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2-Substitution of Adenine Nucleotide Analogues Containing a Bicyclo[3.1.0]hexane Ring System Locked in a Northern Conformation: Enhanced Potency as P2Y₁ Receptor Antagonists

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Abstract

Preference for the northern (*N*) ring conformation of the ribose moiety of adenine nucleotide 3', 5'-bisphosphate antagonists of P2Y₁ receptors was established by using a ring-constrained methanocarba (a bicyclo[3.1.0]hexane) ring as a ribose substitute (Nandan et al. *J. Med. Chem.* **2000**, *43*, 829–842). We have now combined the ring-constrained (*N*)-methanocarba modification with other functionalities at the 2-position of the adenine moiety. A new synthetic route to this series of bisphosphate derivatives was introduced, consisting of phosphorylation of the pseudoribose moiety prior to coupling with the adenine base. The activity of the newly synthesized analogues was determined by measuring antagonism of 2-methylthio-ADP-stimulated phospholipase C (PLC) activity in 1321N1 human astrocytoma cells expressing the recombinant human P2Y₁ receptor and by using the radiolabeled antagonist [³H]2-chloro-*N*⁶-methyl-(*N*)-methanocarba-2'-deoxyadenosine 3',5'-bisphosphate **5** in a newly developed binding assay in Sf9 cell membranes. Within the series of 2-halo analogues, the most potent molecule at the hP2Y₁ receptor was an (*N*)-methanocarba *N*⁶-methyl-2-iodo analogue **12**, which displayed a *K*_i value in competition for binding of [³H]**5** of 0.79 nM and a *K*_B value of 1.74 nM for inhibition of PLC. Thus, **12** is the most potent antagonist selective for the P2Y₁ receptor yet reported. The 2-iodo group was substituted with trimethyltin, thus providing a parallel synthetic route for the introduction of an iodo group in this high-affinity antagonist. The (*N*)-methanocarba-2-methylthio, 2-methylseleno, 2-hexyl, 2-(1-hexenyl), and 2-(1-hexynyl) analogues bound less well, exhibiting micromolar affinity at P2Y₁ receptors. An enzymatic method of synthesis of the 3',5'-bisphosphate from the corresponding 3'-monophosphate, suitable for the preparation of a radiophosphorylated analogue, was explored.

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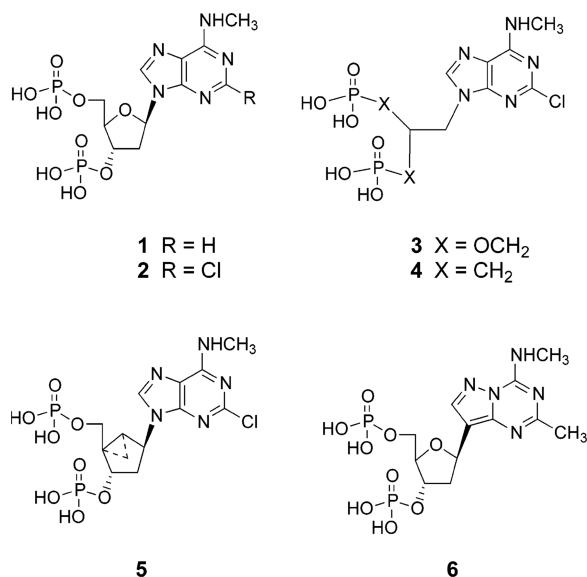
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Introduction

Two families of P2 nucleotide receptors exist: G protein-coupled P2Y receptors, of which eight mammalian subtypes (P2Y_{1,2,4,6,11-14}) are currently sequence-defined, and P2X ligand-gated cation channels, of which seven mammalian subtypes (P2X₁₋₇) have been cloned.¹⁻⁴ Adenine nucleotides are required for activation of the P2Y₁, P2Y₁₁, P2Y₁₂, and P2Y₁₃ subtypes, and uracil nucleotides activate P2Y₂, P2Y₄, and P2Y₆, subtypes. The P2Y₂ receptor is equipotently activated by UTP and ATP. The most recently identified subtypes are the P2Y₁₃ receptor, which is present in the immune system,⁵ and the P2Y₁₄ receptor, which is activated by UDP-glucose.⁶ There is evidence that multiple P2Y receptors may be involved in the activation of eosinophils and neutrophils.⁷

The P2Y₁ receptor is distributed in the heart, skeletal, and various smooth muscles and the prostate, ovary, brain, and circulating blood cells.⁸ This receptor was first cloned from chick brain;⁹ its regional brain distribution and presence in glial cells have been described,¹⁰⁻¹² and abnormalities in P2Y₁ receptor levels may exist in the brains of persons with Alzheimer's disease.¹³ Activation of this receptor by ADP mediates relaxation in the guinea pig aorta.¹⁴ The P2Y₁ receptor plays a major physiological role in platelet aggregation.¹⁵

P2Y receptor ligands are being explored for therapeutic applications in the cardiovascular, endocrine, and other systems. The P2Y₁ and P2Y₁₂ receptors are the furthest advanced in the P2Y family with respect to selective agonists and antagonists.¹⁶⁻²⁶ 3',5'-Bisphosphate nucleotides have been developed as selective antagonists of the P2Y₁ receptor, and high receptor affinities have been achieved in this series.²⁰ The 2'-deoxyribose moiety of widely used P2Y₁ receptor antagonists such as *N*⁶-methyl-2'-deoxyadenosine 3',5'-bisphosphate **1** may be replaced with carbocyclics, smaller and larger rings, acyclics, and conformationally constrained rings,¹⁷⁻¹⁹ resulting in retention or enhancement of affinity for the receptor. A 2-chloro group was found to enhance potency and selectivity in the case of nucleotide **2** and the acyclic bisphosphate analogue **3**.^{19,27} An acyclic bisphosphonate analogue **4** was also shown to be a P2Y₁ receptor antagonist.¹⁹



The most recently introduced P2Y₁ receptor antagonists contain novel substitutions of the ribose moiety or of the adenine ring system. In solution, the conformation of the ribose ring of nucleosides and nucleotides exists in a rapid, dynamic two-state equilibrium between a (*N*) (northern, 2'-*exo*) or (*S*) (southern, 2'-*endo*) conformation, although their target

receptors probably prefer specific conformations. We have replaced the ribose moiety for P2Y₁ antagonists with a carbocyclic ring locked in a preferred conformation, a fusion of cyclopropane and cyclopentane rings known as the methanocarba modification.^{28–30} Two structural variations, depending on the position of the cyclopropane ring, restrict the ring pucker, i.e., hold the riboselike ring (pseudosugar) in either a (*N*)- or (*S*)-envelope conformation, as defined in the pseudorotational cycle. A prototypical ring-constrained P2Y₁ antagonist is the highly potent (*N*)-methanocarba derivative 2-chloro-*N*⁶-methyl-(*N*)-methanocarba-2'-deoxyadenosine 3',5'-bisphosphate **5**.^{18,20} This molecule was also prepared in [³H]-labeled form and provides the first broadly reliable high-affinity antagonist for quantitation of a P2Y receptor in a radioligand binding assay.³¹ The recently reported C-nucleoside pyrazolo[1,5-*a*]-1,3,5-triazine bisphosphate **6** is also a potent P2Y₁ antagonist.²⁵

In the present study, we combined the ring-constrained (*N*)-methanocarba modification of adenine nucleotides with other adenine 2-position functionalities to prepare novel analogues, which were evaluated for affinity at the human P2Y₁ receptor. In one case, a 2-iodo substitution conferred a significant increase in antagonist potency. New synthetic routes to antagonists in this series, including methods for introduction of phosphate groups as either the terminal step or prior to the coupling of sugar and nucleobase, were explored. The 2-iodo substituent could also be introduced at various stages of the synthesis.

Experimental Section

Chemical Synthesis

Nucleosides and synthetic reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and Aldrich (Milwaukee, WI). 2,6-Dichloropurine was obtained from Sigma. The protected intermediate **19** was synthesized in our laboratory as described.⁴⁵ **5** and **9** were prepared as described.¹⁸ **37** and **38** were prepared as described.³⁷

¹H NMR spectra were obtained with a Varian Gemini-300 spectrometer (300 MHz) with D₂O, CDCl₃, CD₃OD, and DMSO-*d*₆ as a solvent. Tetramethylsilane (TMS) was the external standard. ³¹P NMR spectra were recorded at room temperature with a Varian XL-300 spectrometer (121.42 MHz); orthophosphoric acid (85%) was used as an external standard.

Purity of compounds was checked with a Hewlett-Packard 1100 HPLC apparatus equipped with a Luna 5μ C18(2) analytical column (250 × 4.6 mm, Phenomenex, Torrance, CA) in two solvent systems.

System A: linear gradient solvent system, 0.1 M TEAA/CH₃-CN from 95/5 to 40/60 in 20 min; the flow rate was 1 mL/min.

System B: linear gradient solvent system, 5 mM TBAP/CH₃-CN from 80/20 to 40/60 in 20 min; the flow rate was 1 mL/min.

Peaks were detected by UV absorption with a diode array detector. All derivatives tested for biological activity showed 97% purity in the HPLC systems.

Low-resolution CI-NH₃ (chemical ionization) mass spectra were measured with a Finnigan 4600 mass spectrometer, and high-resolution EI (electron impact) mass spectrometry was performed with a VG7070F mass spectrometer at 6 kV. High-resolution FAB (fast atom bombardment) mass spectrometry was performed with a JEOL SX102 spectrometer with 6-kV Xe atoms following desorption from a glycerol matrix.

Purification of the nucleotide analogues for biological testing was carried out on Sephadex-DEAE-A-25 resin columns with a linear gradient (0.01–0.5 M) of 0.5 M ammonium bicarbonate applied as the mobile phase.

(1'*R*,2'*S*,4'*S*,5'*S*)-4-(6-Amino-2-iodo-9*H*-purin-9-yl)-1-[(phosphato)methyl]-2-(phosphato)bicyclo[3.1.0]-hexane Tetraammonium Salt (**7**)

Compound **29** (1.48 mg, 0.0023 mmol) was dissolved in 30% NH₃ in water (0.50 mL) and stirred for 6.5 h at 60 °C in a sealed tube. The solvent was removed under reduced pressure.

Purification of the obtained residue was performed on an ion-exchange column packed with Sephadex-DEAE A-25 resin. A linear gradient (0.01–0.5 M) of 0.5 M ammonium bicarbonate was applied as the mobile phase, and UV and HPLC were used to monitor the elution, which furnished **7** (0.77 mg, 54%): ¹H NMR (D₂O) δ 8.58 (s, 1H), 5.20–4.80 (m, 2H), 4.60–4.40 (m, 1H), 3.60–3.56 (m, 1H), 2.35–2.20 (m, 1H), 2.00–1.88 (m, 1H), 1.88–1.80 (m, 1H), 1.18–1.12 (m, 1H), 0.99–0.91 (m, 1H); ³¹P NMR (D₂O) δ 0.17, –0.25 (2s, 3'-P, 5'-P); MS (*m/e*) (negative-FAB) 546 (M⁺ – 1); HRMS (negative-FAB) calcd for C₁₂H₁₅N₅O₈P₂I 545.9441, found 545.9454; HPLC 8.3 min (99%) (system A), 14.3 min (99%) (system B).

(1'*R*,2'*S*,4'*S*,5'*S*)-4-(6-Amino-2-methylthio-9*H*-purin-9-yl)-1-[(phosphato)methyl]-2-(phosphato)bicyclo[3.1.0]-hexane Tetraammonium Salt (**8**)

To a solution of **26** (1.70 mg, 0.0020 mmol) in CH₂Cl₂ (0.30 mL) was added 1.0 M BCl₃ in CH₂Cl₂ (0.50 mL) and the mixture stirred for 2 d at 5 °C. The reaction was quenched upon addition of 5.0 mL of triethylammonium bicarbonate buffer (1.0 M). The mixture was subsequently frozen and lyophilized, which furnished crude **8** (1.90 mg): MS (*m/e*) (negative-FAB) 485, 487 (peak height ratio = 3:1) (M⁺ – 1); HPLC 15.6 min (99%) (system B).

The crude sample of **8** (1.90 mg) was dissolved in 30% NH₃ in water (3.0 mL) and stirred for 2 h at 70 °C in a sealed tube. The solvent was removed under reduced pressure, the obtained residue was purified on an ion-exchange column packed with Sephadex-DEAE A-25 resin, a linear gradient (0.01–0.5 M) of 0.5 M ammonium bicarbonate was applied as the mobile phase, and UV and HPLC were used to monitor the elution, which furnished homogeneous **8** (1.90 mg, 94%): ¹H NMR (D₂O) δ 8.44 (s, 1H), 5.30–5.20 (m, 1H), 5.09 (d, 1H, *J* = 6.5 Hz), 4.90–4.75 (m, 1H), 3.70–3.55 (m, 1H), 2.60 (s, 3H), 2.30–2.20 (m, 1H), 2.00–1.94 (m, 1H), 1.85–1.80 (m, 1H), 1.17–1.14 (m, 1H), 0.96–0.94 (m, 1H); ³¹P NMR (D₂O) δ 2.03, 1.36 (2s, 3'-P, 5'-P); MS (*m/e*) (negative-FAB) 466 (M⁺ – 1); HRMS (negative-FAB) calcd for C₁₃H₁₈N₅O₈P₂S 466.0374, found 466.0363; HPLC 8.7 min (99%) in solvent system A, 14.1 min (99%) in a solvent system B.

(1'*R*,2'*S*,4'*S*,5'*S*)-4-(2-Fluoro-6-methylamino-9*H*-purin-9-yl)-1-[(phosphato)methyl]-2-(phosphato)bicyclo[3.1.0]-hexane Tetraammonium Salt (**10**)

The mixture of **44** (5 mg, 0.0074 mmol) in 5% TFA/CH₂Cl₂ (5 mL) was stirred at room temperature for 3 h. After solvent was removed, the residue was treated with triethylammonium bicarbonate buffer (1 M, 0.5 mL) and stirred for 30 min. The aqueous layer was washed twice with CH₂Cl₂ and evaporated to dryness under reduced pressure. The residue was purified with ion-exchange column chromatography with the use of Sephadex-DEAE-A-25 resin with a linear gradient (0.01–0.5 M) of 0.5 M NH₄-HCO₃ as the mobile phase. After lyophilization, **10** (1.38 mg, 36%) was obtained as a white solid: ¹H NMR (D₂O) δ 8.25 (s, 1H), 5.25–5.1 (m, 2H), 3.8–3.65 (m, 2H), 3.05 (s, 3H), 2.4–2.1 (1H), 2.1–1.9 (m, 2H), 1.1–0.9 (1H); ³¹P NMR (D₂O) 0.878 (s), 1.2 (s); HRMS (*m/e*) (negative-FAB)

calcd for $C_{13}H_{18}FN_5O_8P_2$ 452.0536, found 452.0548; HPLC 8.2 (99%) min in solvent system A, 16.1 min (99%) in solvent system B.

(1'R,2'S,4'S,5'S)-[4-(2-Bromo-6-methylamino-9H-purin-9-yl)-1-[(phosphato)methyl]-2-(phosphato)bicyclo[3.1.0]-hexane Tetraammonium Salt (11)

The mixture of **45** (3.0 mg, 0.0041 mmol) in 5% TFA/ CH_2Cl_2 (5 mL) was stirred at room temperature for 3 h. After removal of the solvent, the residue was treated with triethylammonium bicarbonate buffer (1 M, 0.5 mL) and stirred for 30 min. The aqueous layer was washed twice with CH_2Cl_2 and evaporated to dryness. The residue was purified with ion-exchange column chromatography with the use of Sephadex-DEAE-A-25 resin with a linear gradient (0.01–0.5 M) of 0.5 M NH_4HCO_3 as the mobile phase. After lyophilization, **11** (1.36 mg, 58%) was obtained as a white solid: 1H NMR (D_2O) δ 8.42 (s, 1H), 5.4–5.1 (m, 2H), 3.8–3.6 (m, 2H), 3.1 (s, 3H), 2.4–2.1 (m, 2H), 2.1–1.8 (m, 2H), 1.1–0.9 (1H); ^{31}P NMR (D_2O) δ 0.4 (s), 0.9 (s); HRMS (*m/e*) (negative-FAB) calcd for $C_{13}H_{18}BrN_5O_8P_2$ 511.9736, found 511.9763; HPLC 9.3 min (99%) in solvent system A, 14.5 min (99%) in solvent system B.

(1'R,2'S,4'S,5'S)-4-(2-Iodo-6-methylaminopurin-9-yl)-1-[(phosphato)methyl]-2-(phosphato)bicyclo[3.1.0]-hexane (12)

Method A—Compound **29** (3.61 mg, 0.0057 mmol) was dissolved in 40% MeNH₂ in water (2.0 mL) and stirred for 26 h at room temperature. The solvent was removed under reduced pressure. Purification of the obtained residue was performed on an ion-exchange column packed with Sephadex-DEAE A-25 resin, a linear gradient (0.01–0.5 M) of 0.5 M ammonium bicarbonate was applied as the mobile phase, and UV and HPLC were used to monitor the elution, which furnished **12** (0.73 mg, 20%).

Method B—A solution of the 2-iodo bis(di-*tert*-butyl phosphate) derivative **46** (7.1 mg, 9.03 μ mol) in 5% trifluoroacetic acid in methylene chloride was stirred for 2 h at room temperature. All volatile material was removed in vacuo. The residue was purified by using ion-exchange column chromatography on Sephadex-DEAE-25 resin and a linear gradient (0–0.5 M) of 0.5 M ammonium bicarbonate as the mobile phase to give the 2-iodo bisphosphate derivative **12** (2.9 mg, 55%) as an ammonium salt: 1H NMR (D_2O) δ 8.54 (bs, 1H), 5.19 (m, 1H), 5.01 (d, 1H, *J* = 6.9 Hz), 4.58 (dd, 1H, *J* = 4.7, 11.3 Hz), 3.73 (dd, 1H, *J* = 4.4, 11.0 Hz), 3.07 (bs, 3H), 2.28 (dd, 1H, *J* = 7.7, 14.6 Hz), 1.92–2.09 (m, 2H), 1.26 (dd, 1H, *J* = 4.1, 6.1 Hz), 1.06 (dd, 1H, *J* = 9.7, 16.3 Hz); ^{31}P NMR (D_2O) 0.651 (s); HRMS (negative-ion FAB) calcd for $C_{13}H_{17}N_5O_8P_2I$ 559.9597, found 559.9604; HPLC 9.8 min (97%) in solvent system A, 15.4 min (97%) in system B.

(1'R,2'S,4'S,5'S)-4-(6-Methylamino-2-methylthio-9H-purin-9-yl)-1-[(phosphato)methyl]-2-(phosphato)bicyclo-[3.1.0]hexane Tetraammonium Salt (13)

To a solution of **28** (4.2 mg, 0.0050 mmol) in CH_2Cl_2 (0.30 mL) was added 1.0 M BCl_3 in CH_2Cl_2 (0.60 mL) and the mixture stirred for 2 d at 5 °C. The reaction was quenched upon the addition of 5.0 mL of triethylammonium bicarbonate buffer (1.0 M). The mixture was subsequently frozen and lyophilized.

Purification of the obtained residue was performed on an ion-exchange column packed with Sephadex-DEAE A-25 resin, a linear gradient (0.01–0.5 M) of 0.5 M ammonium bicarbonate was applied as the mobile phase, and UV and HPLC were used to monitor the elution, which furnished **13** (1.69 mg, 81%): 1H NMR (D_2O) δ 8.45 (s, 1H), 5.25–5.15 (m, 1H), 5.08–5.02 (m, 1H), 4.65–4.60 (m, 1H), 3.65–3.55 (m, 1H), 3.13 (bs, 3H), 2.62 (s, 3H), 2.30–2.20 (m, 1H), 2.00–1.85 (m, 1H), 1.85–1.75 (m, 1H), 1.15–1.10 (m, 1H), 1.00–0.95

(m, 1H); ^{31}P NMR (D_2O) δ 2.21, 2.90 (2s, 3'-P, 5'-P); MS (m/e) (negative-FAB) 480 ($\text{M}^+ - 1$); HRMS (negative-FAB) calcd for $\text{C}_{14}\text{H}_{20}\text{N}_5\text{O}_8\text{P}_2\text{S}$ 480.0488, found 480.0498; HPLC 10.3 min (99%) in solvent system A, 16.4 min (99%) in solvent system B.

(1'*R*,2'*S*,4'*S*,5'*S*)-4-(6-Methylamino-2-methylselenopurin-9-yl)-1-[(phosphato)methyl]-2-(phosphato)bicyclo[3.1.0]hexane Tetraammonium Salt (14)

Compound **30** (0.38 mg, 0.63 μmol) was dissolved in 40% MeNH₂ in water (3.0 mL) and stirred for 8 h at 80 °C. The solvent was removed under reduced pressure. Purification of the obtained residue was performed on an ion-exchange column packed with Sephadex-DEAE A-25 resin, a linear gradient (0.01–0.5 M) of 0.5 M ammonium bicarbonate was applied as the mobile phase, and UV and HPLC were used to monitor the elution, which furnished **14** (0.30 mg, 79%): ^1H NMR (D_2O) δ 8.51 (s, 1H), 5.10–5.00 (m, 1H), 5.00–4.90 (m, 1H), 4.74–4.72 (m, 1H), 3.65–3.50 (m, 1H), 3.14 (bs, 3H), 2.53 (s, 3H), 2.40–2.18 (m, 1H), 2.05–1.85 (m, 1H), 1.85–1.75 (m, 1H), 1.25–1.14 (m, 1H), 1.00–0.95 (m, 1H); ^{31}P NMR (D_2O) δ 3.00, 3.53 (2s, 3'-P, 5'-P); MS (m/e) (negative-FAB) 528 ($\text{M}^+ - 1$), HRMS (negative-FAB) calcd for $\text{C}_{14}\text{H}_{20}\text{N}_5\text{O}_8\text{P}_2\text{Se}$ 527.9948, found 527.9950; HPLC 10.8 min (99%) in solvent system A, 16.8 min (99%) in solvent system B.

(1'*R*,2'*S*,4'*S*,5'*S*)-Phosphoric Acid [4-(2-Methyl-6-methylaminopurin-9-yl)-1-phosphonooxymethylbicyclo[3.1.0]-hex-2-yl] Monoester (15)

A solution of **47** (8.0 mg, 0.012 mmol) in 5% TFA/ CH_2Cl_2 (2.0 mL) was stirred for 3 h at 25 °C. The solvent was removed under reduced pressure and the residue was quenched by addition of 5.0 mL of triethylammonium bicarbonate buffer (1.0 M). The mixture was subsequently frozen and lyophilized. Purification of the obtained residue was performed on an ion-exchange column packed with Sephadex-DEAE A-25 resin, a linear gradient (0.01–0.5 M) of 0.5 M ammonium bicarbonate was applied as the mobile phase, and UV and HPLC were used to monitor the elution, which furnished **15** (4.5 mg, 73%): ^1H NMR (D_2O) δ 8.45 (s, 1H), 5.18 (m, 1H), 5.00 (d, 1H, $J = 5.4$ Hz), 4.57–4.50 (m, 1H), 3.69–3.62 (m, 1H), 3.13 (s, 3H), 2.53 (s, 3H), 2.30–2.20 (m, 1H), 2.24–1.92 (m, 1H), 1.88 (m, 1H), 1.19 (m, 1H), 0.98 (m, 1H); ^{31}P NMR (D_2O) δ 1.05, 0.65 (2s, 3'-P, 5'-P); MS (m/e) (negative-FAB) 448 ($\text{M}^+ - 1$); HRMS (negative-FAB) calcd for $\text{C}_{14}\text{H}_{20}\text{N}_5\text{O}_8\text{P}_2$ 448.0787, found 448.0807; HPLC 7.3 min (99%) (system A), 14.2 min (99%) (system B).

(1'*R*,2'*S*,4'*S*,5'*S*)-4-(2-Hexyl-6-methylaminopurin-9-yl)-1-[(phosphato)methyl]-2-(phosphato)bicyclo[3.1.0]-hexane (16)

The 2-hexanyl bisphosphate derivative **16** (5.6 mg, 69%) was prepared by the same method as above for **11**, except the 2-hexanyl bis(di-*tert*-butyl phosphate) derivative **48** (11.2 mg, 0.015 mmol) was used as the starting material: ^1H NMR (D_2O) δ 8.54 (bs, 1H), 5.23 (m, 1H), 5.10 (d, 1H, $J = 6.6$ Hz), 4.57 (dd, 1H, $J = 5.2, 11.3$ Hz), 3.70 (m, 1H), 3.25 (bs, 3H), 2.85 (dd, 2H, $J = 7.4, 7.7$ Hz), 2.29 (dd, 1H, $J = 7.7, 15.1$ Hz), 2.03 (dt, 1H, $J = 6.6, 16.8$ Hz), 1.92 (dd, 1H, $J = 3.8, 9.1$ Hz), 1.773 (m, 2H), 1.13–1.43 (m, 9H), 1.03 (dd, 1H, $J = 6.6, 7.7$ Hz), 0.85 (m, 3H); ^{31}P NMR (D_2O) 0.827 (s), 0.375 (s); HRMS (negative-ion FAB) calcd for $\text{C}_{19}\text{H}_{30}\text{N}_5\text{O}_8\text{P}_2$ 518.1570, found 518.1544; HPLC 13.2 min (98%) in solvent system A, 16.6 min (97%) in system B.

(1'*R*,2'*S*,4'*S*,5'*S*)-4-(2-Hex-1-ynyl-6-methylaminopurin-9-yl)-1-[(phosphato)methyl]-2-(phosphato)bicyclo[3.1.0]-hexane (17)

The 2-hexenyl bisphosphate derivative **17** (3.2 mg, 68%) was prepared by the same method as above for **11**, except the 2-hexenyl bis(di-*tert*-butyl phosphate) derivative **49** (6.0 mg, 8.08 μmol) was used as the starting material: ^1H NMR (D_2O) δ 8.54 (s, 1H), 7.18 (dt, 1H, $J = 7.1, 15.4$ Hz), 6.45 (m, 1H), 5.22 (m, 1H), 5.08 (d, 1H, $J = 6.3$ Hz), 4.57 (dd, 1H, $J = 4.7,$

11.0 Hz), 3.71 (dd, 1H, $J = 5.0, 10.7$ Hz), 3.22 (bs, 3H), 2.23–2.44 (m, 3H), 2.03 (m, 1H), 1.95 (dd, 1H, $J = 3.7, 8.8$ Hz), 1.32–1.61 (m, 4H), 1.24 (dd, 1H, $J = 4.1, 5.5$ Hz), 1.05 (dd, 1H, $J = 6.3, 8.2$ Hz), 0.94 (m, 3H); ^{31}P NMR (D_2O) 0.601 (s), 0.299 (s); HRMS (negative-ion FAB) calcd for $\text{C}_{19}\text{H}_{28}\text{N}_5\text{O}_8\text{P}_2$ 516.1413, found 516.1403; HPLC 13.5 min (99%) in solvent system A, 16.9 min (99%) in solvent system B.

(1'R,2'S,4'S,5'S)-4-(2-Hex-1-enyl-6-methylaminopurin-9-yl)-1-[(phosphato)methyl]-2-(phosphato)bicyclo[3.1.0]hexane (18)

The 2-hexynyl bisphosphate derivative **18** (4.0 mg, 73%) was prepared by the same method as above for **11**, except the 2-hexynyl bis(di-*tert*-butyl phosphate) derivative **50** (7.0 mg, 9.46 μmol) was used as the starting material: ^1H NMR (D_2O) δ 8.46 (s, 1H), 5.16 (m, 1H), 4.97 (d, 1H, $J = 6.3$ Hz), 4.56 (dd, 1H, $J = 4.7, 11.3$ Hz), 3.70 (dd, 1H, $J = 6.3, 11.3$ Hz), 3.07 (bs, 3H), 2.50 (t, 2H, $J = 7.1$ Hz), 2.24 (dd, 1H, $J = 7.4, 14.6$ Hz), 1.91–2.05 (m, 2H), 1.42–1.71 (m, 4H), 1.23 (dd, 1H, $J = 4.1, 5.8$ Hz), 1.03 (dd, 1H, $J = 6.1, 8.2$ Hz), 0.95 (t, 3H, $J = 7.2$ Hz); ^{31}P NMR (D_2O) 0.777 (s), 0.375 (s); HRMS (negative-ion FAB) calcd for $\text{C}_{19}\text{H}_{26}\text{N}_5\text{O}_8\text{P}_2$ 514.1257, found 514.1234; HPLC 12.4 min (98%) in solvent system A, 12.3 min (99%) in solvent system B.

General (Phosphorylation) Procedure: Synthesis of (M)-Methanocarbaadenosine Derivatives (21–57). (1'R,2'S,4'S,5'S)-4-(2-Amino-6-chloro-9H-purin-9-yl)-1-(benzyloxymethyl)-2-benzyloxybicyclo[3.1.0]hexane (21)

To a solution of triphenylphosphine (0.20 g, 0.76 mmol) in anhydrous tetrahydrofuran (THF, 1.0 mL) was added dropwise diisopropyl azodicarboxylate (DIAD, 0.15 mL, 0.76 mmol) at -20 °C with stirring for 0.5 h. Compound **19** (76 mg, 0.23 mmol) and 2-amino-6-chloropurine **20** (92 mg, 0.54 mmol) in THF (5.0 mL) were added to the reaction mixture and it stirred for 18.5 h at room temperature. The solvent was removed under vacuum, and the residue obtained was purified by using flash chromatography with 1/1 petroleum ether/ethyl acetate to furnish **21** (103 mg, 92%): ^1H NMR (CDCl_3) δ 8.58 (s, 1H), 7.40–7.21 (m, 10H), 5.01 (bs, 1H), 4.97 (d, 1H, $J = 6.6$ Hz), 4.66 (t, 1H, $J = 8.25$ Hz), 4.54 (s, 2H), 4.45 (d, 1H, $J = 11.7$ Hz), 4.38 (d, 1H, $J = 11.7$ Hz), 4.09 (d, 1H, $J = 9.8$ Hz), 3.14 (d, 1H, $J = 9.8$ Hz), 2.09–2.00 (m, 1H), 1.85–1.74 (m, 1H), 1.59–1.54 (m, 1H), 1.11–1.06 (m, 1H), 0.81–0.75 (m, 1H); MS (*m/e*) (positive-FAB) 476, 478 (peak height ratio = 3:1) ($\text{M}^+ + 1$).

(1'R,2'S,4'S,5'S)-4-(2-Amino-6-chloro-9H-purin-9-yl)-1-(hydroxymethyl)-2-hydroxybicyclo[3.1.0]hexane (22)

Compound **21** (169 mg, 0.353 mmol) was dissolved in CH_2Cl_2 (5.0 mL) and treated with BCl_3 (1 M in CH_2Cl_2 , 1.50 mL) for 1 h at -40 °C under argon. *i*-PrOH (0.40 mL) was added to the reaction mixture and it was warmed to room temperature. The solvent was evaporated, and the crude product was purified by flash column chromatography with AcOEt/*i*-PrOH (1/1) to yield **22** as a white solid (72 mg, 69%): ^1H NMR (CD_3OD) δ 8.54 (s, 1H), 4.94 (d, 1H, $J = 6.6$ Hz), 4.26 (d, 1H, $J = 11.7$ Hz), 4.03 (s, 1H), 3.33 (d, 1H, $J = 11.7$ Hz), 2.09–1.98 (m, 1H), 1.84–1.73 (m, 1H), 1.67–1.62 (m, 1H), 1.04–0.99 (m, 1H), 0.79–0.74 (m, 1H); MS (*m/e*) (negative-FAB) 294, 296 (peak height ratio = 3:1) ($\text{M}^+ - 1$).

(1'R,2'S,4'S,5'S)-4-(2-Amino-6-chloro-9H-purin-9-yl)-1-[(dibenzylphosphato)methyl]-2-(dibenzylphosphato)-bicyclo[3.1.0]hexane (24)

To a stirred solution of **22** (56 mg, 0.190 mmol) and 1*H*-tetrazole (130 mg, 1.86 mmol) in 3.0 mL of anhydrous THF was added dibenzyl diisopropylphosphoramidite (**23**, 0.30 mL, 0.89 mmol), and the mixture was stirred for 4.5 h at room temperature. The reaction mixture was cooled to -78 °C and treated with a solution of *m*-chloroperbenzoic acid (*m*-CPBA, 70% max, 350 mg) in CH_2Cl_2 (5.0 mL). The resulting mixture was warmed to room

temperature, and the solvent was removed under reduced pressure. The residue obtained was purified by flash column chromatography (1/1 = AcOEt/CH₂Cl₂), which furnished **24** (52 mg, 35%): ¹H NMR (CDCl₃) δ 7.82 (s, 1H), 7.33–7.25 (m, 20H), 5.41 (dd, 1H, *J* = 7.8, 14.1 Hz), 5.27 (bs, 2H), 5.05–4.91 (m, 8H), 4.74 (d, 1H, *J* = 6.9 Hz), 4.54 (dd, 1H, *J* = 5.7, 11.1 Hz), 3.94 (dd, 1H, *J* = 6.0, 11.1 Hz), 2.14–2.04 (m, 1H), 1.98–1.87 (m, 1H), 1.62–1.52 (m, 1H), 1.00–0.93 (m, 1H), 0.87–0.80 (m, 1H); MS (*m/e*) (positive-FAB) 816, 818 (peak height ratio = 3:1) (M⁺ + 1).

(1'*R*,2'*S*,4'*S*,5'*S*)-4-(6-Chloro-2-iodo-9*H*-purin-9-yl)-1-[(dibenzylphosphato)methyl]-2-(dibenzylphosphato)-bicyclo[3.1.0]hexane (25)

To a solution of **24** (62.1 mg, 0.0761 mmol) in acetonitrile (0.50 mL) were added diiodomethane (2.0 mL) and *tert*-butyl nitrite (0.10 mL, 1.11 mmol) were added, and oxygen was purged by N₂ bubbling. The tube was sealed and stirred at 85 °C for 3.0 h. Diiodomethane was removed upon passage through a short column (silica gel, eluting with CHCl₃, then AcOEt). The residue obtained was purified by preparative thin-layer chromatography (AcOEt), which furnished **25** (25.8 mg, 37%): ¹H NMR (CDCl₃) δ 8.19 (s, 1H), 7.36–7.25 (m, 20H), 5.29 (q, 1H, *J* = 6.9 Hz), 5.10–4.92 (m, 9H), 4.61 (dd, 1H, *J* = 6.3, 12.0 Hz), 3.84 (dd, 1H, *J* = 6.6, 12.0 Hz), 2.05–1.88 (m, 2H), 1.62–1.52 (m, 1H), 1.02 (m, 1H), 0.95–0.84 (m, 1H); MS (*m/e*) (positive-FAB) 928, 930 (peak height ratio = 3:1) (M⁺ + 1).

(1'*R*,2'*S*,4'*S*,5'*S*)-4-(6-Chloro-2-methylthio-9*H*-purin-9-yl)-1-[(dibenzylphosphato)methyl]-2-(dibenzylphosphato)-bicyclo[3.1.0]hexane (26)

To a solution of **24** (4.5 mg, 0.0056 mmol) in MeCN (0.25 mL) were added dimethyl disulfide (0.15 mL, 1.26 mmol) and *tert*-butylnitrite (0.050 mL, 0.56 mmol), and oxygen was purged by nitrogen bubbling. The tube was sealed and stirred at 70 °C for 5.5 h. The solvent was removed under reduced pressure. The residue obtained was purified by preparative thin-layer chromatography (2/1=AcOEt/CH₂Cl₂), which furnished **26** (3.0 mg, 64%): ¹H NMR (CDCl₃) δ 8.15 (s, 1H), 7.33–7.25 (m, 20H), 5.30 (dd, 1H, *J* = 7.8, 14.7 Hz), 5.07–4.91 (m, 9H), 4.62 (dd, 1H, *J* = 6.0, 11.5 Hz), 3.74 (dd, 1H, *J* = 6.6, 11.5 Hz), 2.61 (s, 3H), 2.14–2.04 (m, 1H), 1.98–1.87 (m, 1H), 1.63–1.56 (m, 1H), 1.07–1.03 (m, 1H), 0.88–0.81 (m, 1H); MS (*m/e*) (positive-FAB) 847, 849 (peak height ratio = 3:1) (M⁺ + 1).

(1'*R*,2'*S*,4'*S*,5'*S*)-4-(6-Chloro-2-methylseleno-9*H*-purin-9-yl)-1-[(dibenzylphosphato)methyl]-2-(dibenzylphosphato)bicyclo[3.1.0]hexane (27)

To a solution of **24** (16.0 mg, 0.0196 mmol) in MeCN (0.30 mL) were added dimethyldiselenide (0.10 mL, 1.06 mmol) and *tert*-butyl nitrite (0.100 mL, 1.11 mmol), and oxygen was purged by nitrogen bubbling. The tube was sealed and stirred at 65 °C for 20 h. The solvent was removed under reduced pressure. The residue obtained was purified by preparative thin-layer chromatography (1/1 = AcOEt/CH₂Cl₂), which furnished **27** (6.7 mg, 38%): ¹H NMR (CDCl₃) δ 8.16 (s, 1H), 7.40–7.25 (m, 20H), 5.28 (dd, 1H, *J* = 8.4, 14.4 Hz), 5.10–4.90 (m, 9H), 4.62 (dd, 1H, *J* = 6.0, 11.4 Hz), 3.74 (dd, 1H, *J* = 6.6, 14.4 Hz), 2.53 (s, 3H), 2.15–2.00 (m, 1H), 2.00–1.85 (m, 1H), 1.75–1.65 (m, 1H), 1.45–1.35 (m, 1H), 1.10–1.00 (m, 1H); MS (*m/e*) (positive-FAB) 895, 897 (peak height ratio = 3:1) (M⁺ + 1).

(1'*R*,2'*S*,4'*S*,5'*S*)-4-(6-Methylamino-2-methylthio-9*H*-purin-9-yl)-1-[(dibenzylphosphato)methyl]-2-(dibenzylphosphato)bicyclo[3.1.0]hexane (28)

Compound **26** (3.0 mg, 0.0035 mmol) was dissolved in 2.0 M MeNH₂ in THF (0.50 mL) and stirred for 2.5 h at room temperature. The solvent was removed under reduced pressure. The residue obtained was purified by preparative thin-layer chromatography (AcOEt), which furnished **28** (2.7 mg, 91%): ¹H NMR (CDCl₃) δ 7.77 (s, 1H), 7.30–7.25 (m, 20H), 5.72 (bs,

1H), 5.38 (dd, 1H, $J = 7.5, 14.1$ Hz), 5.03–4.91 (m, 9H), 4.59 (dd, 1H, $J = 6.3, 11.4$ Hz), 3.78 (dd, 1H, $J = 5.1, 11.4$ Hz), 3.19 (bs, 3H), 2.57 (s, 3H), 2.19–2.10 (m, 1H), 1.96–1.82 (m, 1H), 1.60–1.55 (m, 1H), 1.04–1.00 (m, 1H), 0.88–0.78 (m, 1H); MS (m/e) (positive-FAB) 842 ($M^+ + 1$).

(1'*R*,2'*S*,4'*S*,5'*S*)-4-(6-Chloro-2-iodo-9*H*-purin-9-yl)-1-[(phosphato)methyl]-2-(phosphato)bicyclo[3.1.0]-hexane Tetraammonium Salt (29)

To a solution of **25** (13.6 mg, 0.015 mmol) in CH_2Cl_2 (0.80 mL) was added 1.0 M BCl_3 in CH_2Cl_2 (0.80 mL) and the mixture stirred for 24 h at 5 °C. The reaction was quenched upon addition of 3.0 mL of triethylammonium bicarbonate buffer (1.0 M). The mixture was subsequently frozen and lyophilized. Purification of the residue obtained was performed on an ion-exchange column packed with Sephadex-DEAE A-25 resin. A linear gradient (0.01–0.5 M) of 0.5 M ammonium bicarbonate was applied as the mobile phase, and UV and HPLC were used to monitor the elution, which furnished **29** (5.56 mg, 60%): ^1H NMR (D_2O) δ 8.83 (s, 1H), 5.30–5.20 (m, 1H), 5.16 (d, 1H, $J = 6.3$ Hz), 4.60–4.50 (m, 1H), 3.75–3.65 (m, 1H), 2.40–2.20 (m, 1H), 2.10–1.95 (m, 1H), 1.95–1.90 (m, 1H), 1.25–1.20 (m, 1H), 1.05–1.00 (m, 1H); ^{31}P NMR (D_2O) δ 2.02, 1.40 (2s, 3'-P, 5'-P); MS (m/e) (negative-FAB) 565, 567 (peak height ratio = 3:1) ($M^+ - 1$); HPLC 9.8 min (98%) in solvent system A, 16.0 min (98%) in solvent system B.

(1'*R*,2'*S*,4'*S*,5'*S*)-4-(6-Chloro-2-methylseleno-9*H*-purin-9-yl)-1-[(phosphato)methyl]-2-(phosphato)bicyclo[3.1.0]-hexane Tetraammonium Salt (30)

To a solution of **27** (6.7 mg, 0.0075 mmol) in CH_2Cl_2 (0.50 mL) was added 1.0 M BCl_3 in CH_2Cl_2 (0.80 mL) and the mixture stirred for 2 d at 5 °C. The reaction was quenched upon addition of 5.0 mL of triethylammonium bicarbonate buffer (1.0 M). The mixture was subsequently frozen and lyophilized. Purification of the obtained residue was performed on an ion-exchange column packed with Sephadex-DEAE A-25 resin, a linear gradient (0.01–0.5 M) of 0.5 M ammonium bicarbonate was applied as the mobile phase, and UV and HPLC were used to monitor the elution, which furnished **30** (1.28 mg, 28%): ^1H NMR (D_2O) δ 8.78 (s, 1H), 5.35–5.22 (m, 2H), 4.60–4.50 (m, 1H), 3.70–3.65 (m, 1H), 2.61 (s, 3H), 2.45–2.30 (m, 1H), 2.15–1.95 (m, 1H), 1.95–1.85 (m, 1H), 1.25–1.18 (m, 1H), 1.05–0.95 (m, 1H); ^{31}P NMR (D_2O) δ 1.67, 1.09 (2s, 3'-P, 5'-P); MS (m/e) (negative-FAB) 533, 535 (peak height ratio = 3:1) ($M^+ - 1$), HRMS (negative-FAB) calcd for $\text{C}_{13}\text{H}_{16}\text{N}_4\text{O}_8\text{P}_2\text{Se}$ 532.9293, found 532.9295; HPLC 11.0 min (99%) in solvent system A, 15.5 min (99%) in solvent system B.

(1'*R*,2'*S*,4'*R*,5'*S*)-Acetic Acid 4-Hydroxy-5-hydroxymethylbicyclo[3.1.0]hex-2-yl Ester (32)

A mixture of dibenzyl alcohol **19** (137 mg, 0.422 mmol), acetic anhydride (140.4 mg, 1.38 mmol), triethylamine (436 mg, 4.3 mmol), 4-(dimethylamino)pyridine (10 mg, 0.2 mmol), and dichloromethane (5 mL) was stirred at room temperature for 4 h. The resulting mixture was partitioned between ethyl acetate (50 mL) and water/brine (1/1, 20 mL). The organic layer was washed with saturated aqueous sodium bicarbonate (10 mL) and brine (10 mL), dried over sodium sulfate, filtered, and concentrated in vacuo. The residue was used in the next reaction without further purification. The dibenzyl acetate **31** (crude 164 mg) was dissolved in methanol (5 mL). Five percent Pd/C (150 mg) was added. The reaction flask was applied to a vacuum and filled with hydrogen several times. The reaction mixture was stirred for 4 h at room temperature under a hydrogen balloon. The resulting mixture was filtrated through a pad of Celite and washed with methanol (10 mL \times 3). The residue was purified by flash column chromatography (chloroform/methanol = 10/1) to give a diol **32** (76 mg, 97% for two steps yield): ^1H NMR (CDCl_3) δ 5.13 (m, 1H), 4.45 (t, 1H, $J = 8.2$ Hz), 3.96 (d, 1H, $J = 11.8$ Hz), 3.24 (d, 1H, $J = 11.8$ Hz), 2.33 (dt, 1H, $J = 8.0, 13.2$ Hz),

2.01 (s, 3H), 1.66 (m, 1H), 1.27 (dt, 1H, $J = 8.8, 13.5$ Hz), 1.09 (dd, 1H, $J = 3.8, 5.5$ Hz), 0.55 (dd, 1H, $J = 5.5, 8.0$ Hz).

(1'R,2'S,4'R,5'S)-Acetic Acid 4-(Di-*tert*-butoxyphosphoryloxy)-5-(di-*tert*-butoxyphosphoryloxymethyl)-bicyclo[3.1.0]hex-2-yl Ester (33)

To a stirred suspension of diol **32** (25 mg, 0.134 mmol) and diethyl di-*tert*-butylphosphoramidite (100.5 mg, 0.402 mmol) in anhydrous tetrahydrofuran (4 mL) at room temperature was added solid tetrazole (94 mg, 1.34 mmol) in one pot. The reaction mixture was stirred at room temperature for 1 h and then cooled to -78 °C. To this cooled reaction mixture was added solid *m*-CPBA (57–85%, calculated by 57%, 240 mg), and the resulting mixture was stirred at -78 °C for 20 min and at room temperature for 10 min. After removal of tetrahydrofuran in vacuo, the residue was directly purified by flash column chromatography (CHCl₃/MeOH = 10/1) to give **33** (71 mg, 93%): ¹H NMR (CDCl₃) δ 4.87 (dd, 1H, $J = 8.0, 14.6$ Hz), 4.41 (m, 1H), 4.40 (dd, 1H, $J = 7.7, 11.0$ Hz), 3.69 (dd, 1H, $J = 6.9, 11.0$ Hz), 2.46 (dt, 1H, $J = 7.7, 13.5$ Hz), 1.78 (m, 1H), 1.485 (s, 9H), 1.478 (s, 9H), 1.469 (s, 9H), 1.457 (s, 9H), 1.39 (m, 1H), 1.25 (dd, 1H, $J = 5.0, 5.2$ Hz), 0.67 (dd, 1H, $J = 6.3, 7.4$ Hz).

(1'R,2'S,4'R,5'S)-Phosphoric Acid Di-*tert*-butyl Ester 1-(Di-*tert*-butoxyphosphoryloxymethyl)-4-hydroxybicyclo[3.1.0]hex-2-yl Ester (34)

Acetate **33** (70 mg, 0.122 mmol) was dissolved in methanol (3 mL), and then solid potassium carbonate (20 mg, 0.145 mmol) was added. After the reaction mixture was stirred for 2 h, methanol was removed in vacuo and the residue was purified by flash column chromatography (CHCl₃/MeOH = 30/1) to produce the bisphosphate alcohol **34** (51 mg, 79%): ¹H NMR (CDCl₃) δ 5.13 (m, 1H), 4.45 (t, 1H, $J = 8.2$ Hz), 3.96 (d, 1H, $J = 11.8$ Hz), 3.24 (d, 1H, $J = 11.8$ Hz), 2.33 (dt, 1H, $J = 8.0, 13.2$ Hz), 2.01 (s, 3H), 1.66 (m, 1H), 1.27 (dt, 1H, $J = 8.8, 13.5$ Hz), 1.09 (dd, 1H, $J = 3.8, 5.5$ Hz), 0.55 (dd, 1H, $J = 5.5, 8.0$ Hz); MS (*m/e*) (positive-FAB) 529.35 (M⁺ + 1), 551.31 (M⁺ + Na + 1).

2-Bromo-6-chloro-9H-purine (35)

To a stirred solution of **20** (0.35 g, 2 mmol) in CH₂Br₂ (5 mL) was added a solution of SbBr₃ in CH₂Br₂ (5 mL) at -10 °C. This solution was stirred at this temperature for 10 min, and *tert*-butyl nitrite (4.5 mL) was added. The reaction mixture was stirred for 2.5 h at -10 °C and then was poured into a stirred mixture of 2.4 g of NaHCO₃ in 100 mL of water and crushed ice. This mixture was filtered, and the filtrate was extracted with CHCl₃ and dried over Na₂SO₄. After removal of the solvent, the residue was purified by flash chromatography (MeOH/CHCl₃ = 1/10) to provide **35** (0.404 g, 87%): ¹H NMR (DMSO-*d*₆) δ 8.72 (s, 1H); MS (*m/e*) (positive-FAB) 233 (M⁺ + H).

6-Chloro-2-fluoro-9H-purine (36)

Compound **20** (100 mg, 0.572 mmol) was added to 60% HF–pyridine (which was prepared from 1.5 g of 70% HF–pyridine diluted with 0.25 g of pyridine) at -50 °C with stirring. The temperature was allowed to rise from -30 °C, and 0.1 mL of *tert*-butyl nitrite (0.1 mL) was added dropwise. Stirring was continued for 10 min, and the reaction mixture was poured into ice water. The aqueous layer was extracted with CHCl₃ and ethyl acetate. The combined organic layer was washed with H₂O, dried over Na₂SO₄, and filtered, and the filtrate was evaporated. The given residue was purified on a flash column chromatography (MeOH/CHCl₃ = 1/10) to give **36** (90 mg, 91%) as a white solid: ¹H NMR (CD₃OD) δ 8.5 (s, 1H); MS (*m/e*) (positive-FAB) 173 (M⁺ + H).

2-Fluoro-6-methylamino-9H-purine (37)

The mixture of **36** (56 mg, 0.324 mmol) in CH₃NH₂ (2.0 M in THF, 3 mL) was stirred at room temperature overnight. After removal of the solvent, the residue was purified by preparative thin-layer chromatography (MeOH/CHCl₃ = 1/10) to yield **37** (43 mg, 80%): ¹H NMR (DMSO-*d*₆) δ 8.12 (br, 1H), 8.06 (s, 1H), 3.30 (s, 1H), 2.91 (s, 3H); MS (*m/e*) (positive-FAB) 168 (M⁺ + H).

2-Bromo-6-methylamino-9H-purine (38)

The mixture of **35** (130 mg, 0.557 mmol) in CH₃NH₂ (40% in H₂O, 2 mL) was heated to 70 °C and stirred at this temperature for 6 h. The reaction mixture was cooled to room temperature, and the formed white precipitate was filtered and washed with a small amount of water/MeOH. The precipitate was dried in a vacuum to give **38** (118 mg, 93%): ¹H NMR (DMSO-*d*₆) δ 8.08 (s, 1H), 8.06 (bs, 1H), 3.30 (s, 1H), 2.91 (s, 3H); MS (*m/e*) (positive-FAB) 228 (M⁺ + H).

(1'*R*,2'*S*,4'*S*,5'*S*)-4-(2-Fluoro-6-methylamino-9H-purin-9-yl)-1-(di-*tert*-butoxyphosphoryloxymethyl)bicyclo[3.1.0]-hex-2-yl-phosphoric Acid Di-*tert*-butyl Ester (**44**)

To an ice-cold solution of triphenylphosphine (23.6 mg, 0.09 mmol) in dry THF (0.5 mL) was added DIAD (18.7 μL, 0.09 mmol) dropwise under a nitrogen atmosphere, and the mixture was stirred for 20 min until the solution turned red. This mixture was added dropwise to a cold, stirred mixture of **34** (14.4 mg, 0.027 mmol) and **37** (15.03 mg, 0.09 mmol) under a nitrogen atmosphere. The reaction mixture was stirred in an ice bath for 30 min and then allowed to warm to room temperature, and stirring continued for 12 h. Solvent was removed by nitrogen purge, and the residue was purified by preparative thin-layer chromatography (MeOH/CHCl₃ = 1/20) to produce **44** (8.61 mg, 47%) as a thick liquid: ¹H NMR (CDCl₃) δ 8.08 (s, 1H), 6.18 (br, 1H), 5.45–5.25 (m, 1H), 5.10–4.9 (m, 1H), 4.75–4.55 (m, 1H), 4.0–3.8 (m, 1H), 3.17 (s, 3H), 2.45–2.2 (m, 1H), 2.14–1.95 (m, 1H), 1.86–1.75 (m, 1H), 1.49 (s, 18H), 1.47 (s, 18H), 1.2–1.05 (m, 1H), 1.05–0.9 (m, 1H); MS (*m/e*) (positive-FAB) 678 (M⁺ + H).

(1'*R*,2'*S*,4'*S*,5'*S*)-4-(2-Bromo-6-methylamino-9H-purin-9-yl)-1-(di-*tert*-butoxyphosphoryloxymethyl)bicyclo[3.1.0]-hex-2-yl-phosphoric Acid Di-*tert*-butyl Ester (**45**)

To a solution of triphenylphosphine (15 mg, 0.057 mmol) in anhydrous THF (0.5 mL) was added DIAD (12 μL, 0.057 mmol) dropwise at 0 °C under a nitrogen atmosphere, and the mixture was stirred at this temperature for 20 min until the solution turned red. This reaction mixture was added slowly to a cold stirred solution of alcohol **34** (9.2 mg, 0.0174 mmol) and **38** (13.1 mg, 0.057 mmol) under a nitrogen atmosphere. The reaction mixture was stirred in an ice bath for 30 min and then allowed to warm to room temperature while stirring continued for 12 h. Solvent was removed by nitrogen purge, and the residue was purified by preparative thin-layer chromatography (MeOH/CHCl₃ = 1/20) to produce **45** (3.1 mg, 23%) as a thick liquid: ¹H NMR (CDCl₃) δ 8.05 (s, 1H), 5.92 (br, 1H), 5.45–5.25 (m, 1H), 5.15–5.0 (m, 1H), 4.75–4.55 (m, 1H), 3.95–3.80 (m, 1H), 3.18 (s, 3H), 2.4–2.2 (m, 1H), 2.20–2.0 (m, 1H), 1.85–1.7 (m, 1H), 1.58 (s, 18H), 1.49 (s, 18H), 1.2–1.05 (m, 1H), 1.05–0.9 (m, 1H); MS (*m/e*) (positive-FAB) 738 (M⁺ + H).

(1'*R*,2'*S*,4'*S*,5'*S*)-Phosphoric Acid Di-*tert*-butyl Ester 1-(Di-*tert*-butoxyphosphoryloxymethyl)-4-(2-iodo-6-methylaminopurin-9-yl)bicyclo[3.1.0]hex-2-yl Ester (**46**)

The alcohol **34** (10 mg, 0.0189 mmol), triphenylphosphine (20 mg, 0.0756 mmol), and 2-iodo-6-methylaminopurine **39** (10.4 mg, 0.0378 mmol) were mixed and dried under high vacuum for ~30 min. The residue was dissolved in anhydrous tetrahydrofuran (1 mL) and cooled to 0 °C. Diethyl azodicarboxylate (DEAD, 9.9 mg, 0.0567 mmol) was added dropwise via syringe to this solution. After the solution was stirred for 2 h at room temperature (the progress of the reaction was monitored by thin-layer chromatography), tetrahydrofuran was removed in vacuo. The residue was purified by preparative thin-layer chromatography (CHCl₃/MeOH = 20/1) to give the desired 2-iodo bis(di-*tert*-butyl phosphate) derivative **46** (7.1 mg, 48%): ¹H NMR (CDCl₃) δ 7.98 (s, 1H), 5.85 (bs, 1H, –NH), 5.35 (dd, 1H, *J* = 7.7, 14.3 Hz), 5.07 (d, 1H, *J* = 6.9 Hz), 4.66 (dd, 1H, *J* = 4.7, 11.0 Hz), 3.91 (dd, 1H, *J* = 6.6, 11.3 Hz), 3.17 (bs, 3H), 2.31 (dd, 1H, *J* = 8.0, 15.4 Hz), 2.09 (dt, 1H, *J* = 8.2, 14.8 Hz), 1.76 (dd, 1H, *J* = 4.4, 8.5 Hz), 1.32 (s, 18H), 1.29 (s, 9H), 1.26 (s, 9H), 1.10 (dd, 1H, *J* = 4.1, 6.0 Hz), 0.99 (m, 1H); MS (*m/e*) (positive-FAB) 786.3 (M⁺ + 1).

2-Amino-6-chloropurin-9-ylmethyl 2,2-Dimethylpropionate (54)

2-Amino-6-chloropurine **20** (1.00 g, 5.90 mmol) was dissolved in DMSO (5.0 mL) under heating. To this solution were added DMF (20.0 mL), chloromethyl pivalate **53** (1.0 mL, 6.94 mmol), and K₂CO₃ (990 mg, 7.16 mmol), and the mixture was stirred at room temperature for 3 days. The reaction mixture was filtered and the filtrate was evaporated. The obtained residue was purified by flash chromatography (AcOEt/petroleum ether = 2/1) and recrystallized from AcOEt/petroleum ether, which furnished **54** (1.33 g, 79%): ¹H NMR (CDCl₃) δ 8.01 (s, 1H), 6.00 (s, 2H), 5.20 (bs, 2H), 1.18 (s, 9H); MS (*m/e*) (positive-FAB) 284, 286 (peak height ratio 3:1) (M⁺ + 1).

6-Chloro-2-iodopurin-9-ylmethyl 2,2-Dimethylpropionate (55)

To a solution of 2-amino-6-chloropurin-9-yl-methyl 2,2-dimethylpropionate **54** (704 mg, 2.48 mmol) in MeCN (2.0 mL) were added diiodomethane (8.0 mL) and *tert*-butylnitrite (0.90 mL, 9.99 mmol), and oxygen was purged by N₂ bubbling. The tube was sealed and the contents stirred at 80 °C for 2.5 h. The solvent was removed under vacuum and the obtained residue was purified by flash chromatography (AcOEt/petroleum ether = 1/2), which furnished **55** (561 mg, 57%): ¹H NMR (CDCl₃) 8.29 (s, 1H), 6.14 (s, 2H), 1.19 (s, 9H); MS (*m/e*) (positive-FAB) 395, 397 (peak height ratio 3:1) (M⁺ + 1).

6-Chloro-2-iodopurine (43)

To a solution of 6-chloro-2-iodopurin-9-ylmethyl 2,2-dimethylpropionate **55** (125 mg, 0.317 mmol) in *i*-PrOH (5.0 mL) and H₂O (0.500 mL) was added 5 N aq NaOH (0.200 mL) and the mixture stirred at room temperature for 3 days. The solvent was removed under reduced pressure, the residue was poured into saturated aq NaHCO₃ and extracted with THF–AcOEt. The organic layer was washed with brine, dried over Na₂SO₄, and evaporated. The residue obtained was purified by flash chromatography (AcOEt), which furnished **43** (42 mg, 48%): ¹H NMR (DMSO) δ 8.63 (s, 1H); MS (*m/e*) (positive-FAB) 281, 283 (peak height ratio 3:1) (M⁺+1).

(1'*R*,2'*S*,4'*S*,5'*S*)-Phosphoric Acid Di-*tert*-butyl Ester 1-(Di-*tert*-butoxyphosphoryloxymethyl)-4-(6-chloro-2-iodopurin-9-yl)bicyclo[3.1.0]hex-2-yl Ester (**51**)

To a solution of triphenylphosphine (70 mg, 0.26 mmol) in anhydrous THF (0.50 mL) was added DEAD (0.035 mL, 0.22 mmol) dropwise at –20 °C with stirring for 1.0 h. Compound **34** (18 mg, 0.035 mmol) and 6-chloro-2-iodopurine **43** (65 mg, 0.23 mmol) in THF (1.50

mL) were added to the reaction mixture and it stirred at room temperature for 3 days. The solvent was removed under vacuum and the residue obtained was purified by preparative thin-layer chromatography (AcOEt), which furnished **51** (15.5 mg, 57%): $^1\text{H NMR}$ (CDCl_3) δ 8.44 (s, 1H), 5.34 (dd, 1H, $J = 8.1, 15.0$ Hz), 5.16 (d, 1H, $J = 6.9$ Hz), 4.69 (dd, 1H, $J = 5.1, 11.4$ Hz), 3.94 (dd, 1H, $J = 6.6, 11.4$ Hz), 2.40–2.30 (m, 1H), 2.22–2.10 (m, 1H), 1.85–1.80 (m, 1H), 1.50 (s, 9H), 1.49 (s \times 2, 18H), 1.48 (s, 9H), 1.18–1.14 (m, 1H), 1.09–1.03 (m, 1H); MS (m/e) (positive-FAB) 791, 793 (peak height ratio 3:1) ($\text{M}^+ + 1$).

(1'*R*,2'*S*,4'*S*,5'*S*)-Phosphoric Acid Di-*tert*-butyl Ester 1-(Di-*tert*-butoxyphosphoryloxymethyl)-4-(2-iodo-6-methylaminopurin-9-yl)bicyclo[3.1.0]hex-2-yl Ester (46)

To a solution of 6-chloro-2-methylpurin-9-ylmethyl 2,2-dimethylpropionate **51** (13.0 mg, 0.016 mmol) in THF (0.10 mL) was added 2.0 N MeNH_2 in THF (1.0 mL) and the mixture stirred for 3 h. The solvent was removed under reduced pressure and the obtained residue was purified by flash chromatography ($\text{CHCl}_3/\text{MeOH} = 5/1$), which furnished **46** (6.1 mg, 47%).

6-Chloro-2-trimethylstannylpurin-9-ylmethyl 2,2-Dimethylpropionate (56)

To a solution of 6-chloro-2-iodopurin-9-ylmethyl 2,2-dimethylpropionate **55** (323 mg, 0.819 mmol) and $\text{PdCl}_2(\text{PPh}_3)_2$ (30 mg, 0.043 mmol) in dioxane (0.75 mL) was added $\text{Me}_3\text{SnSnMe}_3$ (820 mg, 2.50 mmol) in dioxane (2.0 mL) and the mixture stirred at 100 °C for 5 h. The solvent was removed under vacuum and the residue obtained was purified by flash chromatography (AcOEt/petroleum ether = 1/2), which furnished **56** (310 mg, 88%): $^1\text{H NMR}$ (CDCl_3) δ 8.26 (s, 1H), 6.21 (s, 2H), 1.18 (s, 9H), 0.45 (s, 9H); MS (m/e) (positive-FAB) 431($\text{M}^+ + 1$).

6-Chloro-2-methylpurin-9-ylmethyl 2,2-Dimethylpropionate (57)

A mixture of tris(dibenzylideneacetone)dipalladium(0) chloroform adduct ($\text{Pd}_2(\text{dba})_3$, 16 mg, 0.031 mmol), tri *o*-tolylphosphine ($\text{P}(\text{o-Tol})_3$, 31 mg, 0.102 mmol), CuBr (19 mg, 0.135 mmol), and K_2CO_3 (26 mg, 0.188 mmol) in a flask was dried under reduced pressure, and the vacuum was replaced by dry nitrogen. A solution of 6-chloro-2-trimethylstannylpurin-9-ylmethyl 2,2-dimethylpropionate **56** (109 mg, 0.253 mmol) in DMF (2.0 mL) and MeI (0.15 mL, 2.41 mmol) was added, and the mixture was stirred at 60 °C for 3 h. The resulting reaction mixture was directly pass through a short column (SiO_2 , 2.0 mL, eluent AcOEt) and evaporated. The residue obtained was purified by flash chromatography (AcOEt/petroleum ether = 1/2), which furnished **57** (45 mg, 62%): $^1\text{H NMR}$ (CDCl_3) δ 8.29 (s, 1H), 6.16 (s, 2H), 2.83 (s, 3H), 1.18 (s, 9H); MS (m/e) (positive-FAB) 283, 285 (peak height ratio 3:1) ($\text{M}^+ + 1$).

6-Methylamino-2-methylpurine (40)

To a solution of 6-chloro-2-methylpurin-9-yl-methyl 2,2-dimethylpropionate **57** (34 mg, 0.120 mmol) in THF (1.0 mL) was added 2.0 N MeNH_2 in THF (2.0 mL) and the mixture stirred for 22 h. The solvent was removed under reduced pressure and the obtained residue was purified by preparative thin-layer chromatography (first, AcOEt; second, $\text{CHCl}_3/\text{MeOH} = 5/1$), which also furnished 6-methylamino-2-methylpurine **40** (27 mg, 81%) and 6-methylamino-2-methylpurin-9-ylmethyl 2,2-dimethylpropionate (4 mg, 12%): $^1\text{H NMR}$ (DMSO) δ 7.96 (s, 1H), 7.40 (bs, 1H), 2.94 (s, 3H), 2.40 (s, 3H); MS (m/e) (positive-FAB) 164 ($\text{M}^+ + 1$).

6-Methylamino-2-methylpurin-9-yl-methyl 2,2-dimethylpropionate

$^1\text{H NMR}$ (DMSO) δ 8.11 (s, 1H), 7.65 (bs, 1H), 6.10 (s, 2H), 2.94 (s, 3H), 2.44 (s, 3H), 1.10 (s, 9H); MS (m/e) (positive-FAB) 278 ($\text{M}^+ + 1$).

(1'*R*,2'*S*,4'*S*,5'*S*)-Phosphoric Acid Di-*tert*-butyl Ester 1-(Di-*tert*-butoxyphosphoryloxymethyl)-4-(2-methyl-6-methylaminopurin-9-yl)bicyclo[3.1.0]hex-2-yl Ester (47)

To a solution of triphenylphosphine (52 mg, 0.198 mmol) in anhydrous THF (0.40 mL) was added DEAD (0.030 mL, 0.191 mmol) dropwise at -20°C with stirring for 1.0 h. Compound **34** (16.0 mg, 0.030 mmol) and 6-methylamino-2-methylpurine **40** (27 mg, 0.0975 mmol) in THF (1.50 mL) were added to the reaction mixture, which stirred at room temperature for 3 d. The solvent was removed under vacuum and the residue obtained was purified by preparative thin-layer chromatography (AcOEt), which furnished **47** (10.0 mg, 49%): $^1\text{H NMR}$ (CDCl_3) δ 7.99 (s, 1H), 5.62 (br, 1H), 5.38 (m, 1H), 5.09 (d, 1H, $J = 6.9$ Hz), 4.65 (dd, 1H, $J = 4.5, 11.4$ Hz), 3.92 (dd, 1H, $J = 6.3, 11.4$ Hz), 3.22 (s, 3H), 2.60 (s, 3H), 2.40–2.29 (m, 1H), 2.18–2.05 (m, 1H), 1.80 (m, 1H), 1.49 (s, 18H), 1.47 (s, 9H), 1.45 (s, 9H), 1.13–1.08 (m, 1H), 1.01–0.95 (m, 1H).

(1'*R*,2'*S*,4'*S*,5'*S*)-Phosphoric Acid Di-*tert*-butyl Ester 1-(Di-*tert*-butoxyphosphoryloxymethyl)-4-(2-hexyl-6-methylaminopurin-9-yl)bicyclo[3.1.0]hex-2-yl Ester (48)

2-Hexynyl bis(di-*tert*-butyl phosphate) derivative **50** (13 mg, 0.0176 mmol) was dissolved in methanol (5 mL), and 5% Pd/C (150 mg) was added. The reaction flask was applied to a vacuum and filled with hydrogen several times. The reaction mixture was stirred for 2 h at room temperature under a hydrogen balloon. The resulting mixture was filtrated through a pad of Celite and washed with methanol (5 mL \times 3). The residue was purified by preparative thin-layer chromatography ($\text{CHCl}_3/\text{MeOH} = 20/1$) to give the desired 2-hexanyl bis(di-*tert*-butyl phosphate) derivative **48** (11.5 mg, 88%): $^1\text{H NMR}$ (CDCl_3) δ 8.02 (s, 1H), 5.62 (bs, 1H, $-\text{NH}$), 5.35 (dd, 1H, $J = 7.7, 14.6$ Hz), 5.12 (d, 1H, $J = 6.9$ Hz), 4.64 (dd, 1H, $J = 4.4, 11.0$ Hz), 3.90 (dd, 1H, $J = 6.3, 11.0$ Hz), 3.23 (d, 3H, $J = 4.9$ Hz), 2.81 (dd, 2H, $J = 7.7, 8.0$ Hz), 2.34 (dd, 1H, $J = 7.7, 15.1$ Hz), 2.09 (dt, 1H, $J = 8.0, 15.7$ Hz), 1.23–1.88 (m, 9H), 1.497 (s, 9H), 1.489 (s, 9H), 1.479 (s, 9H), 1.455 (s, 9H), 1.13 (dd, 1H, $J = 4.4, 5.8$ Hz), 0.98 (dd, 1H, $J = 6.3, 8.5$ Hz), 0.89 (m, 3H); MS (m/e) (positive-FAB) 744.5 ($\text{M}^+ + 1$).

(1'*R*,2'*S*,4'*S*,5'*S*)-Phosphoric Acid Di-*tert*-butyl Ester 1-(Di-*tert*-butoxyphosphoryloxymethyl)-4-(2-hex-1-enyl-6-methylaminopurin-9-yl)bicyclo[3.1.0]hex-2-yl Ester (49)

2-Hexenyl bis(di-*tert*-butyl phosphate) derivative **49** (6.1 mg, 44%) was prepared by a method similar to that for **46**, except 2-hexenyl-6-methylaminopurine **41** (10 mg, 0.0189 mmol) was used as the starting material: $^1\text{H NMR}$ (CDCl_3) δ 8.01 (s, 1H), 7.11 (dt, 1H, $J = 6.9, 15.4$ Hz), 6.48 (dt, 1H, $J = 1.4, 15.6$ Hz), 5.62 (bq, 1H, $-\text{NH}$), 5.38 (dd, 1H, $J = 7.7, 14.3$ Hz), 5.14 (d, 1H, $J = 6.9$ Hz), 4.65 (dd, 1H, $J = 4.7, 11.3$ Hz), 3.91 (dd, 1H, $J = 6.3, 11.3$ Hz), 3.24 (d, 3H, $J = 5.0$ Hz), 2.35 (dd, 1H, $J = 8.5, 15.4$ Hz), 2.29 (m, 2H), 2.11 (dt, 1H, $J = 7.7, 15.4$ Hz), 1.80 (dd, 1H, $J = 4.1, 8.5$ Hz), 1.33–1.61 (m, 4H), 1.496 (s, 9H), 1.490 (s, 9H), 1.482 (s, 9H), 1.446 (s, 9H), 1.13 (dd, 1H, $J = 4.4, 5.8$ Hz), 0.98 (dd, 1H, $J = 6.3, 8.5$ Hz), 0.94 (m, 3H); MS (m/e) (positive-FAB) 742.5 ($\text{M}^+ + 1$).

(1'*R*,2'*S*,4'*S*,5'*S*)-Phosphoric Acid Di-*tert*-butyl Ester 1-(Di-*tert*-butoxyphosphoryloxymethyl)-4-(2-hex-1-ynyl-6-methylaminopurin-9-yl)bicyclo[3.1.0]hex-2-yl Ester (50)

2-Hexynyl bis(di-*tert*-butyl phosphate) derivative **50** (20.1 mg, 72%) was prepared by a method similar to that for **44**, except 2-hexynyl-6-methylaminopurine **42** (20 mg, 0.0378 mmol) was used as the starting material: $^1\text{H NMR}$ (CDCl_3) δ 8.14 (s, 1H), 5.77 (bs, 1H), 5.33 (dd, 1H, $J = 8.2, 14.8$ Hz), 5.21 (d, 1H, $J = 6.9$ Hz), 4.67 (dd, 1H, $J = 4.4, 11.3$ Hz), 3.83 (dd, 1H, $J = 6.6, 11.3$ Hz), 3.25 (bs, 3H), 2.46 (t, 2H, $J = 7.1$ Hz), 2.29 (dd, 1H, $J = 8.0, 15.4$ Hz), 2.08 (m, 1H), 1.60–1.82 (m, 5H), 1.499 (s, 9H), 1.488 (s, 9H), 1.472 (s, 9H), 1.462 (s, 9H), 1.12 (dd, 1H, $J = 4.4, 5.8$ Hz), 0.9 (m, 1H), 0.94 (t, 3H, $J = 7.1$ Hz); MS (m/e) (positive-FAB) 740.5 ($M^+ + 1$).

(1'*R*,2'*S*,4'*S*,5'*S*)-Phosphoric Acid Di-*tert*-butyl Ester 1-(Di-*tert*-butoxyphosphoryloxymethyl)-4-(6-chloro-2-trimethylstannylpurin-9-yl)bicyclo[3.1.0]hex-2-yl Ester (52)

To a solution of **51** (2.7 mg, 0.0034 mmol) and $\text{PdCl}_2(\text{PPh}_3)_2$ (3.5 mg, 0.0050 mmol) in dioxane (0.30 mL) was added $\text{Me}_3\text{SnSnMe}_3$ (20 mg, 0.061 mmol) and the mixture stirred at 60 °C for 3.5 h. The solvent was removed under vacuum and the residue obtained was purified by preparative TLC (AcOEt), which furnished **52** (0.7 mg, 25%): $^1\text{H NMR}$ (CDCl_3) δ 8.42 (s, 1H), 5.40–5.20 (m, 2H), 4.65 (m, 1H), 3.90 (m, 1H), 2.40–2.25 (m, 1H), 2.25–2.10 (m, 1H), 1.90–1.80 (m, 1H), 1.50 (s, 9H), 1.48 (s, 18H), 1.44 (s, 9H), 1.25–1.20 (m, 1H), 1.05–1.00 (m, 1H), 0.43 (s, 9H); MS (m/e) (positive-FAB) 827 ($M^+ + 1$), 849 ($M^+ + \text{Na}$).

Small-Scale Conversion of Compound 52 into Compound 12

A 20 μL (0.35 μmol) portion of an iodine solution (4.5 mg of I_2 in 1.00 mL of AcOEt) was added to a solution of **52** (0.15 mg, 0.18 μmol) in AcOEt (0.100 mL) and the mixture stirred at room temperature for 10 min. Then 0.01 mL of 5% aqueous NaHSO_3 was added to stop the reaction, and the solvent was removed under vacuum. The residue (crude **29**) was dissolved in 5% TFA/ CH_2Cl_2 (0.50 mL) and stirred for 3 h at 25 °C. The solvent was removed under reduced pressure, and the residue was quenched by addition of 5.0 mL of triethylammonium bicarbonate buffer (1.0 M). The mixture was subsequently frozen and lyophilized. The residue (crude **29**) was dissolved in 40% MeNH_2 in water (0.50 mL) and stirred for 3 h at room temperature. The solvent was removed under reduced pressure. The residue (crude **12**) was purified by HPLC (system A or system B).

Enzymatic Synthesis of 5

ATP (20 nmol, 2 μL of a 10-mM solution in water) and polynucleotide kinase (Sigma, 10 IU) were added to 20 μL of a buffer consisting of 50 mM Tris HCl, 10 mM MgCl_2 , 5 mM dithiothreitol, 0.1 mM spermidine, and 0.1 mM EDTA. The nucleotide substrate **58**⁴¹ (1 μL of a 10-mM solution in water) was then added, and the mixture was mixed with vortexing and incubated at 37 °C for the time indicated (18 h incubation was found to be optimal). Aliquots (10 μL) were injected for HPLC analysis. A C18 Zorbax column (Agilent) was used, with the flow rate 1 mL/min and the mobile phase consisting of a gradient of acetonitrile/0.1 M triethylammonium acetate (aq) of 10/90 to 15/85 in 10 min, then to 30/70 in 10 min. The product of the enzymatic transformation (**5**) was detected in ~40% yield, as judged with HPLC. The retention time of the product was 6.2 min, compared with the starting material, which had a retention time of 8.1 min. These three individual peaks were collected and lyophilized. Mass spectrometry (FAB) confirmed the identity of the starting material (m/z 387, $M - 1$) and product **5** (m/z 468, $M - 1$). The side product at retention time 11.6 min displayed an m/z of 310, consistent with the corresponding nucleoside ($M + 1$). An unidentified impurity present in the starting material was also detected at 7.7 min.

Compound **58** was found to be chemically stable. The HPLC profile of a solution in H₂O/ acetonitrile/buffer was unchanged following 60 h incubation at 37 °C. However, when enzyme was added in the absence of ATP, ~20% of the compound was dephosphorylated during an incubation of 17 h to the nucleoside **59** (HPLC retention time = 11.6 min). Under the same incubation conditions in the presence of the enzyme, **5** was stable.

Pharmacological Analyses

The affinities of 2-substituted (*N*)-methanocarbo bisphosphate analogues for the human P2Y₁ receptor were directly determined by using [³H]**5** in a radioligand binding assay, as we recently described in detail.³¹ Briefly, the human P2Y₁ receptor was expressed to high levels in Sf9 insect cells with a recombinant baculovirus. Membranes prepared from these cells were incubated for 30 min at 4 °C in the presence of ~20 nM [³H]**5** and a wide range of concentrations of the newly synthesized analogues. Binding reactions were terminated by the addition of ice-cold tris-(hydroxymethyl)aminomethane (Tris) wash buffer (10 mM Tris, pH 7.5, and 145 mM NaCl) to the samples followed by rapid filtration over GF/A glass-fiber filters. Each filter was washed with an additional 4 mL of wash buffer, and radioactivity retained by the filters was quantified by liquid scintillation spectrometry. All assays were carried out in triplicate, and competition curves for all molecules were generated in three separate experiments. IC₅₀ values were determined from each competition curve, and a *K*_i value was calculated for each analogue according to the relationship $K_i = IC_{50}/1 + [^3H]5/K_d$ of [³H]**5**, where the *K*_d of [³H]**5** determined in separate experiments was 8 nM.

P2Y receptor-promoted stimulation of inositol phosphate formation was measured at human P2Y₁ receptors stably expressed in 1321N1 human astrocytoma cells as previously described.^{19,42,43} The *K*_{0.5} values were averaged from three to eight independently determined concentration-effect curves for each compound. Briefly, cells plated in 24-well dishes were labeled in inositol-free medium (DMEM; Gibco, Gaithersburg, MD) containing 1.0 μCi of 2-[³H]*myo*-inositol (20 Ci/mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO) for 18–24 h in a humidified atmosphere of 95% air/5% CO₂ at 37 °C. Phospholipase C activity was measured the following day by quantitating [³H]inositol phosphate accumulation after a 10-min incubation at 37 °C in the presence of 10 mM LiCl. Total [³H]inositol phosphates were quantified by anion-exchange chromatography as previously described.^{42,43}

Data Analysis

IC₅₀ values obtained in radioligand binding assays and in assays of inhibition of 2MeSADP-stimulated inositol phosphate accumulation were calculated by a four-parameter logistic equation and the GraphPad software package (GraphPad, San Diego, CA). All concentration–effect curves were repeated in at least three separate experiments, carried out in duplicate or triplicate with different membrane preparations or different 1321N1 cell cultures.

Results

Chemical Synthesis

We prepared methanocarbo cyclic analogues of various bisphosphate derivatives (Table 1) in which fused cyclopropane and cyclopentane rings were fixed the pseudoribose moiety in a rigid (*N*)-envelope conformation. Synthetic intermediates included *O*-benzyl-protected phosphoesters, carbocyclic derivatives, adenine derivatives, and *O*-*tert*-butyl-protected phosphoesters (Schemes 1–5). The nucleotide analogues **7–18** were prepared in the ammonium salt form according to the methods shown in Schemes 1 and 4 and tested biologically as antagonists at the P2Y₁ receptor (Table 1). Identity was confirmed by NMR

(^1H and ^{31}P) and high-resolution mass spectrometry (HRMS), and purity was demonstrated with high-performance liquid chromatography (HPLC) in two different solvent systems.

To construct the pseudoribose ring leading to the 2'-deoxy-(*N*)-methanocarpa-2'-deoxyadenosine precursors, a bicyclo[3.1.0]hexane intermediate **19** was prepared by the new, optimized approach of Yoshimura et al.,³² which used simple starting materials and enzymatic transformations. Scheme 1 illustrates the usual synthetic route to the bisphosphate derivatives, in which the phosphorylation was carried out on the preformed nucleoside precursor. To prepare that nucleoside, a commercially available 2-substituted adenine intermediate, 6-chloro-2-aminopurine **20**, was condensed with the protected bicyclohexane derivative **19** via a Mitsunobu reaction to give **21**, which was then deprotected to give diol **22**. The 3'- and 5'-hydroxyl groups then were phosphorylated by the phosphoramidite method³³ using dibenzyl diisopropyl phosphoramidite **23** to give the benzyl-protected derivative **24**. The 2-amino group of **24** was replaced with an iodo, methylthio, or methylseleno group (protected intermediates **25–27**) with the use of a neutral purinyl radical, which was generated transiently from the thermal homolysis of the 2-diazonium intermediate. This was accomplished by using the approach introduced by Nair and Fasbender³⁴ for the preparation of 2-methylthio and other 2-derivatives of adenosine. Selenium was selected for inclusion because of its similarity to sulfur and because of its known use in X-ray crystallography of proteins, when incorporated into ligands as a heavy atom.³⁵ To introduce an amino or methylamino group at the 6-position, it was necessary to substitute 6-Cl with the corresponding amine, which could be accomplished either before (to give **13**) or following (to give **7**, **8**, **12**, or **14**) deprotection of the phosphate groups. First we synthesized the *N*⁶-methyl-2-methylthio derivative **13**, following the procedure in Scheme 1. However, we were unable to apply this route for the corresponding *N*⁶-amino derivative **8** because treatment of **26** with ammonia to replace the 6-chloro group caused side reactions. Therefore, we carried out an ammonia replacement reaction following deprotection of the benzyl groups to obtain the *N*⁶-amino derivative **8**. We used similar procedures of phosphoester deprotection followed by replacement of the 6-chloro group for the preparation of the 2-methylseleno derivative **14**.

As an alternate synthetic route to the bisphosphate derivatives, phosphate groups were introduced into a versatile phosphorylated bicyclo[3.1.0]hexane precursor **34**, protected with *tert*-butyl ester groups, prior to the Mitsunobu coupling (Scheme 2).

We first attempted to synthesize 2-fluoro **10** and 2-bromo **11** derivatives by the approach shown in Scheme 1, which used the phosphoramidite route to phosphorylate the corresponding nucleoside diol derivative.¹⁸ However, only traces of the products were obtained due to side reactions, which seemed to result from the high nucleophilicity of the 6-amino group. Thus, an alternate route was needed. For substitution of the 2-position of the purine ring with bromo or fluoro, the corresponding adenine precursors **37** and **38** were prepared (Scheme 3). We used **20**, a commercially available material, to prepare the dihaloadenines **35** and **36** by a method from the literature.³⁶ Subsequent methylation of the 6-position gave **37** and **38**, which were coupled with the methanocarpa moiety **34** by a Mitsunobu reaction. 2-Iodo-*N*⁶-methyladenine **39** was prepared with the method of Nair and Fasbender.³⁴ The two 2-alkene/alkyne-substituted adenines **41** and **42** were prepared as previously reported.³⁷ These 2-haloadenine derivatives and several 2-C-substituted analogues were incorporated into the nucleoside structure as shown in Scheme 4, via the protected bicyclo[3.1.0]-hexane precursor **34**. Following the Mitsunobu reaction, the hexynyl intermediate **50** was reduced to the corresponding alkane to give **48**. Deprotection of the *tert*-butyl groups was carried out with TFA to give the bisphosphate derivatives **10–12** and **15–18**.

The preparation of additional 2-substituted adenine intermediates, including 6-chloro-2-iodopurine **43** used in Scheme 4, required a separate synthetic strategy (Scheme 5). The adenine precursor **20** dissolved in polar organic solvents, e.g. pyridine or DMSO, only to a limited extent, which made the selection of transformation conditions difficult. To proceed further, we applied a protecting group at the N-9 position, expecting an improvement in solubility. The pivaloyloxymethyl group was chosen as the protecting group, and **54** was prepared with the method of Rasmussen and Leonard.⁴¹ Compound **54** dissolved well in the usual organic solvents, allowing the 2-iodination of **54** to be performed by the method of Nair and Fasbender.³⁴

With the success of [³H]**5** as a selective radioligand for P2Y₁ receptor characterization,³¹ we sought to design new antagonists in this series as high-affinity receptor probes. As a route to a novel iodinated analogue that could be potentially radiolabeled, we explored the substitution of the iodo group of **12** (Scheme 4) and its adenine precursors (Scheme 5) with trimethyltin.^{38,39} This provides a means of rapidly obtaining the iodinated ligand **12**. Similar methodology based on trialkyl tin precursors has been used to prepare radioiodinated ligands for SPECT (single photon emission computed tomography).^{39,40} The 2-iodo product **55** was converted to the 2-trimethylstannyl derivative **56** by the method of Baranowska-Kortylewicz et al.⁴² Compound **56** was a useful intermediate because it could serve as a suitable substrate for Stille coupling; thus, we decided to prepare the 2-methyl derivative. Compound **56** reacted with methyl iodide under the conditions of Suzuki et al.⁴³ The product **57** was treated with methylamine to make **40**, which was coupled with the methanocarpa moiety by a Mitsunobu reaction. Deprotection was carried out with TFA to give the bisphosphate derivative **15**.

As an alternate route to introduce iodine in the antagonist **12**, we also attempted stannylation of **46** with the method of Baranowska-Kortylewicz et al., to be followed by reiodination.⁴² However, we obtained only the 2-H derivative, which formed upon replacement of the trimethylstannyl group by hydrogen, which was readily available from an acidic proton such as the 6-NH(Me). As an alternative (Scheme 4), we tried stannylation using a 6-chloro derivative **51**. This intermediate was synthesized from **43**, which was obtained by hydrolysis of **55**, and was coupled with the methanocarpa moiety by a Mitsunobu reaction. Intermediate **51** was successfully stannylated with the method of Baranowska-Kortylewicz et al.⁴² The trimethyl stannyl derivative **52** reacted with iodine (which could be generated rapidly from iodide) and re-formed **51**. By mass spectral analysis of the iodination reaction at various time points (1–40 min), an optimal reaction time was determined to be 10 min. Deprotection of **51** on a small scale with TFA was accomplished to give the bisphosphate derivative **29**, which was then converted to the antagonist **12** (Scheme 1).

We have explored a synthetic methodology that would allow a phosphate group to be incorporated in a potent P2Y₁ receptor antagonist in the final synthetic step. Although chemical phosphorylation methods were used until now to prepare P2Y₁ receptor antagonists in this series, we devised an enzymatic method for the phosphorylation at the 5'-position, leading to compound **5**. This method (Scheme 6) is based on phosphorylation with polynucleotide kinase (EC 2.7.1.78).⁴⁴ The 3'-monophosphorylated intermediate **58**⁴⁵ was treated with the enzyme and ATP as the phosphate source. Following incubation for 60 min, the product **5** was detected in ~40% yield, as judged by using HPLC. The intermediate **58** was found to be chemically stable under the incubation conditions. A side reaction, removal of the 3'-phosphate group of **58** to yield the nucleoside analogue **59**, occurred to a significant degree only in the absence of ATP.

Pharmacological Activity

We recently developed [³H]**5** as a high-affinity and selective radioligand for quantification of the P2Y₁ receptor.³¹ Therefore, the human P2Y₁ receptor can be expressed from a baculovirus to high levels in Sf9 insect cells, and membranes prepared from these cells can be labeled specifically with [³H]**5** to directly assess the affinity of newly synthesized molecules at the P2Y₁ receptor. Therefore, human P2Y₁ receptor-expressing membranes were incubated with 10 nM [³H]**5** and a wide range of concentrations of 2-substituted (N)-methanocarba bisphosphate analogues. As illustrated in Figure 1, all of the bisphosphate derivatives tested interacted with the P2Y₁ receptor, as shown by their capacity to inhibit [³H]**5** binding, and this inhibition occurred with kinetics consistent with interaction by the law of mass action kinetics at a single site. IC₅₀ values were determined (Table 1) from each competition curve, and a K_i value was calculated for each analogue according to the relationship $K_i = IC_{50}/1 + [^3H]5/K_d$ of [³H]**5**.

We also tested the functional activities of (N)-methanocarba bisphosphate analogues as agonists and antagonists at the human P2Y₁ receptor stably expressed in 1321N1 human astrocytoma cells (Table 1). Agonist activity was tested by measuring the capacity of molecules to increase inositol phosphate accumulation by activating the phospholipase C-coupled P2Y₁ receptor, and antagonist activity at the P2Y₁ receptor was assessed by measuring the capacity of these molecules to inhibit 2-methylthio-adenosine 5'-diphosphate (2Me-SADP)-stimulated accumulation of inositol phosphates.

None of these 2-substituted molecules exhibited agonist activity in the 1321N1 cell-test system, but all displayed a capacity to inhibit 2MeSADP-stimulated inositol phosphate accumulation, with an IC₅₀ value consistent with their K_i values determined in binding experiments. Within the series of 2-halo analogues, the most potent antagonist in both functional and binding models was a (N)-methanocarba-2-iodo analogue **12**, and all other 2-halo derivatives were clearly less potent. This derivative displayed a K_i value in competition for binding of [³H]**5** at the hP2Y₁ receptor of 0.79 nM. The N⁶-methyl derivative **12** was 16-fold more potent in binding than the corresponding 6-amino analogue **7**. The corresponding N⁶-methyl 2-bromo and 2-chloro derivatives **11** and **5** exhibited similar K_i values and affinities that were 5–10-fold less than that for **12**, and the fluoro derivative **10** exhibited a much lower affinity (Figure 1). The (N)-methanocarba-2-methylthio-, 2-methylseleno-, 2-hexyl-, 2-(1-hexenyl), and 2-(1-hexynyl) analogues exhibited lower affinities than did most of the 2-halo derivatives, with K_i values in the micromolar range observed for these compounds at P2Y₁ receptors. No significant difference was observed between the alkene and alkyne derivatives **17** and **18**, whereas the corresponding alkane **16** exhibited a 4–5-fold-lower affinity, with a K_i value in binding calculated to be 80 nM. However, the 2-methyl derivative **15** was nearly as potent as the corresponding 2-chloro analogue **5**.

Schild analysis of antagonism by **12** of inositol phosphate accumulation in 1321N1 human astrocytoma cells stably expressing the human P2Y₁ receptor, stimulated by 2-MeS-ADP, was carried out (Figure 2). A K_B value of 1.74 nM was determined, in close agreement with binding data for this compound.

A correlation of binding affinities (expressed as K_i values) and functional antagonistic potencies (expressed as IC₅₀ values) at the human P2Y₁ receptor was examined for nine compounds (Figure 3). The degree of correlation was high, with an r² value of 0.98. Thus, as we showed recently in radioligand binding assays with [³H]**5** and previously studied bisphosphate analogues,³¹ the radioligand binding assay is a readily applicable predictor of the functional effects of the P2Y₁ receptor antagonists.

Discussion

In previous studies,^{18,20} we concluded that substitution of the ribose moiety with the (*N*)-methanocarba ring system provides an enhancement of affinity at the P2Y₁ receptor. We strengthened this conclusion in the present study. For example, the compounds homologous to **8**, **12**, and **13** were reported in the ribose series,¹⁷ and the IC₅₀ values in the same phospholipase C assay in turkey erythrocytes expressing an endogenous P2Y₁ receptor were 1890, 891, and 362 μM, respectively. This is to be compared to the values for **8**, **12**, and **13** of 367, 8.4, and 221 nM, respectively. Thus, the enhancement achieved upon conformationally constraining the ribose-like ring in the preferred (*N*)-conformation was present in all three cases. However, the degree of enhancement ranged from slight, in the case of the *N*⁶-methyl-2-methylthio analogue **13**, to dramatic, i.e., 106-fold, in the case of the *N*⁶-methyl-2-iodo analogue **12**.

In the present study, we focused on optimization of the 2-position substitution. Halo substitution demonstrated a potency order of I > Br, Cl > F, which did not correspond to the relative effects of these substitutions in the ribose series.¹⁷ The 2-alkylthio substitution, which was found to greatly enhance potency in the agonist series, i.e., 5'-mono-, di-, and triphosphates,^{21–23} did not enhance antagonist potency in the present series. This observation is similar to previous findings with other nucleotide antagonists of the P2Y₁ receptor.¹⁷ In contrast with C-substitution at the 2-position of adenine reported previously²⁴ (i.e., 2-methyl in the ribose series), we did not find an enhancement of affinity in the alkene and alkyne analogues synthesized here.

The methods explored in this study for incorporation of iodo and phosphate groups from simple chemical precursors might be suitable for the preparation of radioligands (containing ¹²⁵I, ³³P, etc.) of high affinity for the P2Y₁ receptor.

In conclusion, we have enhanced the potency of ring-constrained P2Y₁ receptor antagonists by 2-position substitution. The structure–activity relationship (SAR) patterns were not entirely in parallel to the SAR observed in the ribose series. Thus, subtle differences in the mode of receptor binding probably occur between ribose-containing and (*N*)-methanocarba nucleotides. A key finding in this study is that compound **12** is the most potent antagonist selective for the P2Y₁ receptor yet reported. These findings promise to be useful in defining the microscopic determinants of the binding sites on these receptors and in designing novel pharmacological probes, including radioligands, and/or therapeutic agents.

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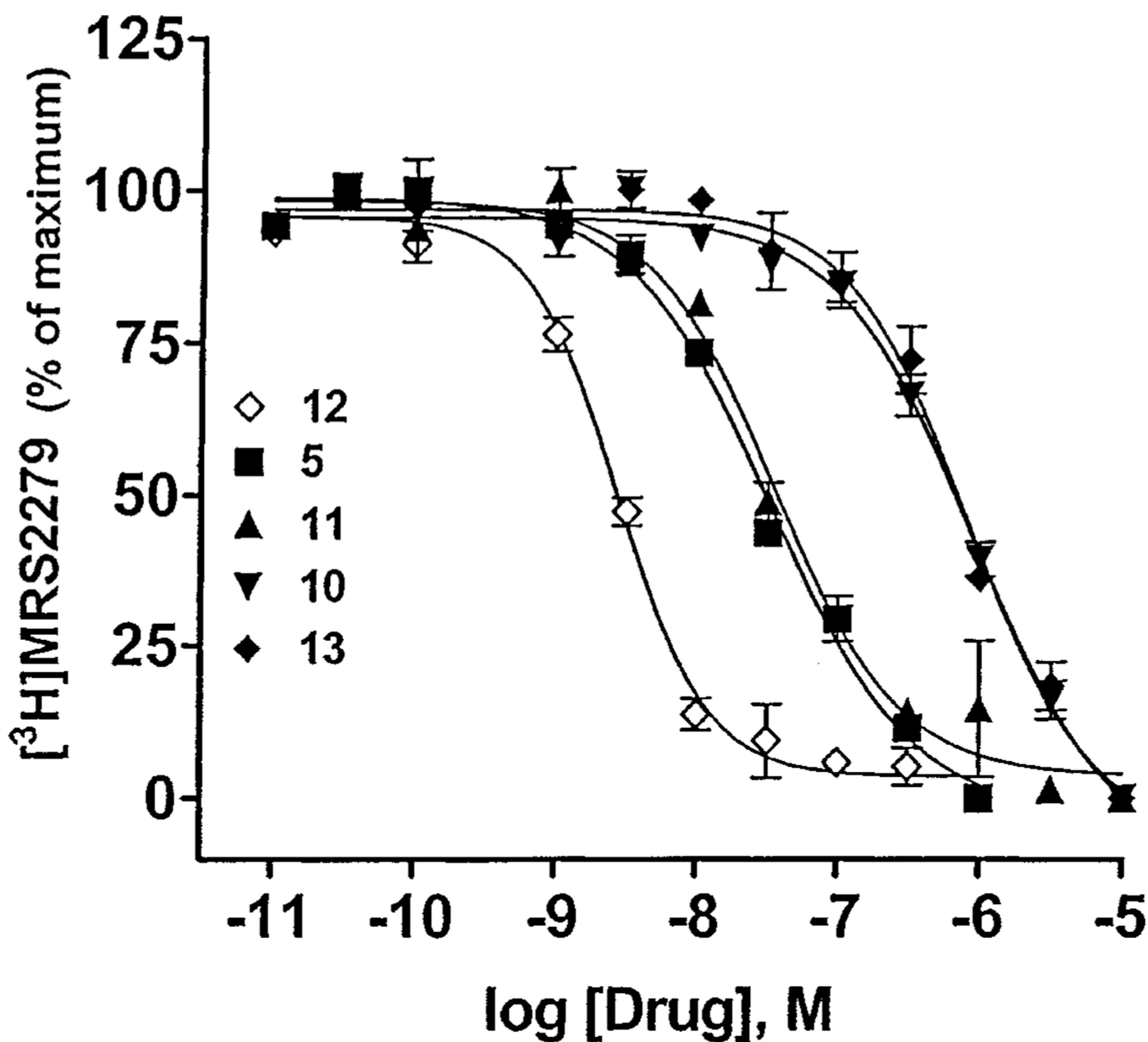


Figure 1. Inhibition of $[^3\text{H}]$ 5 binding to human P2Y_1 receptors by 2-substituted analogues. $[^3\text{H}]$ 5 binding assays were carried out as described in the Experimental Section using Sf9 membranes expressing recombinant human P2Y_1 receptor. The data are presented as percent of total radioligand binding measured in the presence of the indicated concentrations of 2-chloro (**5**), 2-fluoro (**10**), 2-bromo (**11**), 2-iodo (**12**), and 2-methylthio (**13**) N^6 -methyl substituted analogues. The data are the mean of triplicate determinations, and the results are consistent with those obtained in three separate experiments.

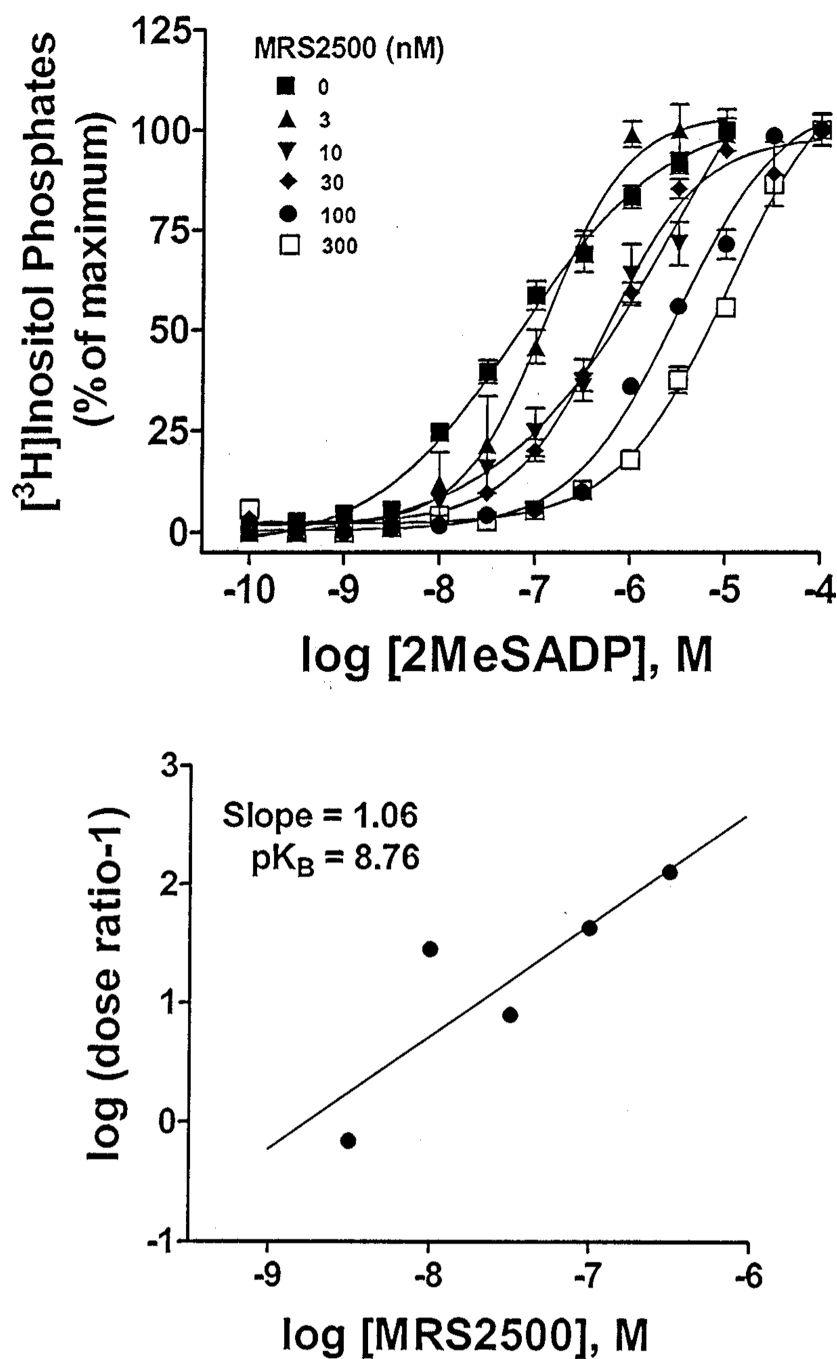


Figure 2. Competitive antagonism of 2MeSADP-promoted activation of the human P2Y₁ receptor. Phospholipase C activity was measured as described in the Experimental Section in 1321N1 human astrocytoma cells stably expressing the human P2Y₁ receptor. Top panel: assays were in the presence of the indicated concentrations of the agonist 2Me-SADP alone or in the presence of the potent antagonist **12** at 3, 10, 30, 100, and 300 nM. The data are the means of triplicate determinations and are representative of results obtained in four separate experiments. Lower panel: Schild analysis of antagonism by **12** of inositol phosphate

accumulation in 1321N1 human astrocytoma cells stably expressing the human P2Y₁ receptor, stimulated by 2-MeS-ADP.

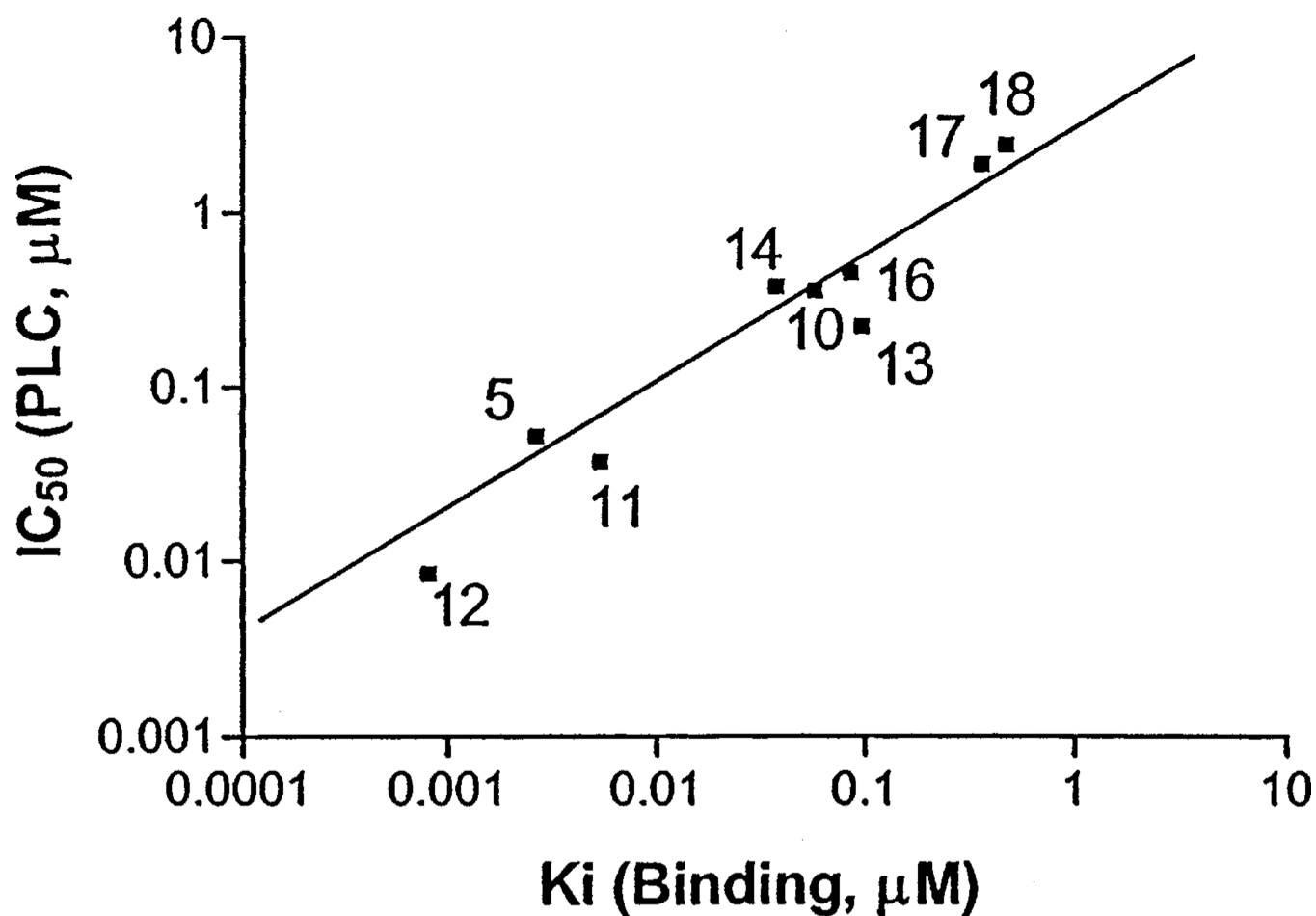
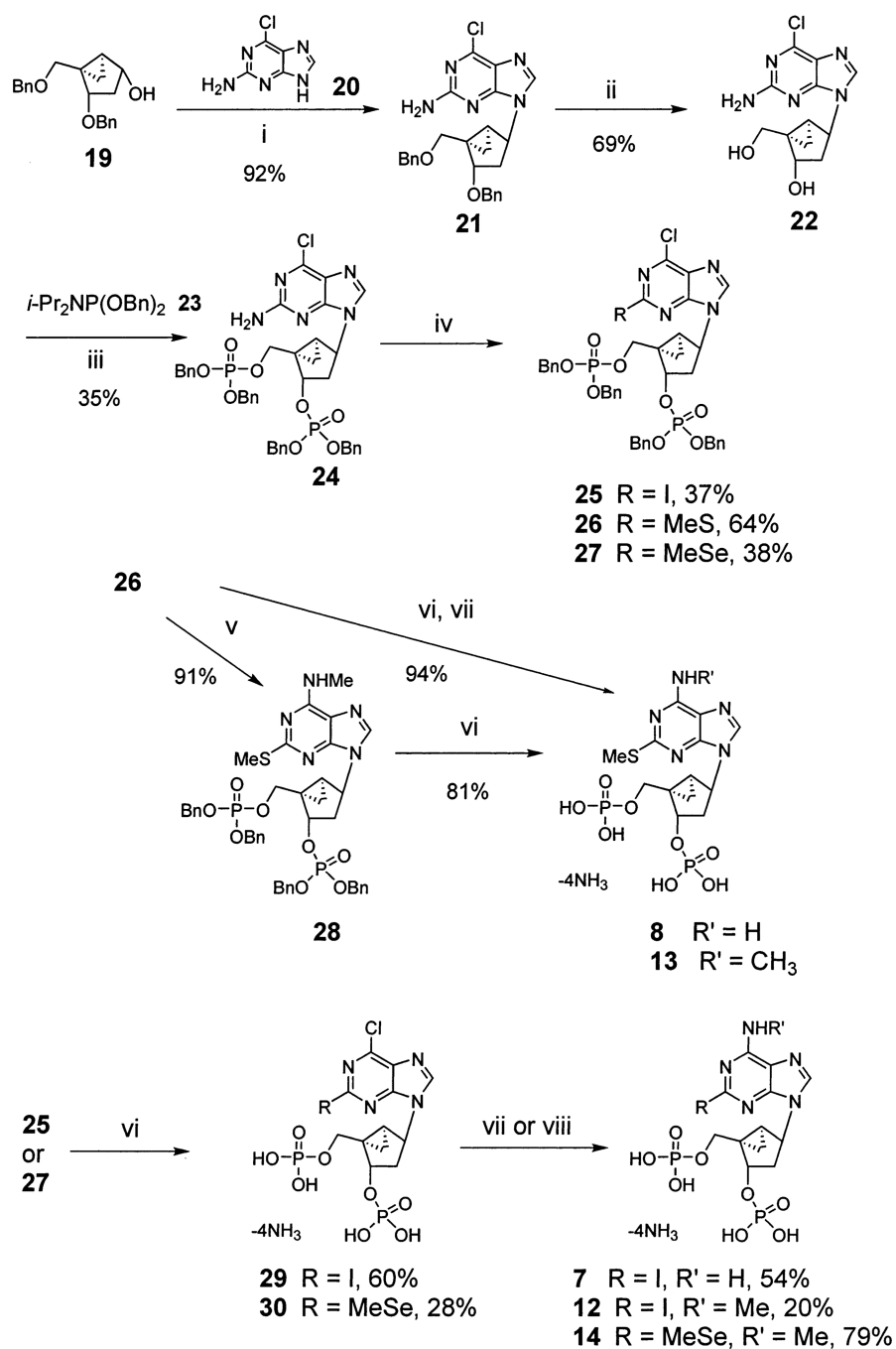
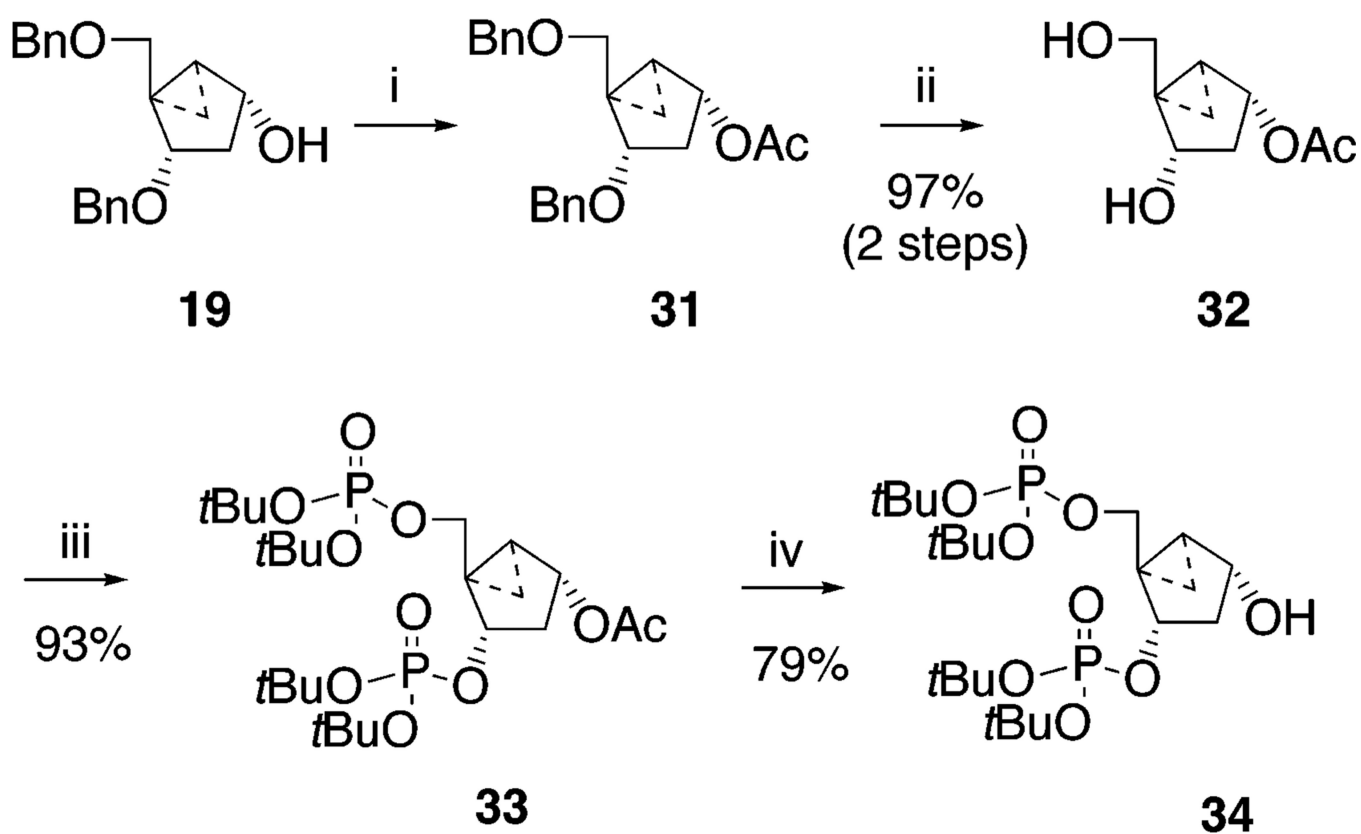


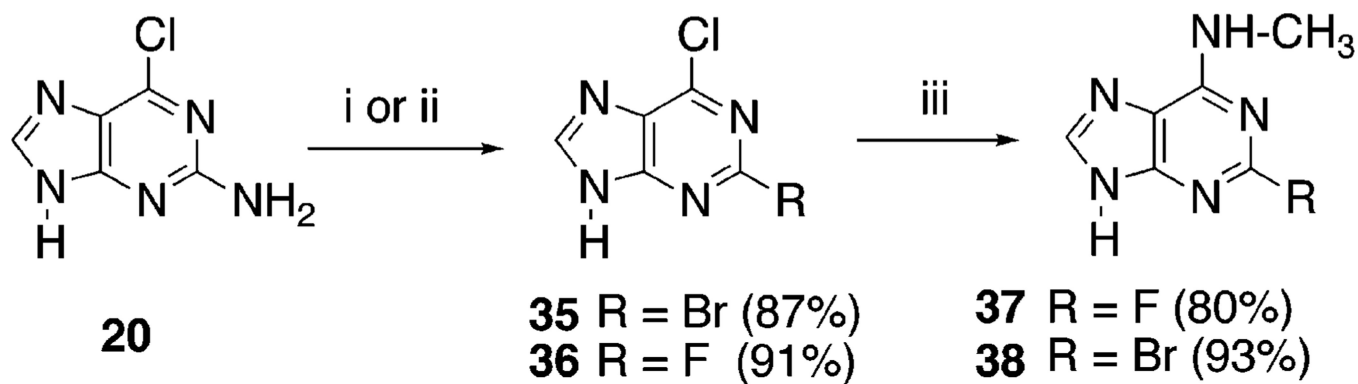
Figure 3. Correlation of binding affinities at the human P2Y₁ receptor expressed in Sf9 cells and functional potencies as P2Y₁ receptor antagonists (measuring inhibition of inositol phosphate accumulation in 1321N1 human astrocytoma cells stably expressing the human P2Y₁ receptor, stimulated by 30 nM 2-MeS-ADP).

**Scheme 1^a**

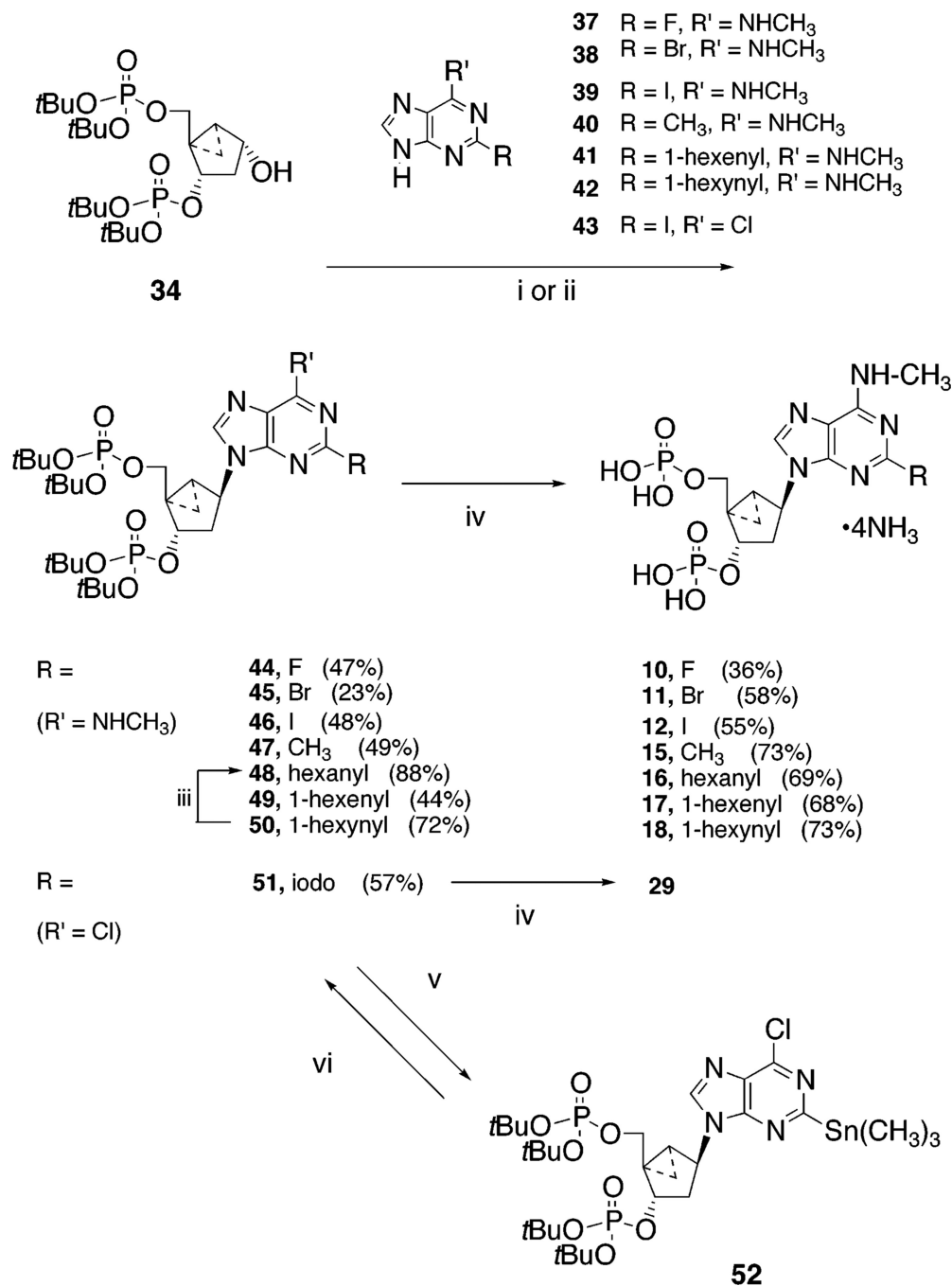
^a Reagents: (i) DIAD, Ph₃P, rt, 2-amino-6-chloropurine; (ii) BCl₃, CH₂Cl₂, -40 °C; (iii) 1*H*-tetrazole then *m*-CPBA; (iv) CH₂I₂, or MeSSMe, or MeSeSeMe, *t*-BuONO, MeCN; (v) MeNH₂/THF; (vi) BCl₃, CH₂Cl₂, 5 °C; (vii) NH₃/H₂O, 70 °C, 2 h; (viii) MeNH₂/H₂O.

**Scheme 2^a**

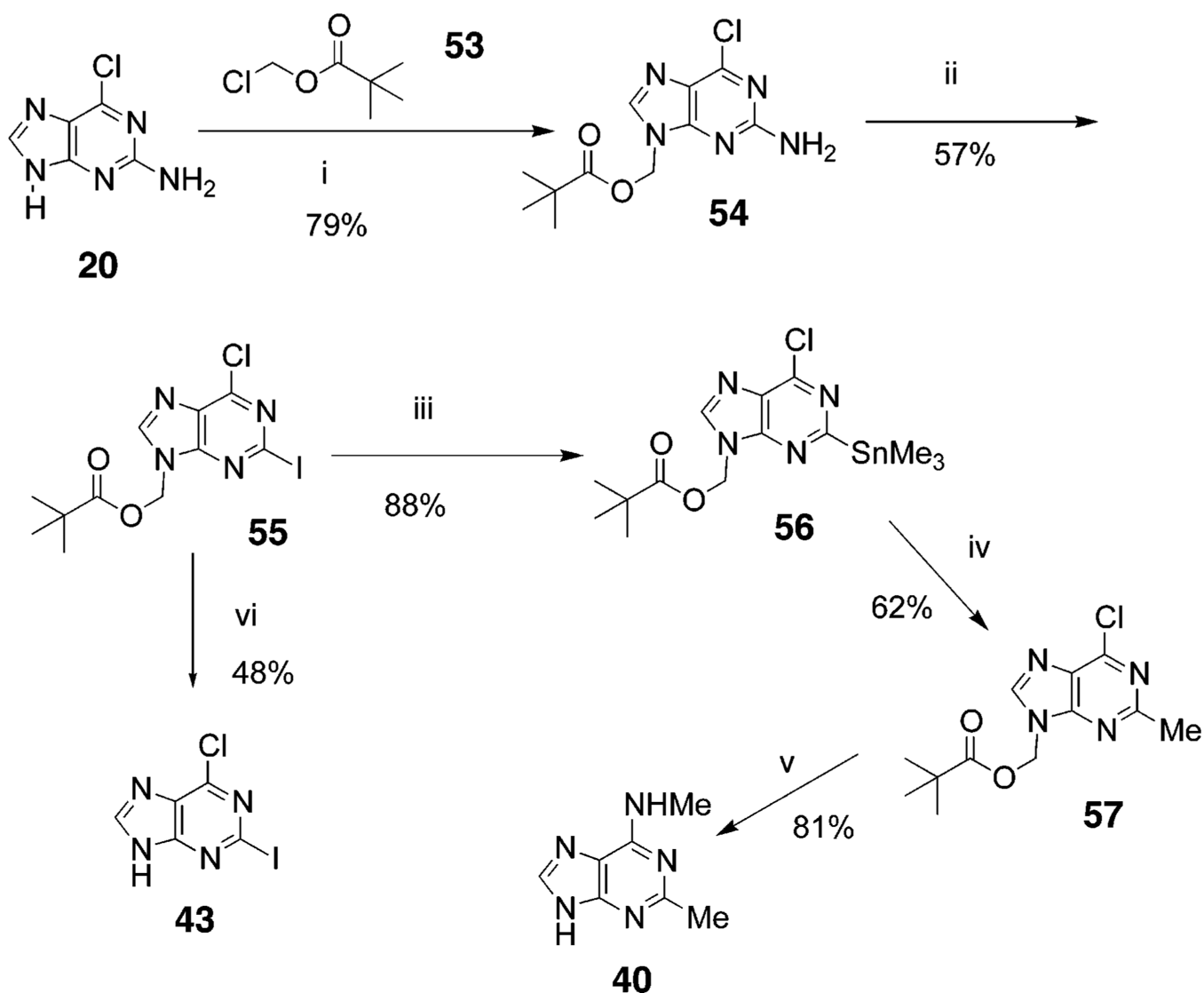
^a Reagents: (i) Ac₂O, cat. DMAP, TEA, CH₂Cl₂, rt, 4 h; (ii) wet 5% Pd/C, H₂, MeOH, rt, 4 h; (iii) (*t*-BuO)₂PNEt, tetrazole, THF, rt, 1 h then *m*-CPBA, -78 to 0 °C, 20 min; (iv) K₂CO₃, MeOH, rt, 2 h.

**Scheme 3^a**

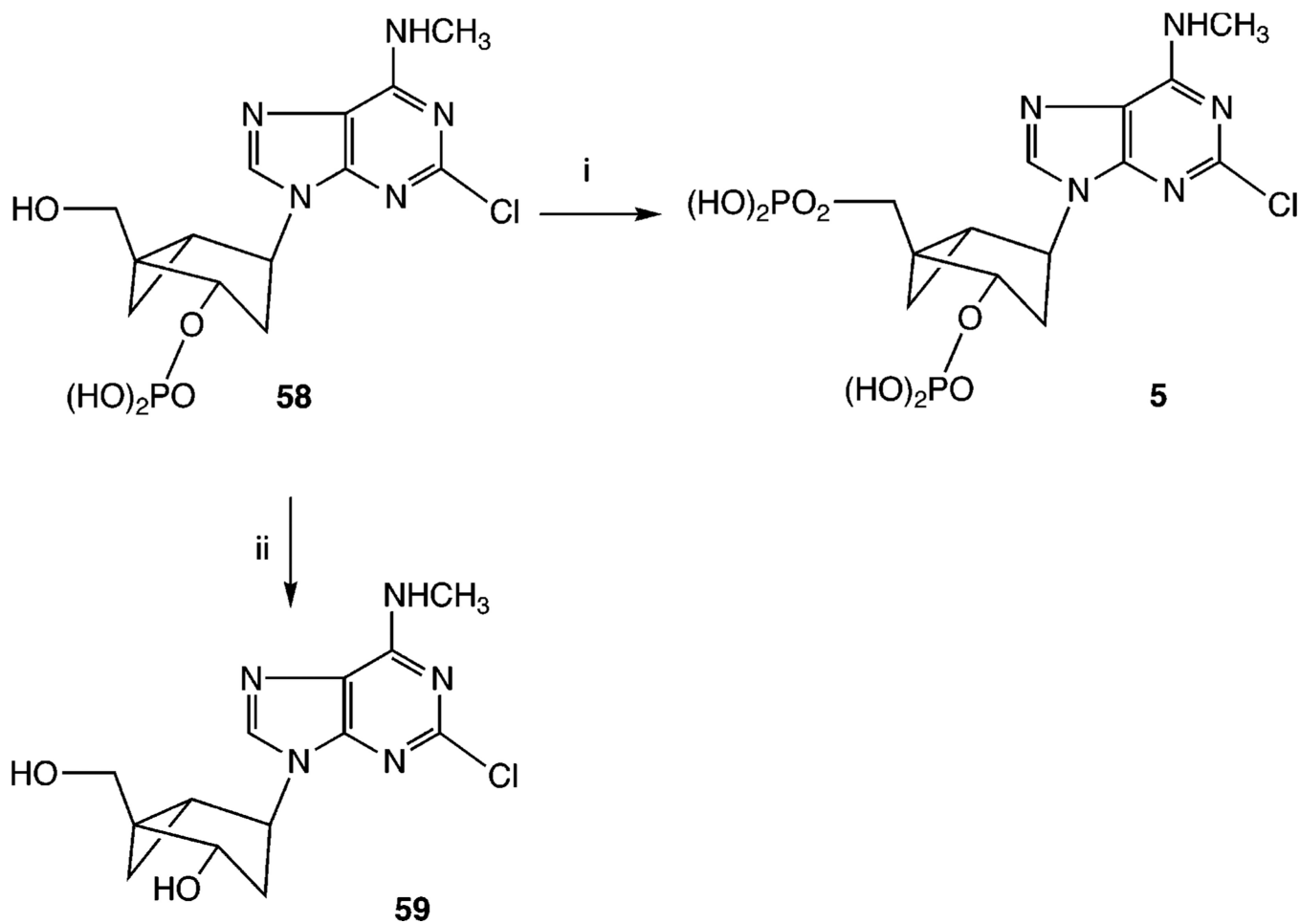
^a Reagents: (i) $\text{SbBr}_3/\text{CH}_2\text{Br}_2$, *t*-Bu nitrite; (ii) HF/pyridine, *t*-Bu nitrite; (iii) $\text{CH}_3\text{NH}_2/\text{THF}$ or H_2O .

**Scheme 4^a**

^a Reagents: (i) 2-substituted (iodo, methyl, 2-hexenyl) 6-methylaminopurine or 2-iodo-6-chloropurine, Ph₃P, DEAD, THF, rt, 2 h; (ii) 2-substituted (fluoro, bromo, or 2-hexynyl) 6-methylaminopurine, Ph₃P, DIAD, THF; (iii) 5% Pd/C, H₂, MeOH, rt, 2 h; (iv) 5% TFA in CH₂Cl₂, rt, 2 h; (v) PdCl₂(PPh₃)₂, Me₃SnSnMe₃, dioxane, 60 °C; (vi) I₂, THF, rt.

Scheme 5^a

^a Reagents: (i) K₂CO₃, DMSO–DMF, rt, 3 d; (ii) CH₂I₂, *t*-BuONO, acetonitrile, 60 °C, 2.5 h; (iii) PdCl₂(PPh₃)₂, Me₃SnSnMe₃, dioxane, 100 °C, 5 h; (iv) Pd₂(dba)₃, P(*o*-tol)₃, CuBr, K₂CO₃, MeI, DMF, 60 °C, 3 h; (v) CH₃NH₂/THF, rt, 22 h; (vi) 5N NaOH, *i*-PrOH, rt, 3 d.

Scheme 6^a

^a Reagents: (i) polynucleotide kinase, ATP; (ii) polynucleotide kinase, alone.

Table 1

In Vitro Pharmacological Data at Human P2Y₁ Receptors for Inhibition of PLC Elicited by 30 nM 2-MeSADP (receptor expressed in 1321N astrocytoma cells) and in Binding (receptor expressed in Sf9 cells)^{31 d}

compd	R	R'	binding, K _i , nM ^a	antagonism, IC ₅₀ , nM ^b
7	I	H	12.6 ± 0.3	87 ± 1
8	SCH ₃	H	863 ± 400	367 ± 194
9	H	CH ₃	17.6 ± 2.7	157 ± 60
10	F	CH ₃	45 ± 21	356 ± 122
5 ^c	Cl	CH ₃	2.5 ± 0.4	52 ± 1
11	Br	CH ₃	5.1 ± 0.6	37 ± 6
12 ^c	I	CH ₃	0.78 ± 0.08	8.4 ± 0.8
13	SCH ₃	CH ₃	91 ± 12	221 ± 30
14	SeCH ₃	CH ₃	35 ± 4	377 ± 154
15	CH ₃	CH ₃	3.6 ± 0.7	49 ± 1
16	(CH ₂) ₅ CH ₃	CH ₃	80 ± 10	452 ± 221
17	<i>trans</i> -CH=CH-(CH ₂) ₃ CH ₃	CH ₃	330 ± 70	1870 ± 590
18	C≡C-(CH ₂) ₃ CH ₃	CH ₃	430 ± 200	2400 ± 600

^aThe affinities were determined by using [³H]5 in a radioligand binding assay, as recently described.³¹ The human P2Y₁ receptor was expressed to high levels in Sf9 insect cells with a recombinant baculovirus. Membranes prepared from these cells were incubated for 30 min at 4 °C in the presence of ~20 nM [³H]5.

^bAntagonist IC₅₀ values (mean ± standard error) represent the concentration needed to inhibit by 50% the effect elicited by 30 nM 2-MeSADP. The percent of maximal inhibition is equal to 100 minus the residual fraction of stimulation at the highest antagonist concentration. *N* = 3, unless otherwise indicated in parentheses.

^c5, MRS 2279; 12, MRS 2500.

^dMean ± SEM given for three separate determinations. None of the compounds displayed agonist effects. R and R' are defined in Scheme 1.