 12 h. The volatile component of the mixture was removed in vacuo. The residue was triturated with a mixture of *n*-hexane:Et_2O. The precipitate was removed by suction. The filtrate was evaporated. Column chromatography of the residue on silica gel eluted with *n*-hexane:EtOAc = 60:1 gave *cis*-enriched **13a** (2.6 g; *cis*:*trans* = 64:36; 68% yield). Preparative HPLC separation [Inertsil (GL-science), $CHCl_3$:MeOH = 20:1, flow rate: 5 mL/min, UV-detector (254 nm)] of the mixture gave diastereomerically pure **13a** (1.1 g, 43% yield) as an oil: IR (film) 1769, 1267, 1165 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 8.68 (1H, s), 8.13 (1H, s), 4.35 (1H, dd, J = 13.8, 4.5 Hz), 4.28–4.13 (1H, m), 4.24 (4H, t, J = 7.3 Hz), 4.05 (1H, t, J = 8.4 Hz), 3.64–3.59 (2H, m), 3.54 (1H, dd, J = 9.3, 2.5 Hz), 2.96–2.81 (2H, m), 2.51–2.35 (1H, m), 2.24–2.07 (1H, m), 1.34 (6H, t, J = 7.1 Hz); ^{13}C NMR ($CDCl_3$) δ 151.9, 151.8, 151.0, 145.1, 131.5, 120.0 (dt, J_{CP} = 216.0 Hz, J_{CF} = 260.8 Hz), 71.4, 70.0, 65.1 (d, J_{CP} = 6.9 Hz), 42.5, 41.9, 34.3, 31.9 (dt, J_{CP} = 15.0 Hz, J_{CF} = 20.8 Hz), 16.4 (d, J_{CP} = 5.3 Hz); ^{19}F NMR ($CDCl_3$) δ −48.4 (1F, dddd, J_{FF} = 105.5 Hz, J_{FP} = 105.5 Hz, J_{FH} = 29.8, 0.5 Hz), −49.7 (1F, dddd, J_{FF} = 105.8 Hz, J_{FP} = 105.8 Hz, J_{FH} = 28.8, 10.8 Hz); ^{31}P NMR ($CDCl_3$) δ 6.78 (t, J_{PF} = 105.5 Hz). EIMS *m/z* 438 (M^+). HREIMS calcd for $C_{16}H_{22}ClF_2N_4O_4P$ (M^+): 438.1035. Found: 438.1046.

Diethyl 2-{(3*S,4*S**)-3-[(6-chloro-9*H*-purin-9-yl)methyl]-tetrahydro-2*H*-pyran-4-yl}-1,1-difluoroethylphosphonate (13b).** This compound was prepared from the *cis*-enriched alcohol **12b** (*cis*:*trans* = 84:16) in an analogous manner to that for the preparation of **13a** as an oil after preparative HPLC separation [Inertsil (GL-science), $CHCl_3$:MeOH = 20:1, flow rate: 5 mL/min, UV-detector (254 nm)]. Yield: 75%; IR (film) 1258, 1028 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 8.75 (1H, s), 8.15 (1H, s), 4.64 (1H, dd, J = 13.7, 11.2 Hz), 4.39–4.23 (5H, m), 4.12–4.03 (1H, m), 3.75–3.45 (2H, m), 3.44–3.36 (1H, m), 2.62–2.10 (4H, m), 1.80–1.65 (2H, m), 1.42 (6H, *t*, J = 7.1 Hz); ^{13}C NMR ($CDCl_3$) δ 151.9, 150.9, 145.7, 131.4, 120.8 (dt, J_{CP} = 215.6 Hz, J_{CF} = 260.9 Hz), 68.1 (d, J_{CP} = 6.3 Hz), 64.7, 64.6, 40.5, 38.8, 36.7 (dt, J_{CP} = 14.5 Hz, J_{CF} = 20.2 Hz), 30.9, 28.7, 16.3 (d, J_{CP} = 6.9 Hz); ^{19}F NMR ($CDCl_3$) δ −53.0 (2F, dt, J_{FP} = 118.0 Hz, J_{FH} = 22.0 Hz); ^{31}P NMR ($CDCl_3$) δ 6.96 (t, J_{PF} = 106.7 Hz); EIMS *m/z* 452 (M^+). Anal. calcd for $C_{17}H_{24}ClF_2N_4O_4P$: C, 45.09; H, 5.34; N, 12.38. Found: C, 45.01; H, 5.55; N, 11.95.

Diethyl 2-{(3*S,4*R**)-4-[(6-chloro-9*H*-purin-9-yl)methyl]-tetrahydro-3-furanyl}-1,1-difluoroethylphosphonate (14a).** This compound was prepared as an oil from the *trans*-enriched alcohol **12a** (*cis*:*trans* = 15:85) in an analogous

manner to that for the preparation of **13a** after preparative HPLC separation [Inertsil (GL science), $CHCl_3$:MeOH = 20:1, flow rate: 5 mL/min; UV detector (254 nm)]. Yield: 57%; IR (film) 1593, 1561, 1272, 1184, 1127 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.72 (1H, s), 8.16 (1H, s), 4.42 (1H, dd, J = 14.1, 6.2 Hz), 4.35–4.15 (4H, m), 3.78 (1H, dd, J = 9.4, 7.0 Hz), 3.70 (1H, dd, J = 9.4, 4.7 Hz), 3.51–3.43 (1H, m), 2.71–2.63 (1H, m), 2.51–2.43 (1H, m), 2.29–2.08 (4H, m), 1.38 (6H, t, J = 7.1 Hz); ^{13}C NMR ($CDCl_3$) δ 152.0, 151.9, 151.1, 145.2, 131.4, 121.4 (dt, J_{CP} = 215.2 Hz, J_{CF} = 260.3 Hz), 73.8, 70.4, 64.8–64.6 (m), 46.4, 45.7, 37.0 (dt, J_{CP} = 14.6 Hz, J_{CF} = 20.4 Hz), 36.5–36.0 (m), 16.4 (d, J_{CP} = 5.3 Hz); ^{19}F NMR ($CDCl_3$) δ −48.2 (1F, dddd, J_{FF} = 105.8 Hz, J_{FP} = 105.8 Hz, J_{FH} = 26.3 Hz, 13.3 Hz), −48.7 (1F, dddd, J_{FF} = 105.3 Hz, J_{FP} = 105.3 Hz, J_{FH} = 25.8, 14.8 Hz); ^{31}P NMR ($CDCl_3$) δ 7.45 (t, J_{PF} = 105.6 Hz); EIMS m/z 439 ($M^+ + 1$). HREIMS m/z calcd for $C_{16}H_{22}ClF_2N_4O_4P$ ($M^+ + 1$): 439.1135. Found: 439.1147.

Diethyl 2-{(3*S,4*R**)-3-[(6-chloro-9*H*-purin-9-yl)methyl]-tetrahydro-2*H*-pyran-4-yl}-1,1-difluoroethylphosphonate (14b).** This compound was prepared from the *trans*-enriched alcohol **12b** (*cis*:*trans* = 50:50) in an analogous manner to that for the preparation of **13a** as an oil after preparative HPLC separation. Yield: 34%; IR (film) 1260, 1147 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.76 (1H, s), 8.11 (1H, s), 4.50 (1H, dd, J = 14.5, 4.4 Hz), 4.35–4.17 (5H, m), 3.85–3.77 (1H, m), 3.72–3.63 (1H, m), 3.59–3.47 (1H, m), 3.28 (1H, dd, J = 11.9, 7.4 Hz), 2.63–2.39 (1H, m), 2.27–2.00 (5H, m), 1.40 (6H, t, J = 7.0 Hz); ^{13}C NMR ($CDCl_3$) δ 152.0, 151.1, 145.3, 131.4, 121.6 (dt, J_{CP} = 215.5 Hz, J_{CF} = 260.7 Hz), 68.2, 66.4, 64.7 (d, J_{CP} = 6.8 Hz), 44.5, 40.4, 36.9 (dt, J_{CP} = 14.7 Hz, J_{CF} = 19.8 Hz), 31.1, 29.6, 16.4 (d, J_{CP} = 6.8 Hz); ^{19}F NMR ($CDCl_3$) δ −53.7 (1F, dddd, J_{FF} = 119.0 Hz, J_{FP} = 107.0 Hz, J_{FH} = 31.0, 15.0 Hz), −54.6 (1F, dddd, J_{FF} = 119.0 Hz, J_{FP} = 107.0 Hz, J_{FH} = 30.8, 14.8 Hz); ^{31}P NMR ($CDCl_3$) δ 7.09 (J_{PF} = 107.0 Hz); EIMS m/z 452 (M^+). Anal. calcd for $C_{17}H_{24}ClF_2N_4O_4P$: C, 45.09; H, 5.34; N, 12.38. Found: C, 44.79; H, 5.75; N, 11.83.

1,1-Difluoro-2-{(3*S,4*S**)-4-[(6-oxo-1,6-dihydro-9*H*-purin-9-yl)methyl]tetrahydro-3-furanyl}ethylphosphonic acid ((±)-*cis*-4a).** To a stirred solution of **13a** (100.4 mg, 0.12 mmol) in CH_2Cl_2 (6 mL) was added bromotrimethylsilane (0.16 mL, 0.6 mmol) at room temperature. The mixture was stirred for 18 h and evaporated under reduced pressure. The residue was treated with H_2O (6 mL) at room temperature for 1 h. The mixture was portioned between $CHCl_3$ and H_2O. The aqueous layer was washed with $CHCl_3$. Lyophilization of the aqueous layer gave (±)-*cis*-**4a** (70.4 mg, 82%) as an amorphous powder. UV (H_2O) λ_{max} 250.7 nm (ε = 11854); IR (KBr) 2341, 1710, 1571, 1029 cm^{-1}; ^1H NMR (CD_3OD) δ 9.33 (1H, brs), 8.27 (1H, brs), 4.61–4.47 (1H, m), 4.47–4.31 (1H, m), 4.12–4.01 (1H, m), 3.84–3.61 (3H, m), 3.05 (1H, brs), 2.93 (1H, brs), 2.63–2.39 (1H, m), 2.39–2.07 (1H, m); ^{13}C NMR (CD_3OD) δ 154.8, 150.5, 148.8, 140.8, 122.6 (dt, J_{CP} = 258.5 Hz, J_{CF} = 210.0 Hz), 117.7, 73.1, 71.4, 62.1, 61.9, 47.0, 46.0, 43.4, 36.1, 33.6, 32.7 (dt, J_{CP} = 15.2 Hz, J_{CF} = 21.0 Hz); ^{19}F NMR (CD_3OD) δ −47.8 (1F, dddd, J_{FF} = 286.2 Hz, J_{FP} = 104.1 Hz, J_{FH} = 28.9, 9.3 Hz), −50.9 (1F, dddd, J_{FF} = 286.2 Hz, J_{FP} = 104.1 Hz, J_{FH} = 27.3, 12.8 Hz); ^{31}P NMR (CD_3OD) δ 4.10 (t, J_{PF} = 104.1 Hz); FABMS m/z 365 (MH^+). HRFABMS m/z calcd for $C_{12}H_{14}F_2N_4O_5P$ (MH^+): 365.0826. Found: 365.0811.

1,1-Difluoro-2-{(3*S,4*S**)-4-[(6-oxo-1,6-dihydro-9*H*-purin-9-yl)methyl]tetrahydrofuran-3-yl}ethylphosphonic acid ((±)-*trans*-4a).** This compound was prepared as an amorphous powder from **14a** in an analogous manner to that for preparation of (±)-*cis*-**4a**. Yield: 56%; UV (H_2O) λ_{max} 250.2 nm (ε = 8815); IR (KBr) 2432, 1771, 1571, 1024 cm^{-1}; ^1H NMR (CD_3OD) δ 9.34 (1H, brs), 8.27 (1H, brs); 4.53–4.46 (1H, m), 4.19–4.12 (1H, m), 3.74–3.58 (3H, m), 3.50–3.43 (1H, m), 2.75–2.66 (1H, m), 2.54–2.40 (1H, m), 2.38–2.03 (2H, m); ^{19}F NMR (CD_3SOCD_3) δ −47.9 (1F, dd with small splits, J_{FF} = 285.3 Hz, J_{FP} = 98.8 Hz), −50.2 (1F, dd with small splits, J_{FF} = 285.3 Hz, J_{FP} = 98.8 Hz); ^{31}P NMR (CD_3SOCD_3) δ 3.90 (t, J_{PF} = 98.8 Hz); FABMS m/z 365 (MH^+). HRFABMS m/z calcd for $C_{12}H_{14}F_2N_4O_5P$ (MH^+): 365.0826. Found: 365.0801.

1,1-Difluoro-2-{(3*S,4*S**)-3-[(6-oxo-1,6-dihydro-9*H*-purin-9-yl)methyl]tetrahydro-2H-pyran-4-yl}ethylphosphonic acid ((±)-*cis*-4b).** This compound was prepared as amorphous powder from **13b** in an analogous manner to that for preparation of (±)-*cis*-**4a**. Yield: 90%; UV (H_2O) λ_{max} 250.0 nm (ε = 9818); IR (KBr) 1709, 1566, 1169, 1022 cm^{-1}; ^1H NMR (CD_3OD) δ 8.9 (1H, s), 7.86, (1H, s), 4.34 (1H, dd, J = 12, 10 Hz), 4.08 (1H, d, J = 12 Hz), 3.57 (1H, d, J = 9 Hz), 3.25–2.98 (3H, m), 2.18–1.70 (4H, m), 1.49–1.19 (2H, m); ^{19}F NMR (CD_3OD) δ −47.5 (1F, dd with small splits, J_{FF} = 285.8 Hz, J_{PF} = 101.1 Hz), −48.8 (1F, dd with small splits, J_{FF} = 285.8 Hz, J_{PF} = 101.1 Hz); ^{31}P NMR (CD_3OD) δ 4.29 (t, J_{PF} = 101.1 Hz); FABMS m/z 379 (MH^+); HRFABMS m/z calcd for $C_{13}H_{17}F_2N_4O_5P$ (M^+): 378.0905. Found: 378.0904.

1,1-Difluoro-2-{(3*S,4*S**)-3-[(6-oxo-1,6-dihydro-9*H*-purin-9-yl)methyl]tetrahydro-2H-pyran-4-yl}ethylphosphonic acid ((±)-*trans*-4b).** This compound was prepared as an amorphous powder from **14b** in an analogous manner to that for preparation of (±)-*cis*-**4a**. Yield: 67%; UV (H_2O) λ_{max} 250.0 nm (ε = 2680); IR (KBr) 1709, 1566, 1169, 1022 cm^{-1}; ^1H NMR (CD_3OD) δ 9.55 (1H, s), 8.32 (1H, s), 4.67 (1H, dd, J = 14.2, 4.98 Hz), 4.41 (1H, dd, J = 14.2, 9.2 Hz), 3.82–3.72 (2H, m), 3.58–3.50 (1H, m), 3.48–3.40 (1H, m), 2.78–2.45 (1H, m), 2.25–2.0 (4H, m), 1.52–1.40 (1H, m); ^{19}F NMR (CD_3OD) δ −46.2 (1F, dddd, J_{FF} = 294.0 Hz, J_{PF} = 104.8 Hz, J_{HF} = 25.6, 12.0 Hz), −49.2 (1F, dddd, J_{FF} = 294.0 Hz, J_{FP} = 104.8 Hz, J_{FH} = 25.4, 15.8 Hz); ^{31}P NMR (CD_3OD) δ 4.29 (t, J_{FP} = 104.8 Hz); FABMS m/z 379 (MH^+); HRFABMS m/z calcd for $C_{13}H_{17}F_2N_4O_5P$ (M^+): 378.0905. Found: 378.0904.

Assay and inhibition of PNP. PNP activity was measured by the xanthine oxidase couple assay of Stoeckler et al.[12] with minor modification. Briefly, the assay mixture contained either 5 or 500 mM potassium phosphate buffer (pH 7.5, 300 μL), 0.2 U/mL PNP (Sigma, St.

Louis, 300 μL), 0.12 U/mL xanthine oxidase (Sigma, St. Louis, 300 μL), 0.05–10 μM inhibitor (300 μL), and distilled water (1.5 mL), and was incubated at 30 °C for 5 min. To the reaction mixture was added 0.1, 1.0 and 10 mM inosine (Wako Pure Chemical Co., Osaka, 300 μL), and the increase in absorbance at 293 nm based on the formation of uric acid was monitored for 1 min with a Shimadzu UV-1600 spectrophotometer. PNP activity was calculated by using the molecular extinction coefficient of uric acid (1.25×10^4), and the specific activity was expressed as μmol of uric acid/min/mg protein. IC_{50} was the concentration of the compounds giving 50% of enzyme inhibition. K_i values were determined by using a Dixon plot and a computer developed in-house for linear regression analysis. It was verified that the compounds had no inhibitory activity toward xanthine oxidase in this assay.

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