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5'-Phosphate and 5'-Phosphonate Ester Derivatives of (N)-Methanocarba Adenosine with in Vivo Cardioprotective Activity

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Abstract

Activation of a cardiac myocyte P2X4 receptor protects in heart failure. 5'-Phosphonate and 5'-phosphate analogues of AMP containing a (N)-methanocarba (bicyclo[3.1.0]hexane) system could protect from heart failure by potentially activating this cardioprotective channel. Phosphoesters and phosphonodiesters were synthesized and administered in vivo via a mini-osmotic pump in a mouse ischemic heart failure model; most significantly increased intact heart contractile function (echocardiography) compared to vehicle-infusion. Several new thio and deuterated phosphate derivatives were protective in a calsequestrin (CSQ)-overexpressing heart failure model. Diethyl (**7**, MRS4084) and diisopropyl (**8**, MRS4074) phosphotriesters were highly protective in the ischemic model. Substitution of 2-Cl with iodo reduced protection in the CSQ model. Diisopropyl ester **16** (MRS2978) of (1'S,2'R,3'S,4'R,5'S)-4'-(6-amino-2-chloropurin-9-yl)-2',3'-(dihydroxy)-1'-(phosphonoethylene)-bicyclo[3.1.0]hexane was highly efficacious (CSQ), while lower homologue 1'-phosphonomethylene derivative **14** was inactive. Thus, we identified uncharged carbocyclic nucleotide analogues that represent potential candidates for the treatment of heart failure, suggesting this as a viable and structurally broad approach.

Introduction

Activation of cardiac P2X receptors, which are trimeric ligand-gated ion channels,[1] has been explored as a target for the treatment of heart failure.[2–4] A P2X receptor on the cardiomyocyte activated by ATP or other potent nucleotide analogues mediates cardioprotection. The P2X4 receptor (P2X4R) is an important subunit of the native cardiac P2X receptor, which mediates an ionic current induced by extracellular ATP, although the precise subunit composition of this receptor channel is unknown.[4] Cardiac myocyte-specific overexpression of the P2X4R can mimic the beneficial effects following chronic infusion of P2X agonist analogues, such as (1'S,2'R,3'S,4'R,5'S)-4-(6-amino-2-chloro-9H-purin-9-yl)-1-[phosphoryloxy-methyl]bicyclo[3.1.0]hexane-2,3-diol, (MRS2339, **1**, Chart 1). The continuous activation of this ATP-gated, nonselective cation channel under the resting or negative membrane potentials would produce an inward current, while its activation during depolarized portions of the action potential should lead to an outward current. These ionic

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Supporting Information Available: NMR spectral data and HPLC traces of selected nucleotide derivatives, stability data for selected phosphonates, in vivo assay methods and results for the CSQ model are included. This material is available free of charge via the Internet at http://pubs.acs.org.

currents represent a possible ionic mechanism by which the cardiomyocyte P2X channel achieves its protective effect.

Other nucleotides, 5'-monophosphates (related to AMP), were also shown to be cardioprotective by the same mechanism. The cardioprotective ability of chronically administered **1** and its phosphonate derivatives **2** (MRS2776) and **3** (MRS2925) was demonstrated using a transgenic mouse overexpressing calsequestrin (CSQ) as a genetic model of heart failure.[5] Compound **1** is a methanocarba monophosphate derivative of 2-chloro-AMP that contains a rigid bicyclic ring system (bicyclo[3.1.0]hexane) of the North (N) conformation in place of ribose. For other nucleotide receptors, such as the $P2Y_1$ G protein-coupled receptor for ADP, this constrained bicyclic system consisting of fused cyclopropane and cyclopentane rings maintained a receptor-preferred conformation of the ribose-like moiety of the nucleotide.[6] At various P2X receptors, the (N) conformation was also shown to be preferred in receptor activation using rigid methanocarba nucleotide analogues,[7] consistent with the high cardioprotective (P2X receptor-dependent) potency of compound **1**.

In cardiomyocytes obtained from the CSQ mouse, compound **1** induced a current that is characteristic of the action of the cardiac P2X4R and similar to the response to ATP.[4] Compound **1** rescued the hypertrophic and heart failure phenotype in the CSQ-overexpressing mouse.[4] It significantly improved standard measures of cardiac function and increased longevity as compared to vehicle-injected mice. The improvement in survival was associated with decreases in the heart weight/body weight ratio and in the cross-sectional area of the cardiac myocytes. Compound **1** was also devoid of any vasodilator action in aorta ring preparations indicating that its salutary effect in heart failure was not due to any vascular unloading.

The active phosphonate derivatives, such **2** and **3**, are expected to add an extra measure of stability *in vivo*. The 5'-monophosphate group of **1**, which is associated with P2X receptor recognition, would be subject to cleavage by nucleotidases (although less efficiently than the native riboside[6]), but this action would be prevented with phosphonate derivatives.[5] Nevertheless, the cardioprotective effects of compound **1** chronically administered *in vivo* were evident in spite of the expected gradual removal of the phosphate moiety.

In this study, we further explored the structure activity relationship (SAR) of **1** and its phosphonate analogues and various protected ester derivatives. We have approached the issues of short half-life and low oral bioavailability typical of charged nucleotide derivatives through the synthesis of simple uncharged phosphoester derivatives. The new derivatives were studied in a phenotypic screen *in vivo*, i.e. in a model of ischemic heart failure produced by permanent ligation of the left coronary artery in mice. The charged and uncharged nucleotides were administered through chronic infusion via an Alzet mini-osmotic pump, and oral bioavailability was not tested. Most of the derivatives tested alleviated the characteristic heart failure in the ischemic heart failure mouse, suggesting this as a viable and structurally broad approach to use of nucleotides for cardioprotection. Data for some derivatives were also available using the previous CSQ mouse model.

Results

Chemical Synthesis

The reference compounds **1–3** (Chart 1, all containing a 2-chloroadenine nucleobase) were synthesized by the reported methods,[5] and their previously reported cardioprotective activity in the CSQ mouse model is listed in Table S1 (Supporting Information).[5] Novel, charged 5'-phosphates and phosphonate (compound **4**, Table 1 and compounds **5** and **6**, Tables 2 and

S2) and masked (uncharged) nucleotide phosphoesters (**7 – 16**, Table 1) in the (N)-methanocarba series were synthesized by the methods shown in Schemes 1–4. Modifications of compound **1** include the introduction of deuterium at the 5' position (**4**, Table 1) and substitution of S in place of an O atom (**5**), and we anticipate that these derivatives might improve stability *in vivo*.[8,9] Compound **6** was prepared as the 2-iodo equivalent of reference 2-chloro 5'-phosphonate **2** (Chart 1). The corresponding masked ester nucleotides (**7 – 16**) of the reference compounds **1–3** were synthesized using the previously described intermediates **17, 24, 30**, and **36**[5] (Schemes 1–4). A series of alkyl, i.e. ethyl, isopropyl and *t*-butyl, masked 5'-*O*-phosphate ester derivatives (**7 – 9**) was synthesized to evaluate their effects on the well-characterized cardioprotective effect of **1**.[10] In addition, various other modifications on the atoms of 5'-*O*-phosphate group of reference compound **1**, such as dideutero (**10**), thioate (**11, 12**) and dithioate (**13**), have been made in the form of diethyl esters. Finally, we also decided to evaluate the cardioprotective ability of ester analogues **14, 15** and **16** of phosphonate derivatives **2, 6** and **3**, respectively.

Synthesis of 2-chloro-5'-dideutero-(N)-methanocarba-adenosine 5'-monophosphate **4** and the corresponding diethyl ester **10** was achieved from previously reported nucleoside **17**[5] and shown in Scheme 1. Initial amination at the C6 position of 2,6-dichloropurine using 2M NH_3/*i*-ProH afforded 6-amino nucleoside **18** in 68% yield. Reduction of the ethyl ester of **18** using $LiBD_4$ in refluxing anhydrous THF provided the 5'-hydroxydideutero nucleoside **19** in 72% yield. The nucleoside **19** was phosphorylated by either with di-*t*-butyl *N,N*-diethylphosphoramidite and tetrazole followed by *m*-chloroperbenzoic acid or with diethylchlorophosphate in pyridine to afford the corresponding di-*t*-butyl or di-ethyl phosphotriester derivatives **20** and **21**, respectively. Both *t*-butyl groups and acetonide group of **20** were deprotected simultaneously by Dowex-50 resin in a MeOH:H_2O mixture with heating at 70 °C to afford the 5'-dideutero monophosphate derivative **4** in 32% yield. On the other hand, diethyl phosphate ester **10** was prepared by chemoselective deprotection of the acetonide group of nucleotide **21** using the same Dowex reaction conditions except heating at 55 °C (Scheme 1). The synthesis of the 5'-dideutero phosphate diethyl ester derivative **10** was also accomplished by an alternative route, in which we used $LiAlD_4$ to reduce the ethyl ester of nucleoside intermediate **18** and incorporate two D atoms (Scheme 1). This route involves initial deprotection of the isopropylidine group of nucleoside **18** to afford 2',3'-dihydroxy nucleoside **22** in 77% yield. Reduction of 5'-ethyl ester of **22** using $LAlD_4$ in THF afforded the 2',3',5'-trihydroxy-5'-dideuteronucleoside **23** in 51% yield. To our surprise, the phosphorylation on this tri-ol nucleoside **23** using diethylchlorophosphate in pyridine yielded the desired 5'-diethylphosphate ester **10** in satisfactory 55% yield (Scheme 1).

Synthesis of various thiophosphate derivatives was achieved from the previously reported common key intermediate **24**[5] and is shown in Scheme 2. 5'-Iodination of the nucleoside **24** using PPh_3, I_2 and imidazole in THF afforded the 5'-iodo nucleoside **25** in 74% yield. Subsequent deprotection of the acetonide group of **25** using 10% aqueous trifluoroacetic acid afforded the 5'-iodo-2',3'-dihydroxy nucleoside **26** in 60% yield. Treatment of nucleoside **26** with trisodium thiophosphate in H_2O for 3 d afforded the target 5'-*S*-phosphorothiate **5** in 57% yield. 5'-*O*-Phosphorothioate diethyl ester **12** was synthesized from nucleoside **24** by initial phosphorylation using *O,O'*-diethyl chlorothiophosphate in pyridine to get 5'-*O*-phosphorothioate diethyl ester **27** in very poor yield (13%). Several attempts to improve the yield of this reaction, such as changing the solvent and base, were unsuccessful (data not shown). The target 2',3'-dihydroxy-5'-*O*-phosphorothioate diethyl ester **12** was achieved by chemo-selective deprotection of acetonide of compound **27** using acidic Dowex-50 resin at rt. Performing this reaction at 55°C resulted in deprotection of the phosphorothioate moiety to form the corresponding 2',3',5'-trihydroxy nucleoside (results

not shown). 5'-*S*-phosphorothioate diester **11** and 5'-*S*-phosphorodithioate diester **13** were obtained from the common key intermediate **25** using similar reaction conditions. Treating the 5'-iodo nucleoside **25** with *O,O*-diethyl thiophosphate potassium salt or with *O,O′*-diethyl dithiophosphate in CH_3CN and Et_3N as base at rt afforded the phosphorothioate **28** and phosphorodithioate **29**, respectively. Deprotection of the acetonide groups of these compounds using Dowex-50 resin resulted in formation of the target 2',3'-dihydroxy phosphorothioate **11** and phosphorodithioate **13** (Scheme 2), repsectively.

Synthesis of various phosphate diethyl esters from the previously reported 2,6-dichloropurine nucleoside **30**[5] was carried out as shown in Scheme 3. First, the 5'-hydroxyl of the nucleoside **30** was phosphorylated using diethylchlorophosphate in pyridine at rt to obtain 5'-phosphate diethyl ester **31** in 69% yield. Then, compound **31** was subjected to 2 M NH_3 in *i*-PrOH at 70 °C to give the corresponding 6-amino compound **32** in 65% yield. The target 2',3'-dihydroxy phosphate diethyl ester **7** was realized upon treating compound **32** with Dowex-50 resin in a mixture of MeOH:H_2O (1:1, v/v) at 55°C in 59% yield. Alternately, the corresponding diisopropyl **8** and di-*t*-butyl **9** esters were synthesized from nucleoside **24**. Reaction of nucleoside **24** with diisopropylchlorophosphate in pyridine at rt resulted in the formation of 5'-phosphate diisopropyl ester **33** in 49% yield. Subsequent deprotection of the acetonide of **33** yielded 2',3'-dihydroxy phosphate diisopropyl ester **8** in 43% yield. To minimize cleaving labile *t*-butyl groups, we deprotected the acetonide of the previously reported compound **34**[5] using Dowex-50 resin at rt. Even after performing this reaction rt, we observed the formation of substantial amounts of the corresponding monophosphate derivate **1** (Chart 1). As a result we isolated the target 2',3'-dihydroxy phosphate di-*t*-butyl ester **8** in very poor yield (9%). The long chain phosphonate diethyl ester **16** was synthesized by deprotection of isopropylidine group of previously reported compound **35**[5] using Dowex-50 resin (Scheme 3).

Synthesis of phosphonate derivatives containing 2-iodo or 2-chloroadenine was performed using the previously reported compound **36**[5] as shown in Scheme 4. Initially, the 6-chloro-2-iodo purine nucleobase was installed at the 1'-position of the (N)-methanocarba sugar **36**[5,6] by a Mitsunobu reaction using PPh_3, diisopropyl azodicarboxylate, and 6-chloro-2-iodopurine to generate 6-chloro-2-iodo-purine methanocarba nucleotide **37** in 70% yield. Next, amination at the C6 position of compound **37** using 2M NH_3 in *i*-PrOH afforded the nucleotide **38** in 64% yield. Finally, treating compound **38** either with freshly opened iodotrimethylsilane in CH_2Cl_2 or with Dowex-50 resin in a mixture of MeOH:H_2O resulted in the formation of the target 2',3'-dihydroxy-5'-phosphonate **6** and corresponding diethyl ester **15**, respectively. Similar to the other derivatives, the corresponding 2-chloro derivative of phosphonate diethyl ester **14** was obtained using Dowex-50.

The aqueous solubility of two representative phosphonate esters, diethyl ester **14** and diisopropyl ester **16**, was determined to be 60 and 7.25 mM, respectively. Thus, the solubility in this series is substantial and would not be a limiting factor in in vivo application. The stability of **14** and **16** was examined under various conditions (Supporting Information). Compound **16** was stable throughout an incubation of 24 h in aqueous medium at pH 1 and pH 11 (50°C) and in the presence of rat plasma[19] or microsomes from mouse liver or rat liver (37°C).[20,21] Similarly, compound **14** was stable when incubated in the presence of rat plasma.

Biological Evaluation

Various 5'-phosphate and 5'-phosphonate derivatives were tested for cardioprotection in mice in which heart failure was induced by ligation of the left anterior descending coronary artery (LAD).[10]

A nucleotide derivative was infused subcutaneously for one month via an Alzet minipump in each mouse, beginning within 24 h of ligation. The *in vivo* heart function was assessed using echocardiography-derived fractional shortening (FS),[5] which is the ratio of the change in the diameter of the left ventricle between the contracted and relaxed states (Table 1). Thus, a higher percentage represents a protection of function. These FS values are not directly comparable to the FS values in the earlier CSQ model (Supporting Information).

The effects of the infused nucleotide derivatives were compared with vehicle-infused control mice and with WT mice overexpressing the P2X4R in the heart. The WT control mice served as the baseline for testing improvement in cardiac function by nucleotide derivatives. The cardiac-specific P2X4R overexpressing transgenic mice are known to be protected in this model of ischemic heart failure and served as a positive control.[2] The protective effect was determined as an improvement in left ventricular FS after the coronary ligation. An increase of in vivo echocardiographically-derived FS is an established indication of improved cardiac function in heart failure. Among the compounds tested in the ligation model, only the dideuterated compound **10** did not fully protect as indicated by the absence of a significant increase in FS. There was no difference in infarct size in mice infused with any of the derivatives as compared to vehicle-infused control mice (Fig. 1).

Further studies were performed using the *ex vivo* isolated working heart preparation to investigate improvement in cardiac function by selected derivatives. In the same ischemic heart failure model (Table 2), both charged (compounds **5** and **6**) and masked (compounds **7** and **9**) nucleotides were able to improve function as indicated by a more favorable left ventricular rate of pressure change (+dP/dt). There was no significant change in infarct size for any of these analogues, suggesting that any benefit seen was due to the effect on heart failure progression, consistent with what we previously reported for this class of nucleotides.[5] Short chain masked phosphonate **14** did not protect using the *ex vivo* model to assess protection. This derivative was not evaluated in the LAD model.

Selected analogues were also examined for cardioprotection in the previously examined CSQ mouse model of genetically-induced severe heart failure (Tables S1 and S2, Supporting information). Two-week infusion of the 2-iodo phosphonate derivative **6** (n=5 mice) did not improve FS (Table S2) or prevent LV wall thinning in CSQ-overexpressing mice with heart failure (data not shown). Thus, 2-Cl substitution of the adenine moiety as in phosphonate **2** was essential for activity in this model; substitution with iodo in **6** abolished protection, although the same compound was protective in the *ex vivo* heart model. A 2-week infusion of thiophosphate **5** (n=5), containing a 5'-thioester, could protect the CSQ mice with a better preservation of LV septal (0.492 ± 0.012 mm) and posterior (0.493 ± 0.016 mm) wall thickness, as compared those obtained in NS-infused (both septal and posterior: 0.450 ± 0.007 mm) CSQ mice ($P<0.05$, data not shown). Thus, substitution of 5'-oxygen with sulfur was tolerated.

The SAR of the masked (uncharged) nucleotide analogs was explored using the same experimental model. Only some of the ester derivatives of the previously characterized cardioprotective agents **1–3** were shown to act *in vivo*. These findings implied that there may be an *in vivo* cleavage step to liberate the charged nucleotide that is known to be active. Among the new derivatives, diisopropyl ester **16** of the longer chain phosphonate (1'S,2'R, 3'S,4'R,5'S)-4'-(6-amino-2-chloropurin-9-yl)-2',3'-(dihydroxy)-1'-(phosphonoethylene)-bicyclo[3.1.0]hexane **3** was highly efficacious in the CSQ model. This phosphonate diester resulted in an improved FS as compared to vehicle (Figure S1, Table S2). In mice infused with **16**, the LV posterior wall thickness and septal thickness during systole and the LV posterior wall thickness during diastole were greater than those in NS-infused CSQ mice (Figure S2). The activity of diethyl ester **7** was compared to its analogues: isomeric

phosphorothioates **11** and **12** and the corresponding dithiophosphate triester **13**. The corresponding dideuterated ester **10** was not evaluated in the CSQ mice, but it did not provide statistically significant protection in the LAD model (Table 1).

In order to determine a potential P2X agonist-like activity of charged compounds that were cardioprotective,[5] the contractile response to two representative unmasked compounds (5'-monophosphate **1** and 4'-phosphonoethylene derivative **3**) was measured in adult WT murine cardiac ventricular myocytes. Both compounds caused a modest increase in contraction shortening (CS), consistent with an agonist-like activity (Fig. 2). Compound **1** was previously shown in murine ventricular myocytes to elicit a P2X-like current.[4] Although it is possible that the contractile response to **1** was due to a non-specific effect, an increase of P2X-like current and of CS in ventricular myoctes of the same species (mouse) suggests an agonist effect of this agent. We have not tested these nucleotides in other models of P2X ion channel activity.

Discussion

Initially, we were considering prodrug schemes for in vivo bioavailability of the uncharged phosphate and phosphonate derivatives. Thus, in principle a prodrug could be absorbed intestinally and cleaved to liberate the free drug either in circulation, in the liver, or intracellularly at the site of action. In our case, internalization into the cell is undesirable, because the P2X receptors are ion channels on the surface that are stimulated by extracellular ligands. However, model studies of phosphotriesters and phosphonate diesters have shown that simple esters similar to our uncharged alkyl esters are not readily cleaved *in vivo*.[11–14] Therefore, we examined the stability of representative nucleotide derivatives under biological conditions. Consistently, we were unable to demonstrate any hydrolysis of phosphonate diesters **14** and **16** under various conditions of pH extremes (1 and 11) and the presence of rat plasma or liver microsomes.[19–21] Thus, we have no evidence that the protected esters are acting in vivo as prodrugs of the charged derivatives, although we have not examined stability in the presence of cardiac and other tissues. Also, 5'-phosphate **1** was stable to acidic conditions (pH 1.5, 37°C, 24 h) representative of stomach acid (data not shown).

Thus, pronounced *in vivo* cardioprotective activity of many of the derivatives occurs without evidence of in vivo cleavage. The oral route of administration and the *in vivo* pharmacokinetics of this series remain to be studied.

The synthetic nucleotide 5'-monophosphate **1** activates a native cardiac P2X receptor, as indicated in electrophysiological experiments with normal cardiac myocytes and those that overexpress CSQ and based on its *in vivo* ability to improve the heart failure phenotype of these animals. Other agonists of this receptor are 5'-triphosphates,[2] and it is not certain if the action of **1** is competitive at the site that recognizes 5'-triphosphates or is allosteric. The rigid carbocyclic ring system contained in this derivative stabilizes nucleotides toward the action of nucleotidases, such as 5'-nucleotidase (CD73), which converts AMP to adenosine.[6a]

The SAR of the lead molecules was explored, for example with the introduction of a 2-iodoadenine in **6**. Also, substitution of monophosphate esters with phosphorothioate groups of various ligands has been found to provide resistance to phosphatase-catalyzed hydrolysis without reducing binding affinity.[8] Two sulfur-containing ester derivatives **11** and **13** were protective in the LAD model.

Also, introduction of deuterium in place of hydrogen at strategic locations on labile receptor ligands and other drugs has been shown to increase biological lifetime due to an isotope effect without reducing binding affinity.[9] Thus, the 5'-dideutero analogue **4** may prove to be more stable than **1**. Curiously, the free phosphate **4** in this series, but not the ester derivative **10**, was significantly protective in the LAD model (although **10** was tending toward protection with $P<0.1$). Further studies will be needed to explore the role of a deuterium isotope effect in the biological activity of these derivatives.

The spacing of the phosphorus relative to the methanocarba ring was important for *in vivo* activity in the CSQ model. Among phosphonate derivatives, only the longer spacer of 2 carbons in **16** resulted in effective cardioprotection by ester derivatives, even though the precursors or unblocked nucleotides, i.e. **2** and its higher homologue **3**, were protective in both cases.[5] Thus, both the masked diester **16** and its charged precursor **3** were clearly protective *in vivo*.

In conclusion, we have chemically modified carbocyclic nucleotide analogues with blocking groups such that the negative charges have been masked. All but one agent evaluated in the LAD model, i.e. the deuterated analogue **4**, were able to protect against ischemic heart failure. Using the *ex vivo* working heart preparation to determine cardiac function in heart failure, only one of the compounds examined, i.e. **14**, did not protect. In the CSQ model, *in vivo* cardioprotective activity was seen with masked nucleotides e.g. **7** and **16**, as was observed previously for corresponding unmasked species, e.g. **1 – 3**.[5] Among the ester derivatives, diethyl (**7**) and diisopropyl (**8**) phosphotriesters of were highly protective. Substitution of 2-Cl with iodo reduced protection in the CSQ-overexpressing mouse model. Diisopropyl ester **16** of (1'S,2'R,3'S,4'R,5'S)-4'-(6-amino-2-chloropurin-9-yl)-2',3'-(dihydroxy)-1'-(phosphonoethylene)-bicyclo[3.1.0]-hexane was highly efficacious (CSQ), while lower homologue 1'-phosphonomethylene derivative **14** was inactive. Thus, the uncharged compounds often preserved heart contractile function when compared to their unmasked counterparts in the same models of heart failure, e.g. **8** versus **1** in ischemic heart failure and **16** versus **3** in the CSQ genetic heart failure. Both compounds **1** and **3** appear to have agonist-like effect in adult murine cardiac myocytes, implicating a possible role of cardiac P2X receptors in the protective effect of these two compounds. The lack of difference in infarction size may be multi-factorial, one of which may be an absence of a vascular effect of the compounds. Therefore, these phosphonodiester and phosphotriester derivatives represent potential candidates for the treatment of heart failure and can now be explored in additional models of cardiac failure and cardiomyopathy and in pharmacokinetic/metabolism models.

Experimental Procedures

General methods

All reagents and solvents (regular and anhydrous) were of analytical grade and obtained from commercial suppliers and used without further purification. Compounds **34** and **35** were prepared as described.[5,6b] Reactions were conducted under an atmosphere of argon whenever anhydrous solvents were used. All reactions were monitored by thin-layer chromatography (TLC) using silica gel coated plates with a fluorescence indicator which were visualized: a) under UV light, b) by dipping in 5% conc. H_2SO_4 in absolute ethanol (v/v) followed by heating, or c) by dipping in a solution of anisaldehyde:H_2SO_4 (1:2, v/v) in MeOH followed by heating. Silica gel column chromatography was performed with silica gel (SiO_2, 200–400 mesh, 60Å) using moderate air pressure. Evaporation of solvents was carried out under reduced pressure at a temperature below 40 °C. After column chromatography, appropriate fractions were pooled, evaporated and dried at high vacuum

for at least 12 h to give the obtained products in high purity. ^{1}H NMR and ^{31}P NMR ascertained sample purity. No corrections in yields were made for solvent of crystallization. ^{1}H NMR and ^{31}P NMR spectra were recorded at 400 MHz and 161.9 MHz, respectively. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane or deuterated solvent as the internal standard (™H: $CDCl_3$ 7.26; MeOD-d_4 3.31 ppm). Systematic compound names for bicyclic nucleosides are given according to the von Baeyer nomenclature.[16] High resolution mass spectroscopic (HRMS) measurements were performed on a proteomics optimized Q-TOF-2 (Micromass-Waters) using external calibration with polyalanine. Observed mass accuracies are those expected on the basis of known performance of the instrument as well as the trends in masses of standard compounds observed at intervals during the series of measurements. Reported masses are observed masses uncorrected for this time-dependent drift in mass accuracy. Purification of the nucelotide derivatives (**4–6**) for biological testing was performed by HPLC with a Luna 5μ RP-C18(2) semipreparative column (250 × 10.0 mm; Phenomenex, Torrance, CA) under the following conditions: flow rate of 2 mL/min; 10 mM triethylammonium acetate (TEAA)-CH_3CN from 100:0 (v/v) to 70:30 (v/v) in 30 min and isolated in the triethylammonium salt form. Analytical purity of compounds was checked using a Hewlett–Packard 1100 HPLC equipped with Zorbax SB-Aq 5 μm analytical column (50 × 4.6 mm; Agilent Technologies Inc, Palo Alto, CA). Mobile phase: linear gradient solvent system: 5 mM TBAP (tetrabutylammonium dihydrogenphosphate)-CH_3CN from 80:20 to 40:60 in 13 min; the flow rate was 0.5 mL/min. Peaks were detected by UV absorption with a diode array detector at 254, 275, and 280 nm. All derivatives tested for biological activity showed >99% purity by HPLC analysis (detection at 254 nm).

***(1'S,2'R,3'S,4'R,5'S)*-4-(6-Amino-2-chloro-9H-purin-9-yl)-1-[phosphoryloxydideuteromethyl]-2,3-diol-bicyclo[3.1.0]hexane (4)—**To a solution containing nucleoside **20** (7.0 mg, 12.8 μmol) in MeOH and H_2O (2 mL, 1:1, v/v) was added Dowex-50 resin (~50 mg). The mixture was stirred for 3 h at 70 °C and the resin removed by filtration. The filtration was then treated with 1 M triethylammonium bicarbonate buffer (1 mL) and lyophilized to dryness. The resulted mixture was purified by purified by semi preparative HPLC (retention time 16.5 min) to get 5'-monophosphate **4** (1.62 mg, 32%) as white solid. ESI-HRMS m/z 392.0493 ($[M - H]^+$ ($C_{12}H_{12}D_2N_5O_6ClP$, calcd 392.0496). ^{1}H NMR (D_2O) δ 8.45 (s, 1H), 4.73 (s, 1H), 3.97 (d, 1H, J = 6.5 Hz), 1.72–1.77 (m, 1H), 1.39–1.43 (m, 1H), 0.84–0.90 (m, 1H). ^{31}P NMR (D_2O) δ 3.73. Purity >99% by HPLC (retention time: 2.83 min).

(*1'S,2'R,3'S,4'R,5'S*)-4-(6-Amino-2-chloro-purin-9-yl)-2,3-(dihydroxy)-1-[monophosporothioate]-bicyclo-[3.1.0]hexane (5)—To the suspension of 5'-iodo nucleoside **26** (3.0 mg, 7.12 μmol) and H_2O (0.5 mL), trisodium thiophosphate (10 mg, 55 μmol) was added. After stirring the reaction mixture for 3 d at room temperature under argon atmosphere, reaction mixture was lyophilized and purified by semi preparative HPLC (retention time 19.5 min) to get 5'-monophosphorothioate **5** (1.65 mg, 57%) as a white solid. ESI-HRMS m/z 406.0159 ($[M - H]^+$ ($C_{12}H_{14}N_5O_5SPCl$, calcd 406.0165). ^{1}H NMR (D_2O) δ 8.39 (s, 1H), 4.62 (s, 1H), 4.63 (s, 1H), 3.97 (d, 1H, J = 6.5 Hz), 3.19–3.26 (m, 1H), 2.79–2.87 (m, 1H), 1.69–1.74 (m, 1H), 1.39–1.43 (m, 1H), 0.84–0.90 (m, 1H).

***(1'S,2'R,3'S,4'R,5'S)*-4'-(6-Amino-2-iodoropurin-9-yl)-2',3'-(dihydroxy)-1'-(phosphonomethylene)-bicyclo[3.1.0]hexane (6)—**Nucleoside **38** (10.0 mg, 0.017 mmol) was coevaporated with anhydrous toluene (3 × 3 mL) and dissolved in anhydrous CH_2Cl_2 (2 mL). Iodotrimethylsilane (25 μl, 0.17 mmol) was added and after stirring for 17 h, the reaction mixture was cooled to 0 °C followed by the addition of ice-cold H_2O (20 mL) and CH_2Cl_2 (25 mL). The phases were separated and the aqueous phase washed with

CH_2Cl_2 (2 × 35 mL) and diethyl ether (4 × 35 mL). The resulting aqueous phase lyophilized to dryness and purified by HPLC (retention time: 20 min) to afford **6** (4.1 mg, 49%) as a white solid. ESI-HRMS *m/z* 465.9777 [M - H]$^-$, $C_{12}H_{14}IN_5O_5P^-$: Calcd. 465.9771); ^{1}H NMR (D_2O) δ 8.19 (s, 1H), 4.71 (s, 1H), 4.58 (d, 1H, *J* = 5.8 Hz), 3.98 (d, 1H, *J* = 5.8 Hz), 2.17 (t, 1H, 15.5 Hz), 1.90 (t, 1H, 15.5 Hz), 1.68–1.74 (m, 1H), 1.35–1.42 (m, 1H), 0.88–0.94 (m, 1H). ^{31}P NMR (D_2O) δ 25.39. Purity >99% by HPLC (retention time: 5.93 min).

***(1'S,2'R,3'S,4'R,5'S)*-4-(6-Amino-2-chloro-9H-purin-9-yl)-1-[(diethylphosphate) methyl]-2,3-(dihydroxy)-bicyclo[3.1.0]hexane (7)—**To a solution containing **32** (7.0 mg, 14.3 μmol) in MeOH:H_2O (2 mL, 1:1, v/v) was added Dowex-50 resin (~50 mg). The mixture was stirred for 3 h at 55°C and the resin removed by filtration. Filtrate was evaporated to dryness and resulting crude purified by silica gel column chromatography (0–10% MeOH in CH_2Cl_2, v/v) to afford nucleoside **7** (3.8 mg, 59%) as a white solid. R_f = 0.5 (8% MeOH in CH_2Cl_2, v/v); ESI-HRMS m/z 448.1156 ([M + H] $^+$ ($C_{16}H_{23}N_5O_6ClP$, calcd 448.1153). ^{1}H NMR (MeOD-d_4) δ 8.23 (s, 1H), 4.83 (s, 1H), 4.75 (d, 1H, *J* = 7.2 Hz), 4.79 (d, 1H, *J* = 7.2 Hz), 4.63–4.69 (m, 1H), 4.13–4.23 (m, 4H), 3.94–4.05 (m, 2H), 1.76–1.82 (m, 1H), 1.66–1.70 (m, 1H), 1.37 (t, 6H,*J* = 7.2 Hz), 0.91–0.95 (m, 1H). ^{31}P NMR (MeOD-d_4) δ −1.25. Purity >99% by HPLC (retention time: 2.52 min).

***(1'S,2'R,3'S,4'R,5'S)*-4-(6-Amino-2-chloro-9H-purin-9-yl)-1-[(diisopropylphosphate) methyl]-2,3-(dihydroxy)-bicyclo[3.1.0]hexane (8)—**To a solution containing **33** (4.0 mg, 14.3 μmol) in MeOH:H_2O (2 mL, 1:1, v/v) was added Dowex-50 resin (~50 mg). The mixture was stirred for 3 h at 55°C and the resin removed by filtration. Filtrate was evaporated to dryness and resulting crude purified by silica gel column chromatography (0–10% MeOH in CH_2Cl_2, v/v) to afford nucleoside **8** (1.63 mg, 43%) as a white solid. R_f = 0.4 (6% MeOH in CH_2Cl_2, v/v); ESI-HRMS m/z 476.1474 ([M + H] $^+$ ($C_{18}H_{28}N_5O_6ClP$, calcd 476.1466). ^{1}H NMR (MeOD-d_4) δ 8.23 (s, 1H), 4.83 (s, 1H), 4.74 (d, 1H, *J* = 7.2 Hz), 4.63–4.71 (m, 3H), 3.93–3.99 (m, 2H), 1.75–1.81 (m, 1H), 1.65–1.70 (m,1H), 1.35–1.41 (m, 12H), 0.88–0.96 (m, 1H). ^{31}P NMR ($CDCl_3$) δ −3.03. Purity >99% by HPLC (retention time: 4.69 min).

***(1'S,2'R,3'S,4'R,5'S)*-4-(6-Amino-2-chloro-9H-purin-9-yl)-2,3-(dihydroxy)-1-[(t-butylphosphate)methyl]-bicyclo[3.1.0]hexane (9)—**To a solution containing **34** (15.0 mg, 36.8 μmol) in MeOH:H_2O (3 mL, 2:1, v/v) was added Dowex-50 resin (~50 mg). The mixture was stirred for 3 h at rt and the resin removed by filtration. Filtrate was evaporated to dryness and resulting crude purified by silica gel column chromatography (0–6% MeOH in CH_2Cl_2, v/v) to afford nucleoside **9** (1.61 mg, 9%) as a white solid. R_f = 0.4 (6% MeOH in CH_2Cl_2, v/v); ESI-HRMS m/z 504.1780 ([M + H] $^+$ ($C_{20}H_{32}N_5O_6ClP$, calcd 504.1779). ^{1}H NMR ($CDCl_3$) δ 7.89 (s, 1H), 5.83 (s, 2H), 5.47 (s, 1H), 5.03 (d, 1H, *J* = 6.5 Hz), 4.83 (s, 1H), 4.72 (t, 1H, *J* = 11.6 Hz), 4.12 (d, 1H, *J* = 6.5 Hz), 3.92 (t, 1H, *J* = 11.6 Hz), 3.65 (s, 1H), 1.65–1.70 (m, 1H), 1.55 (s, 18H), 0.90–1.02 (m, 1H). ^{31}P NMR ((MeOD-d_4) δ −10.29. Purity >99% by HPLC (retention time: 5.30 min).

***(1'S,2'R,3'S,4'R,5'S)*-4-(6-Amino-2-chloro-9H-purin-9-yl)-2,3-(dihydroxyl)-1-[(di-ethylphosphate)dideuteromethyl]bicyclo[3.1.0]hexane (10)**

Method A: To a solution containing **21** (8.0 mg, 16.3 μmol) in MeOH:H_2O (2 mL, 1:1, v/v) was added Dowex-50 resin (~50 mg). The mixture was stirred for 2 h at 55°C and the resin removed by filtration. Filtrate was evaporated to dryness and resulting crude purified by silica gel column chromatography (0–7% MeOH in CH_2Cl_2, v/v) to afford nucleoside **10** (4.2 mg, 55%) as a white solid.

Method B: Nucleoside **23** (2.5 mg, 7.9 μmol) was coevaporated with anhydrous toluene (3 × 2 mL), dissolved in anhydrous pyridine (0.5 mL) and diethylchlorophosphate (6 μL, 39.8 μmol) was added to this. After stirring at rt for 8 h, the reaction mixture was evaporated to dryness. The resulting crude residue was purified by silica gel column chromatography (0–4% MeOH in CH_2Cl_2, v/v) to afford nucleoside **10** (1.9 mg, 55%) as a white solid. R_f = 0.3 (6% MeOH in CH_2Cl_2, v/v); ESI-HRMS m/z 450.1263 ($[M + H]^+$ ($C_{16}H_{21}D_2N_5O_6Cl{\cdot}H^+$, calcd 450.1278). ^{1}H NMR (MeOD-d_4) δ 8.14 (s, 1H), 5.35 (d, 1H, J = 6.8 Hz), 4.96 (s, 1H), 4.77 (d, 1H, J = 7.2 Hz), 4.11–4.23 (m, 4H), 1.75–1.80 (m, 1H), 1.53 (s, 3H), 1.33–1.39 (m, 6H), 1.27 (s, 3H), 1.21–1.24 (m, 1H), 1.10–1.16 (m, 1H). ^{31}P NMR (D_2O) δ 1.22. Purity >99% by HPLC (retention time: 2.92 min).

***(1'S,2'R,3'S,4'R,5'S)*-4-(6-Amino-2-chloro-purin-9-yl)-1-[*O,O*-diethyl *S*-methyl phosphorothioate]- 2,3-(dihydroxyl)-bicyclo-[3.1.0]hexane (11)—**To a solution containing nucleoside **28** (7.0 mg, 13.9 μmol) in MeOH and H_2O (2 mL, 1:1, v/v) was added Dowex-50 resin (~50 mg). The mixture was stirred for 2 h at 55 °C and the resin removed by filtration. The filtrate was then evaporated to dryness and the resulted mixture was purified by purified by silica gel column chromatography (0–7% MeOH in CH_2Cl_2, v/v) to afford 2',3' dihydroxy, 5'-phosphorothioate **11** (3.1 mg, 48%). R_f = 0.4 (7% MeOH in CH_2Cl_2, v/v). ESI-HRMS m/z 464.0925 ($[M+H]^+$ ($C_{16}H_{24}N_5O_5PSCl{\cdot}H$, calcd 464.0924). ^{1}H NMR (MeOD-d_4) δ 8.25 (s, 1H), 4.78 (d, 1H, J = 6.9 Hz), 4.75 (s, 1H), 4.14–4.22 (m, 4H), 4.07 (d, 1H, J = 6.9 Hz), 3.45 (t, 1H, J = 12.9 Hz), 3.24–3.30 (m, 1H), 1.72–1.76 (m, 1H), 1.59–1.63 (m, 1H), 1.30–1.40 (m, 6H), 0.90–0.96 (m, 1H). ^{31}P NMR (MeOD-d_4) δ 28.98. Purity >99% by HPLC (retention time: 3.88 min).

***(1'S,2'R,3'S,4'R,5'S)*-4-(6-Amino-2-chloro-purin-9-yl)-1-[*O,O*-diethyl *O*-methyl phosphorothioate]- 2,3-(*O*-isopropylidine)-bicyclo-[3.1.0]hexane (12)—**To a solution containing nucleoside **27** (3.0 mg, 5.9 μmol) in MeOH and H_2O (1 mL, 1:1, v/v) was added Dowex-50 resin (~30 mg). The mixture was stirred for 6 h at rt and the resin removed by filtration. The filtrate was then evaporated to dryness and the resulted mixture was purified by purified by silica gel column chromatography (0–7% MeOH in CH_2Cl_2, v/v) to afford 2',3' dihydroxy, 5'-diethyl phosphorothioate **12** (0.31 mg, 12%). R_f = 0.4 (7% MeOH in CH_2Cl_2, v/v). ESI-HRMS m/z 464.0926 ($[M+H]^+$ ($C_{16}H_{24}N_5O_5PSCl^+$ calcd 464.0924). ^{1}H NMR (MeOD-d_4) δ 8.31 (s, 1H), 4.84 (s, 1H), 4.69–4.75 (m, 2H), 4.13–4.28 (m, 4H), 3.87–3.93 (m, 2H), 1.76–1.81 (m, 1H), 1.67–1.72 (m, 1H), 1.30–1.40 (m, 6H), 0.90–0.96 (m, 1H). ^{31}P NMR (MeOD-d_4) δ 67.9 Purity >99% by HPLC (retention time: 6.41 min).

***(1'S,2'R,3'S,4'R,5'S)*-4-(6-Amino-2-chloro-purin-9-yl)-1-[*O,O*-diethyl *S*-methyl phosphorodithioate]- 2,3-(dihydroxyl)-bicyclo-[3.1.0]hexane (13)—**To a solution containing nucleoside **29** (6.0 mg, 11.5 μmol) in MeOH and H_2O (2 mL, 1:1, v/v) was added Dowex-50 resin (~50 mg). The mixture was stirred for 3 h at 45 °C and the resin removed by filtration. The filtrate was then evaporated to dryness and the resulted mixture was purified by purified by silica gel column chromatography (0–5% MeOH in CH_2Cl_2, v/v) to afford 2',3' dihydroxy, 5'-phosphorothioate **13** (3.27 mg, 59%). R_f = 0.3 (5% MeOH in CH_2Cl_2, v/v). ESI-HRMS m/z 480.0703 ($[M+H]^+$ ($C_{16}H_{24}N_5O_4PS_2Cl{\cdot}H$, calcd 480.0696). ^{1}H NMR (MeOD-d_4) δ 8.27 (s, 1H), 4.76 (s, 1H), 4.73 (d, 1H, J = 7.2 Hz), 4.09–4.22 (m, 4H), 4.01 (d, 1H, J = 6.9 Hz), 3.56 (t, 1H, J = 13.6 Hz), 3.26 (t, 1H, J = 13.6 Hz), 1.72–1.76 (m, 1H), 1.59–1.63 (m, 1H), 1.30–1.40 (m, 6H), 0.90–0.94 (m, 1H). ^{31}P NMR (MeOD-d_4) δ 95.25. Purity >99% by HPLC (retention time: 7.54 min).

Diethyl-*(1'S,2'R,3'S,4'R,5'S)*-4'-(6-amino-2-chloropurin-9-yl)-2',3'-(hydroxy)-bicyclo[3.1.0]hexane phosphonate (14)—To a solution containing **39** (25 mg, 0.053

mmol) in MeOH (3 mL) and water (3 mL) was added Dowex-50 resin (~100 mg). The mixture was stirred for 3 h at 70°C and the resin removed by filtration. Filtrate was evaporated to dryness and resulting crude purified by silica gel column chromatography (10% MeOH in EtOAc, v/v) to afford nucleoside **14** (8.3 mg, 40%) as a white solid. R_f = 0.4 (10% MeOH in CH_2Cl_2, v/v); ESI-HRMS *m/z* 432.1197 $[M + H]^+$, $C_{16}H_{24}ClN_5O_5P^+$: Calcd. 432.1204); ^{1}H NMR (MeOD-d_4) δ 8.31 (s, 1H), 4.79 (s, 1H), 4.76 (d, 1H, *J* = 7.1 Hz), 4.10–4.19 (m, 4H), 3.98 (d, *J* = 7.1 Hz), 2.44–2.54 (m, 1H), 2.27–2.36 (m, 1H), 1.72–1.78 (m, 1H), 1.51–1.57 (m, 1H), 1.28–1.35 (m, 6H), 0.80–0.89 (m, 1H). ^{31}P NMR (MeOD-d_4) δ 30.86. Purity >99% by HPLC (retention time: 5.17 min).

Diethyl-*(1'S,2'R,3'S,4'R,5'S)*-4'-(6-amino-2-iodopurin-9-yl)-2',3'-(dihydroxy)-bicyclo[3.1.0]hexane phosphonate (15)—To a solution containing **38** (6.0 mg, 11.2 μmol) in MeOH:H_2O (2 mL, 1:1, v/v) was added Dowex-50 resin (~50 mg). The mixture was stirred for 3 h at 70°C and the resin removed by filtration. Filtrate was evaporated to dryness and resulting crude purified by silica gel column chromatography (0–8% MeOH in CH_2Cl_2, v/v) to afford nucleoside **15** (4.5 mg, 49%) as a white solid. R_f = 0.2 (5% MeOH in CH_2Cl_2, v/v); ESI-HRMS *m/z* 524.0562 $[M + H]^+$, $C_{16}H_{24}IN_5O_5P^+$: Calcd. 524.0560); ^{1}H NMR (MeOD-d_4) δ 8.25(s, 1H), 4.77–4.81 (m, 2H), 4.10–4.19 (m, 4H), 3.98 (d, 1H, *J* = 6.8 Hz), 2.35–2.52 (m, 1H), 1.68–1.73 (m, 1H), 1.49–1.52 (m, 1H), 1.29–1.35 (m, 6H), 0.85–0.91 (m, 1H).

***(1'S,2'R,3'S,4'R,5'S)*-4'-(6-Amino-2-chloropurin-9-yl)-1'-[diisopropyl-(E)-ethenylphosphonate]-2,3-(dihydroxy)-bicyclo-[3.1.0]-hexane (16)—**To a solution containing **35** (4.0 mg, 7.7 μmol) in MeOH:H_2O (1 mL, 1:1, v/v) was added Dowex-50 resin (~25 mg). The mixture was stirred for 3 h at 55 °C and the resin removed by filtration. Filtrate was evaporated to dryness and resulting crude purified by silica gel column chromatography (0–10% MeOH in CH_2Cl_2, v/v) to afford nucleoside **16** (1.8 mg, 49%) as a white solid. R_f = 0.3 (10% MeOH in CH_2Cl_2, v/v); ESI-HRMS *m/z* 474.1679 $[M + H]^+$, $C_{19}H_{30}ClN_5O_5P{\cdot}H^+$: Calcd. 474.1673); ^{1}H NMR (MeOD-d_4) δ 8.06 (s, 1H), 4.76 (d, 1H, *J* = 6.9 Hz), 4.65–4.71 (m, 3H), 4.07 (d, 1H, *J* = 6.9 Hz), 3.67–3.72 (m, 1H), 3.55–3.59 (m, 1H), 2.10–2.16 (m, 2H), 1.80–2.03 (m, 2H), 1.47–1.52 (m, 1H), 1.28–1.41 (m, 13H), 0.66–0.72 (m, 1H). ^{31}P NMR (MeOD-d_4) δ 31.22. Purity >99% by HPLC (retention time: 4.69 min).

Ethyl *(1'S,2'R'3'S,4'R,5'S)*-4-(6-Amino-2-chloropurin-9-yl]-2,3-*O*-(isopropylidene)-bicyclo[3.1.0]hexanecarboxylate (18)—Nucleoside **17** (53 mg, 0.13 mmol) was treated with 2M NH_3 in *i*-PrOH (5 mL) and heated to 70 °C. After stirring the reaction for 16 h, reaction mixture evaporated to dryness. The resulting residue purified by silica gel column chromatography (0–4% MeOH in CH_2Cl_2, v/v) to afford nucleoside **18** (35 mg, 68%) as a white solid. R_f = 0.4 (5% MeoH in CH_2Cl_2, v/v). ESI-HRMS m/z 394.1282 ($[M + H]^+$ ($C_{17}H_{21}N_5O_4Cl$, calcd 394.1282). ^{1}H NMR (MeOD-d_4) δ 8.09 (s, 1H), 5.85 (d, 1H, *J* = 7.1 Hz), 4.98 (s, 1H), 4.81 (d, 1H, *J* = 7.1 Hz), 4.20–4.26 (m, 1H), 2.23–2.28 (m, 1H), 1.64–1.67 (m, 1H), 1.52 (s, 3H), 1.34 (t, 3H, *J* = 7.2 Hz), 1.20–1.29 (m, 4H).

***(1'S,2'R,3'S,4'R,5'S)*-4-(6-Amino-2-chloro-purin-9-yl)-1-[hydroxydeuteromethyl]-2,3-(*O*-isopropylidine)bicyclo-[3.1.0]hexane (19)—** Nucleoside **18** (9.0 mg, 23 μmol) was coevaporated with anhydrous toluene (3 × 10 mL), dissolved in anhydrous THF (10 mL). $LiBD_4$ (3 mg, 115 μmol) was added and after stirring the reaction mixture for 4 h at 70 °C, it is cooled to room temperature and quenched with slow addition of MeOH (3 mL). The resulting reaction mixture was evaporated to dryness, and purified by silica gel column chromatography (0–8% MeOH in CH_2Cl_2, v/v) to afford

nucleoside **19** (6.0 mg, 72%) as a white solid. R_f = 0.4 (10% MeOH in CH_2Cl_2, v/v). ESI-HRMS m/z 354.1291 ([M + H] $^+$ ($C_{15}H_{17}D_2N_5O_3Cl$, calcd 354.1302). ^{1}H NMR (MeOD-d_4) δ 8.26 (s, 1H), 5.37 (d, 1H, J = 6.7 Hz), 4.95 (s, 1H), 4.70 (d, 1H, J = 7.2 Hz), 1.67–1.73 (m, 1H), 1.51 (s, 3H), 1.25 (s, 3H), 1.10–1.15 (m, 1H), 0.94–1.00 (m, 1H).

***(1'S,2'R,3'S,4'R,5'S)*-4-(6-Amino-2-chloro-9H-purin-9-yl)-2,3-(*O*isopropylidene)-1-[(di-tert-butylphosphate)dideuteromethyl]-bicyclo[3.1.0]hexane (20)—**Nucleoside **19** (6.0 mg, 17 μmol) was coevaporated with anhydrous toluene (3 × 10 mL), dissolved in anhydrous THF (1 mL). Di-*t*-butyl-*N,N'*-diethylphosphoramidite (24 μL, 85 μmol) and tetrazole (12 mg, 169 μmol) were added. After stirring at rt for 4 h, the reaction mixture was cooled to −70 °C followed by the addition of *m*-chloroperbenzoic acid (25 mg, 77%). The reaction mixture was warmed to 0 °C and allowed to stir for 15 min followed by the addition of triethylamine (0.5 mL). The reaction mixture was evaporated to dryness, and the resulting crude residue was purified by silica gel column chromatography (0–4% MeOH in CH_2Cl_2, v/v) to afford nucleoside **20** (7.0 mg, 76%) as a white solid. R_f = 0.3 (5% MeOH in CH_2Cl_2, v/v); ESI-HRMS m/z 546.2234 ([M + H] $^+$ ($C_{23}H_{34}D_2N_5O_6Cl$, calcd 546.2217). ^{1}H NMR (MeOD-d_4) δ 8.15 (s, 1H), 5.34 (d, 1H, J = 6.8 Hz), 4.98 (s, 1H), 4.77 (d, 1H, J = 7.2 Hz), 1.74–1.77 (m, 1H), 1.48–1.55 (m, 21H), 1.25 (s, 3H), 1.21–1.24 (m, 1H), 1.09–1.13 (m, 1H).

***(1'S,2'R,3'S,4'R,5'S)*-4-(6-Amino-2-chloro-9H-purin-9-yl)-2,3-(*O*isopropylidene)-1-[(di-ethylphosphate)dideuteromethyl]-bicyclo[3.1.0]hexane (21)—**Nucleoside **19** (20 mg, 54 μmol) was coevaporated with anhydrous toluene (3 × 10 mL), dissolved in anhydrous pyridine (2 mL) and diethylchlorophosphate (24 μL, 161 μmol) was added to this. After stirring at rt for 8 h, the reaction mixture was diluted with EtOAc (25 mL) and washed with aqueous $NaHCO_3$ (2 × 10 mL). The aqueous phase was back extracted with EtOAc (1 × 10 mL) and combined organic phase was evaporated to dryness. The resulting crude residue was purified by silica gel column chromatography (0–4% MeOH in CH_2Cl_2, v/v) to afford nucleoside **21** (17 mg, 65%) as a white solid. R_f = 0.5 (10% MeOH in CH_2Cl_2, v/v); ESI-HRMS m/z 489.1537 ([M + H] $^+$ ($C_{19}H_{27}D_2N_5O_6Cl$, calcd 489.1529). ^{1}H NMR (MeOD-d_4) δ 8.22 (s, 1H), 4.82 (s, 1H), 4.74 (d, 1H, J = 6.8 Hz), 4.15–4.23 (m, 4H), 3.96 (d, 1H, J = 6.7 Hz), 1.76–1.82 (m, 1H), 1.65–1.70 (m, 3H), 1.37 (t, 6H, J = 7.1 Hz), 0.89–0.95 (m, 1H). ^{31}P NMR (MeOD-d_4) δ 1.30.

Ethyl *(1'S,2'R'3'S,4'R,5'S)*-4-(6-Amino-2-chloropurin-9-yl]-2,3-(dihydroxyl)-bicyclo[3.1.0]hexanecarboxylate (22)—To a solution containing **18** (19 mg, 48.3 μmol) in MeOH:H_2O (4 mL, 1:1, v/v) was added Dowex-50 resin (~100 mg). The mixture was stirred for 2 h at 55°C and the resin removed by filtration. Filtrate was evaporated to dryness and resulting crude purified by silica gel column chromatography (0–8% MeOH in CH_2Cl_2, v/v) to afford nucleoside **22** (13.1 mg, 77%) as a white solid. R_f = 0.6 (10% MeOH in CH_2Cl_2, v/v); ESI-HRMS m/z 354.0957 ([M + H] $^+$ ($C_{14}H_{16}N_5O_4Cl{\cdot}H^+$, calcd 354.0969). ^{1}H NMR (MeOD-d_4) δ 8.01 (s, 1H), 5.23 (d, 1H, J = 6.7 Hz), 4.77 (s, 1H), 4.25 (q, 2H, J = 7.1 Hz), 4.10 (d, 1H, J = 6.7 Hz), 2.14–2.20 (m, 1H), 1.84–1.86 (m, 1H), 1.60–1.65 (m, 2H), 1.32 (t, 3H, J = 7.1 Hz).

***(1'S,2'R,3'S,4'R,5'S)*-4-(6-Amino-2-chloro-purin-9-yl)-1-2,3-(dihydroxyl)-1-[hydroxydeuteromethyl]-bicyclo-[3.1.0]hexane (23)—**Nucleoside **22** (6.0 mg, 16.9 μmol) was coevaporated with anhydrous toluene (3 × 10 mL), dissolved in anhydrous THF (3 mL) and cooled to 0 °C. $LiAlD_4$ (3 mg, 115 μmol) was added to ice cold reaction mixture and it is allowed to warm up to room temperature. After stirring the reaction mixture for 6 h and it was quenched with slow addition of MeOH (3 mL). The resulting reaction mixture

was evaporated to dryness, and purified by silica gel column chromatography (0–10% MeOH in CH_2Cl_2, v/v) to afford nucleoside **23** (2.7 mg, 51%) as a white solid. R_f = 0.4 (10% MeOH in CH_2Cl_2, v/v). ESI-HRMS m/z 314.0982 ($[M + H]^+$ ($C_{12}H_{12}D_2N_5O_3Cl$, calcd 314.0989). ^1H NMR ($CDCl_3$) δ 7.82 (s, 1H), 5.92 (s, 2H) 5.60 (d, 1H, *J* = 6.4 Hz), 4.78 (s, 1H), 4.68 (d, 1H, *J* = 7.2 Hz), 3.87 (s, 1H), 1.72–1.76 (m, 1H), 1.70 (s, 2H), 1.56 (s, 3H), 1.27 (s, 3H), 1.13–1.18 (m, 1H), 0.96–1.02 (m, 1H).

***(1'S,2'R,3'S,4'R,5'S)*-4-(6-Amino-2-chloro-purin-9-yl)-1-[iodomethyl]-2,3-(*O*-isopropylidine) bicyclo-[3.1.0]hexane (25)—**Nucleoside **24** (45 mg, 0.128 mmol) was coevaporated with anhydrous toluene (3 × 10 mL) and dissolved in anhydrous THF (3 mL). I_2 (66 mg, 0.256 mmol), triphenylphosphine (68 mg, 0.256 mmol), and imidazole (18 mg, 0.256 mmol) were added. After stirring for 17 h, the reaction mixture was diluted with EtOAc (30 mL) and washed with saturated aqueous $Na_2S_2O_3$ (2 ×15 mL). The phases were separated and aqueous phase was extracted with EtOAc (3 × 25 mL). The combined organic phase was evaporated to dryness, and the resulting residue was purified by silica gel column chromatography (0–80% EtOAc in petroleum ether, v/v) to afford 5'-iodo nucleoside **25** (41 mg, 74%). R_f = 0.4 (5% MeOH in CH_2Cl_2, v/v). ESI-HRMS m/z 462.0205 ($[M + H]^+$ ($C_{15}H_{19}N_5O_2Cl$, calcd 462.0194). ^1H NMR ($CDCl_3$) δ 8.09 (s, 1H), 6.11 (s, 2H), 5.32 (d, 1H, *J* = 6.9 Hz), 4.89 (s, 1H), 4.70 (d, 1H, *J* = 6.9 Hz), 3.63–3.69 (d, 1H, *J* = 10.5 Hz), 3.53–3.56 (d, 1H, *J* = 10.5 Hz), 1.65–1.74 (m, 1H), 1.56 (s, 3H), 1.45–1.50 (m, 1H), 1.24–1.32 (s, 4H), 1.10–1.15 (m, 1H).

(*1'S,2'R,3'S,4'R,5'S*)-4-(6-Amino-2-chloro-purin-9-yl)-2,3-(dihydroxy)-1-[iodomethyl]bicyclo-[3.1.0]hexane (26)—5-Iodo nucleoside **25** (106 mg, 0.229 mmol) was dissolved in THF (1 mL) followed by the addition of 10% aqueous trifluoroacetic acid (3.5 mL, v/v). After stirring the reaction mixture at 65 °C for 15 h, reaction mixture was evaporated to dryness and the resulting residue was purified by silica gel column chromatography (0–80% EtOAc in petroleum ether, v/v) to afford 2',3'-dihydroxy-5'-iodo nucleoside **26** (41 mg, 60%). R_f = 0.3 (10% MeOH in CH_2Cl_2, v/v). ESI-HRMS m/z 421.9883 ($[M + H]^+$ ($C_{12}H_{14}N_5O_2ClI$, calcd 421.9881). ^1H NMR (MeOD-d_4) δ 8.41 (s, 1H), 4.75 (dd, 1H, *J* = 1.8 Hz), 4.72 (s, 1H), 4.10 (dt, 1H, *J* = 8 Hz, 2.8 Hz), 3.87–3.91 (d, 1H, *J* = 10.5 Hz), 3.46–3.52 (d, 1H, *J* = 10.5 Hz), 1.91–1.95 (m, 1H), 1.671.72 (m, 1H), 1.04–1.09 (m, 1H).

***(1'S,2'R,3'S,4'R,5'S)*-4-(6-Amino-2-chloro-purin-9-yl)-1-[*O,O*-diethyl *O*-methyl phosphorothioate]- 2,3-(*O*-isopropylidine)-bicyclo-[3.1.0]hexane (*27*)—**Nucleoside **24** (18 mg, 51.2 μmol) was dissolved in anhydrous pyridine (3 mL) and *O,O'*-diethyl chlorothiophosphate (24 μL, 153 μmol) was added to this. After stirring at room temperature for 17 h, the reaction mixture was evaporated to dryness. The resulting residue was purified by silica gel column chromatography (0–5% MeOH in CH_2Cl_2, v/v) to afford 5'-diethyl phosphorothioate nucleoside **27** (3.2 mg, 13%). ESI-HRMS m/z 504.1234 ($[M + H]^+$ ($C_{19}H_{28}ClN_5O_5P$, calcd 504.1237). R_f = 0.3 (5% MeoH in CH_2Cl_2, v/v). ^1H NMR (MeOD-d_4) δ 8.25 (s, 1H), 5.35 (d, 1H, *J* = 6.2 Hz), 4.94 (s, 1H), 4.67 (d, 1H, *J* = 6.2 Hz), 4.06–4.14 (m, 4H), 3.96 (d, 1H, *J* = 11.5 Hz), 3.63 (d, 1H, *J* = 11.5 Hz), 1.66–1.71 (m, 1H), 1.51 (s, 3H), 1.30–1.40 (m, 6H), 1.27 (s, 3H), 1.10–1.16 (m, 1H), 0.94–0.99 (m, 1H).

***(1'S,2'R,3'S,4'R,5'S)*-4-(6-Amino-2-chloro-purin-9-yl)-1-[*O,O*-diethyl *S*-methyl phosphorothioate]-2,3-(O-isopropylidine) bicyclo-[3.1.0]hexane (28)—**5'-iodo nucleoside **25** (11 mg, 0.024 mmol) was dissolved in anhydrous CH_3CN (2 mL), *O,O*-Diethyl thiophosphate potassium salt (24.8 mg, 0.12 mmol) and Et_3N (34 μL, 0.24 mmol) were added. After stirring the reaction at room temperature for 22 h, reaction mixture was evaporated to dryness and the resulting crude residue was purified by silica gel column

chromatography (0–5% MeOH in CH_2Cl_2, v/v) to afford 5'-phosphorothioate **28** (7.3 mg, 52%). R_f = 0.4 (5% MeOH in CH_2Cl_2, v/v). ESI-HRMS m/z 504.1235 ([M + H] $^+$ ($C_{19}H_{27}N_5O_5SClP$·H, calcd 504.1237). ^{1}H NMR (MeOD-d_4) δ 8.14 (s, 1H), 5.32 (d, 1H, J = 7.2 Hz), 4.91 (s, 1H), 4.81 (d, 1H, J = 7.2 Hz), 4.16–4.27 (m, 4H), 3.74 (t, 1H, J = 13.5 Hz), 2.93–3.01 (m, 1H), 1.72–1.76 (m, 1H), 1.53 (s, 3H), 1.30–1.40 (m, 6H), 1.27 (s, 3H), 1.19–1.23 (m, 1H), 1.06–1.11 (m, 1H). ^{31}P NMR (MeOD-d_4) δ 28.91.

***(1'S,2'R,3'S,4'R,5'S)*-4-(6-Amino-2-chloro-purin-9-yl)-1-[*O,O*-diethyl *S*-methyl phosphorodithioate]-2,3-(O-isopropylidine) bicyclo-[3.1.0]hexane (29)—**5'-iodo nucleoside **25** (11.0 mg, 0.024 mmol) was dissolved in anhydrous CH_3CN (2 mL), *O,O*′-Diethyl dithiophosphate (19.9 μL, 0.12 mmol) and Et_3N (34 μL, 0.24 mmol) were added. After stirring the reaction at room temperature for 22 h, reaction mixture was evaporated to dryness and the resulting crude residue was purified by silica gel column chromatography (0–4% MeOH in CH_2Cl_2, v/v) to afford 5'-phosphorodithioate **29** (6.8 mg, 49%). R_f = 0.4 (5% MeOH in CH_2Cl_2, v/v). ESI-HRMS m/z 520.1024 ([M + H] $^+$ ($C_{19}H_{27}N_5O_4S_2ClP$·H, calcd 520.1009). ^{1}H NMR (MeOD-d_4) δ 8.15 (s, 1H), 5.30 (d, 1H, J = 7.2 Hz), 4.90 (s, 1H), 4.80 (d, 1H, J = 7.2 Hz), 4.11–4.24 (m, 4H), 3.69 (t, 1H, J = 13.5 Hz), 3.06 (t, 1H, J = 13.5 Hz), 1.69–1.75 (m, 1H), 1.53 (s, 3H), 1.30–1.38 (m, 6H), 1.27 (s, 3H), 1.19–1.23 (m, 1H), 1.06–1.11 (m, 1H). ^{31}P NMR (MeOD-d_4) δ 94.27

***(1'S,2'R,3'S,4'R,5'S)*-4-(2,6-Dichloro-9H-purin-9-yl)-1-[(diethylphosphate) methyl]-2,3-(*O*-isopropylidene)-bicyclo[3.1.0]hexane (31)—**Nucleoside **30** (20 mg, 54 μmol) was coevaporated with anhydrous toluene (3 × 10 mL), dissolved in anhydrous pyridine (2 mL) and diethylchlorophosphate (24 μL, 161 μmol) was added to this. After stirring at rt for 17 h, the reaction mixture was diluted with EtOAc (25 mL) and washed with aqueous $NaHCO_3$ (2 × 10 mL). The aqueous phase was back extracted with EtOAc (1 × 10 mL) and combined organic phase was evaporated to dryness. The resulting crude residue was purified by silica gel column chromatography (0–4% MeOH in CH_2Cl_2, v/v) to afford nucleoside **31** (19 mg, 69%) as a white solid. R_f = 0.5 (5% MeOH in CH_2Cl_2, v/v); ESI-HRMS m/z 507.0963 ([M + H] $^+$ ($C_{19}H_{26}N_4O_6Cl_2P$, calcd 507.0967). ^{1}H NMR ($CDCl_3$) δ 8.10 (s, 1H), 5.35 (d, 1H, J = 7.2 Hz), 5.06 (s, 1H), 4.62 (d, 1H, J = 7.2 Hz), 4.43–4.50 (m, 1H), 4.14–4.30 (m, 5H), 1.72–1.77 (m, 1H), 1.57 (s, 3H), 1.35–1.43 (m, 6H), 1.27–1.33 (m, 1H), 1.26 (s, 3H), 1.11–1.16 (m, 1H). ^{31}P NMR ($CDCl_3$) δ −1.30.

***(1'S,2'R,3'S,4'R,5'S)*-4-(6-Amino-2-chloro-9H-purin-9-yl)-1-[(diethylphosphate) methyl]-2,3-(*O*-isopropylidene)-bicyclo[3.1.0]hexane (32)—**Nucleoside **31** (17 mg, 33.5 μmol) was dissolved in 2M NH_3/i-PrOH (5 mL). After stirring at 70 °C for 17 h, the reaction mixture was evaporated to dryness and resulting crude residue was purified by silica gel column chromatography (0–6% MeOH in CH_2Cl_2, v/v) to afford nucleoside **32** (10 mg, 65%). R_f = 0.5 (8% MeOH in CH_2Cl_2, v/v); ESI-HRMS m/z 488.1466 ([M + H] $^+$ ($C_{19}H_{27}N_5O_6ClP$, calcd 488.1466). ^{1}H NMR (MeOD-d_4) δ 8.14 (s, 1H), 5.35 (d, 1H, J = 7.2 Hz), 4.96 (s, 1H), 4.79 (d, 1H, J = 7.2 Hz), 4.58–4.62 (m, 1H), 4.12–4.21 (m, 4H), 4.02–4.08 (m, 2H), 1.75–1.79 (m, 1H), 1.53 (s, 3H), 1.29–1.39 (m, 6H), 1.27–1.33 (m, 4H), 1.11–1.16 (m, 1H). ^{31}P NMR (MeOD-d_4) δ −1.34.

***(1'S,2'R,3'S,4'R,5'S)*-4-(6-Amino-2-chloro-9H-purin-9-yl)-1-[(diisopropylphosphate) methyl]-2,3-(*O*-isopropylidene)-bicyclo[3.1.0]hexane (33)—**Nucleoside **24** (15 mg, 38 μmol) was coevaporated with anhydrous toluene (3 × 10 mL), dissolved in anhydrous pyridine (2 mL) and diisopropyl chlorophosphate (90 μL, 190 μmol) was added to this. After stirring at rt for 17 h, the reaction mixture was diluted with EtOAc (25 mL) and washed with aqueous $NaHCO_3$ (2 × 10 mL). The aqueous phase was back extracted with EtOAc (1 × 10 mL) and combined organic phase was evaporated to

dryness. The resulting crude residue was purified by silica gel column chromatography (0–4% MeOH in CH_2Cl_2, v/v) to afford nucleoside **33** (9.6 mg, 49%) as a white solid. R_f = 0.3 (5% MeOH in CH_2Cl_2, v/v); ESI-HRMS m/z 516.1790 ($[M + H]^+$ ($C_{21}H_{32}N_5O_6ClP$, calcd 516.1779). ^1H NMR ($CDCl_3$) δ 8.08 (s, 1H), 6.16 (s, 2H), 5.33 (d, 1H, *J* = 6.9 Hz), 5.01 (s, 1H), 4.65–4.73 (m, 2H), 4.60 (d, 1H, *J* = 7.2 Hz), 4.42–4.48 (m, 1H), 4.09–4.15 (m, 5H), 1.69–1.75 (m, 1H), 1.57 (s, 3H), 1.33–1.42 (m, 12H), 1.22–1.29 (m, 4H), 1.07–1.12 (m, 1H). ^{31}P NMR ($CDCl_3$) δ −2.65.

***Diethyl-(1'S,2'R,3'S,4'R,5'S)*-4'-(6-chloro-2-iodo-purin-9-yl)-2',3'-*O*-(isopropylidene)-bicyclo[3.1.0]hexane phosphonate (37)—**Diisopropyl azodicarboxylate (86 μL, 0.44 mmol) was added at rt to a mixture of triphenylphosphine (115 mg, 0.44 mmol) and 6-chloro-2-iodopurine (122 mg, 0.44 mmol) in anhydrous THF (4 mL). After stirring for 45 min, a solution of the compound **36** (70 mg, 0.22 mmol) in THF (4 mL) was added to the mixture. After stirring for 36 h, the reaction mixture was evaporated to dryness. The resulting residue was purified by silica gel column chromatography (0–3% MeOH in CH_2Cl_2, v/v) to afford nucleoside **37** (89 mg, 70%) as a white solid. R_f = 0.5 (5% MeOH in CH_2Cl_2, v/v); ESI-HRMS *m/z* 583.0374 $[M + H]^+$, $C_{19}H_{26}ClIN_4O_5P^+$: Calcd. 583.0373); ^1H NMR ($CDCl_3$) δ 8.64 (s, 1H), 5.39 (d, 1H, *J* = 7.5 Hz), 5.07 (s, 1H), 4.64 (d, 1H, *J* = 7.5 Hz), 4.15–4.21 (m, 4H), 2.41 (t, 1H, *J* = 16.0 Hz), 2.11–2.22 (m, 1H), 1.73–1.79 (m, 1H), 1.59–1.64 (m, 1H), 1.54 (s, 3H), 1.35 (t, 3H, *J* = 7.1 Hz), 1.28 (t, 3H, *J* = 7.1 Hz), 1.25 (s, 3H), 1.04–1.09 (m, 1H).

Diethyl-*(1'S,2'R,3'S,4'R,5'S)*-4'-(6-amino-2-iodopurin-9-yl)-2',3'-*O*-(isopropylidene)-bicyclo[3.1.0]hexane phosphonate (38)—Nucleoside **37** (80 mg, 0.14 mmol) was treated with 2M NH_3 in *i*-PrOH (8 mL) and the mixture was heated to 70 °C and stirred for 17 h. The reaction mixture was evaporated to dryness, and the resulting residue was purified by silica gel column chromatography (0–6% MeOH in CH_2Cl_2, v/v) to afford nucleoside **38** (48 mg, 64%) as a white solid. R_f = 0.4 (7% MeOH in CH_2Cl_2, v/v); ESI-HRMS *m/z* 564.0873 $[M + H]^+$, $C_{19}H_{28}IN_5O_5P^+$: Calcd. 564.0856); ^1H NMR ($CDCl_3$) δ 8.15(s, 1H), 5.74 (s, 2H), 5.34 (d, 1H, *J* = 6.1 Hz), 4.94 (s, 1H), 4.64 (d, 1H, *J* = 6.1 Hz), 4.12–4.19 (m, 4H), 2.19–2.42 (m, 2H), 1.69–1.74 (m, 1H), 1.55 (s, 3H), 1.33 (t, 3H, *J* = 7.1 Hz), 1.26 (t, 3H, *J* = 7.1 Hz), 1.22 (s, 3H), 1.18–1.21 (m, 1H), 1.03–1.09 (m, 1H).

Determination of aqueous solibility of selected phosphonate triesters—To 5 mg of solid compound **14** was added water in a drop-wise fashion with agitation at room temperature to prepare a saturated solution, i.e. with solid remaining. Total volume of water added was 50 μL. The solution was allowed to stand at room temperature for 1 h, and then it was centrifuged. The supernatant was removed and lyophilized. The amount of solid obtained was 1.3 mg, providing an aqueous solubility of 60 mM. A similar procedure was used with 35 mg of compound **16** (2 mL water added, with 6.75 mg remaining after lyophilization) to provide an aqueous solubility of 7.25 mM.

Biological evaluation

Post-infarction ischemic heart failure model—An ischemic heart failure model was carried out by permanent ligation of the LAD in mice using a procedure similar to that described previously.[17] Adult 12-week old WT (BL6) mice or transgenic mice with cardiac-specific overexpression of the P2X4R were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitoneally. After endotracheal intubation and left intercostal thoracotomy, myocardial Infarction was produced by ligating the LAD with an 8-0 nylon suture within 2 mm below the edge of left atrium near the origin of the artery. All animal procedures were performed according to guidelines of and approved by the University of Connecticut School of Medicine Review Board.

Measurements of intact heart function by in vivo echocardiography or by ex vivo working heart model and infarct size—Transthoracic echocardiography was performed using a linear 30-MHz transducer according to the manufacturer's instructions (Vevo 660 High Resolution Imaging System from VisualSonics, Toronto, Canada) similar to previously described methods.[5] Two-dimensional-targeted M-mode echocardiographic measurements were carried out at mid-papillary muscle level after anesthesia with 1% isoflurane. FS was determined as LVEDD - LVESD/LVEDD where LVEDD and LVESD represented left ventricular end diastolic and end systolic dimensions in mm respectively. Measurements were averaged from more than three cardiac cycles. Another measure of intact heart function was the left ventricular rate of pressure development (+dP/dt) using an ex vivo working heart preparation as previously described[10]. The afterload against which buffered perfusate solution was ejected from left ventricle was constant at 56 mmHg of hydrostatic pressure from a column of buffer connected to the aorta. Hemodynamic parameters from the various groups of mice were obtained under the same afterload. Data were analyzed by computer software (WorkBench for Windows+, Kent Scientific Corp., Torrington, CT).

The infarct size was quantified as previously described.[17] After fixing in 10% formalin, sections were embedded in paraffin, cut into 4-μm slices, and stained with Masson's trichrome to measure area of infarcted myocardium as fibrosis. Infarct size was calculated as the ratio of infarct length to the circumference of both the endocardium and the epicardium after tracing with a planimeter image analyzer (ImageProPlus). Contractile response was measured in adult WT murine cardiac ventricular myocytes in which contraction shortening in the presence of drugs was determined as previously described[18].

Drug administration

Compound **1** and its analogues were dissolved in phosphate-buffered saline, pH=7.4 at 10 μM (200 μL total volume), filtered for sterility for *in vivo* administration at 6 μL per d for 28 d via a mini-osmotic pump (Alzet) in mice. Intact heart function *in vivo* was assessed by echocardiography following infusion of nucleotide or vehicle.

Data analysis

Unless otherwise indicated, data were provided as mean ± standard error of the mean. For analysis of multiple groups, one-way ANOVA and Newman Keuls post-test comparison were used. Student's t-test for paired or unpaired samples was used to evaluate the effects of experimental interventions; $P<0.05$ was taken as statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

5'-AMP	adenosine 5'-monophosphate
CS	contraction shortening

CSQ	calsequestrin
DIBAL-H	diisobutylaluminum hydride
DMEM	Dulbecco's modified Eagle medium
DMF	dimethylformamide
FS	fractional shortening
HEPES	*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectroscopy
LAD	left anterior descending coronary artery
LV	left ventricular
NS	normal saline
rt	room temperature
SAR	structure activity relationship
TBAP	tributylammonium phosphate
THF	tetrahydrofuran
TEAA	triethylammonium acetate

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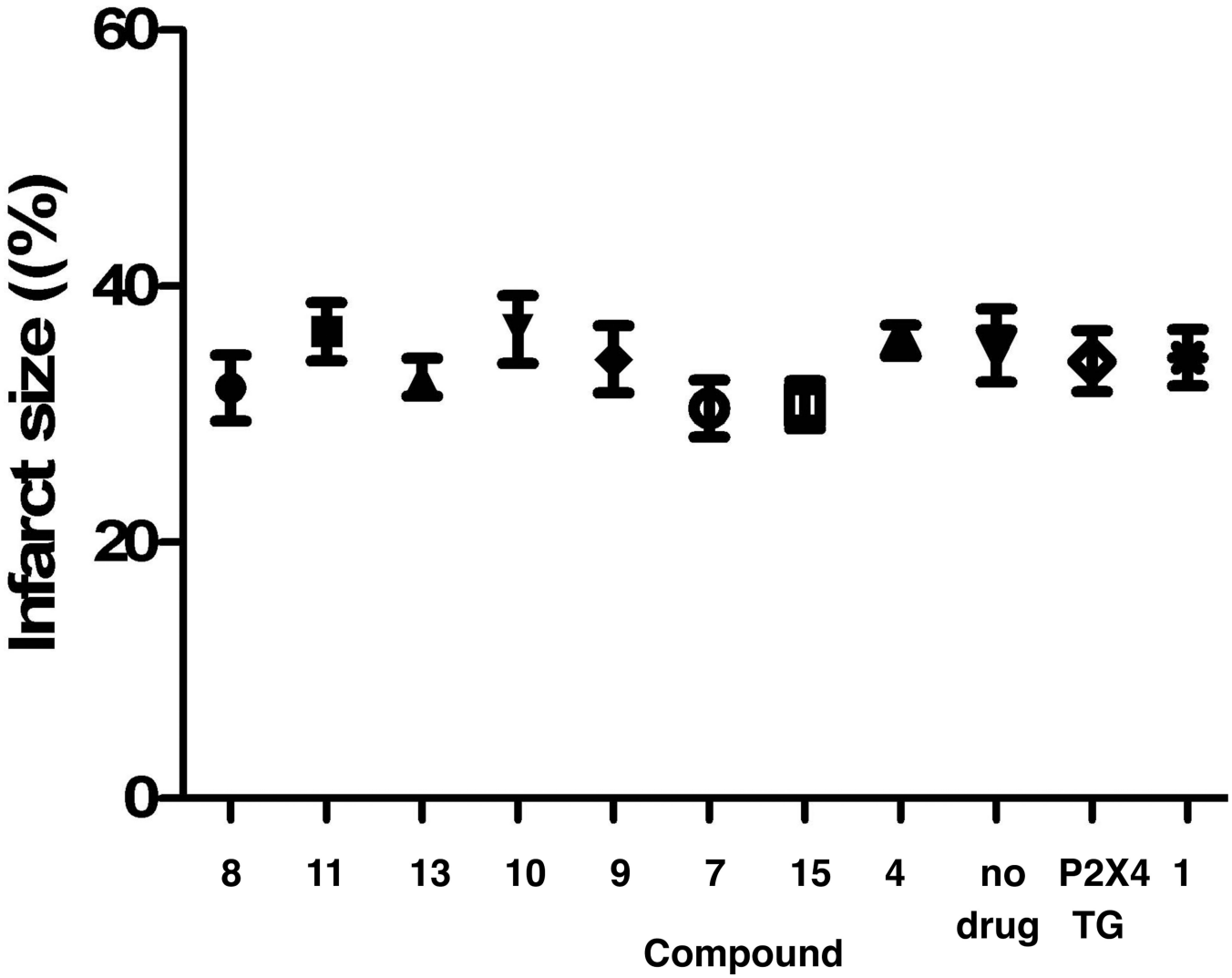


Figure 1. Effects on infarct size following chronic infusion of various agents

Within 24 hours of LAD ligation, various agents were infused for one month and infarct size determined after measurement of intact heart function as described in Experimental Procedures. As controls, data were also obtained in both vehicle-infused wild type mice and transgenic mice with cardiac specific P2X4 receptor overexpression. There were no differences in the infarct size under any of the drug interventions or in the transgenic mice (one-way ANOVA and post-test comparison, $P>0.05$).

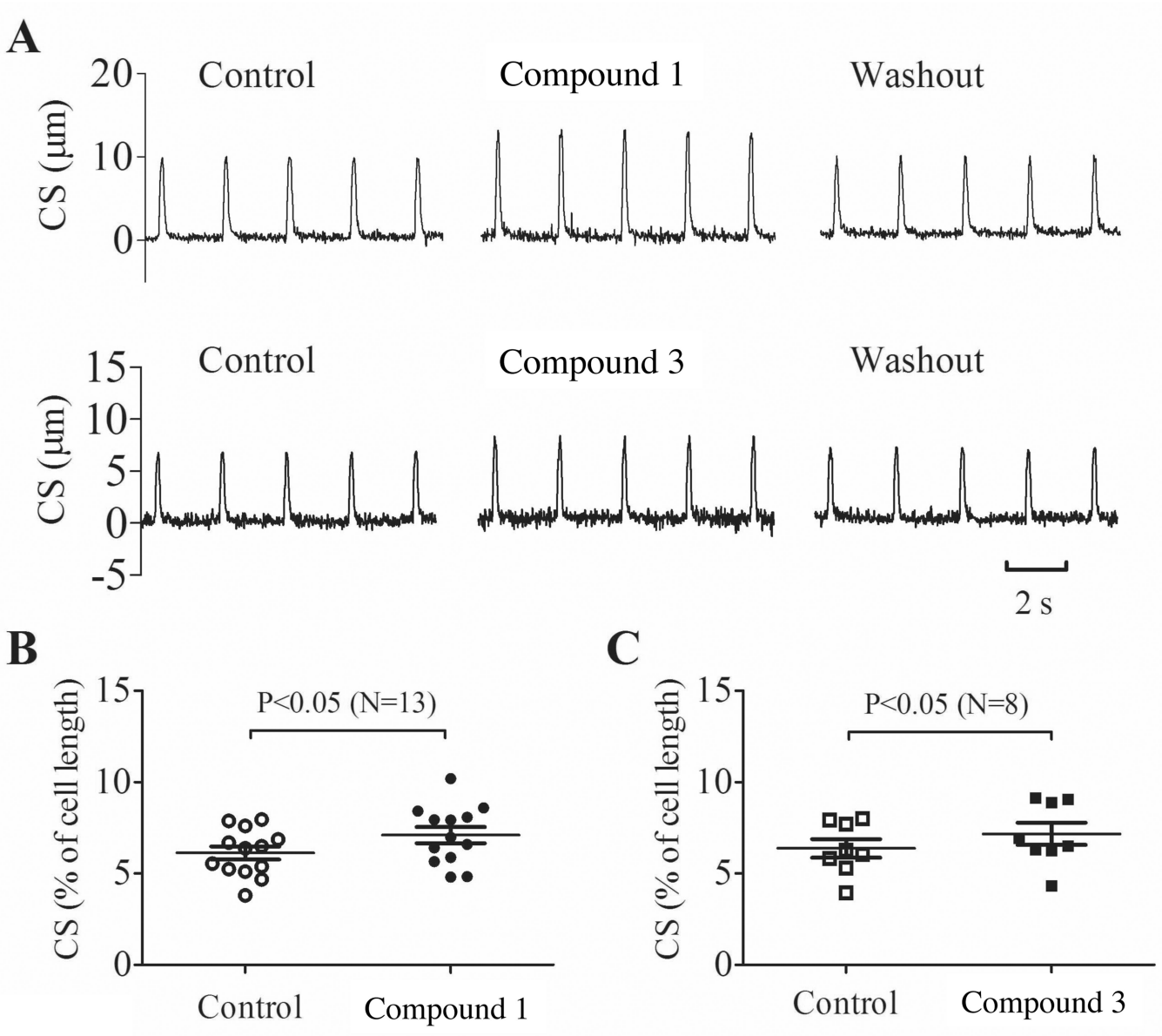
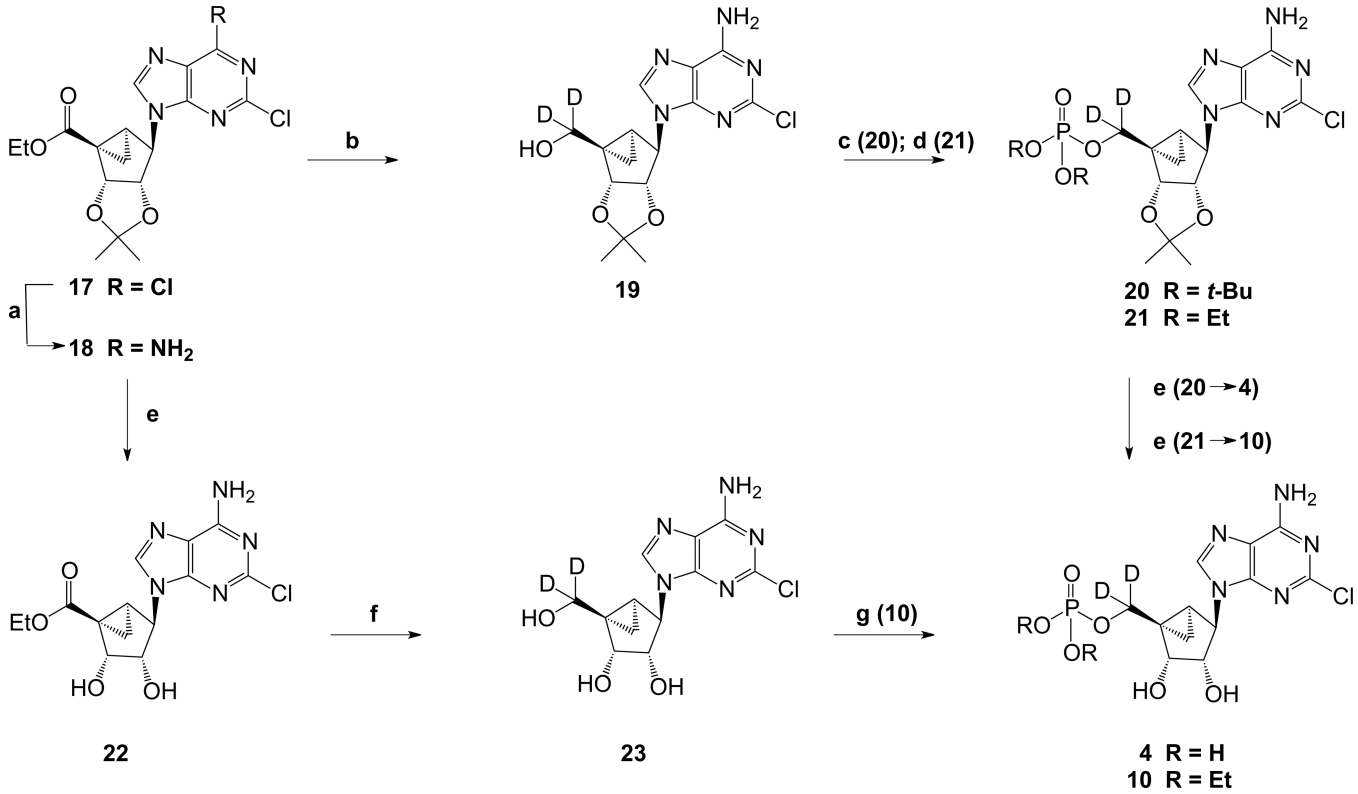


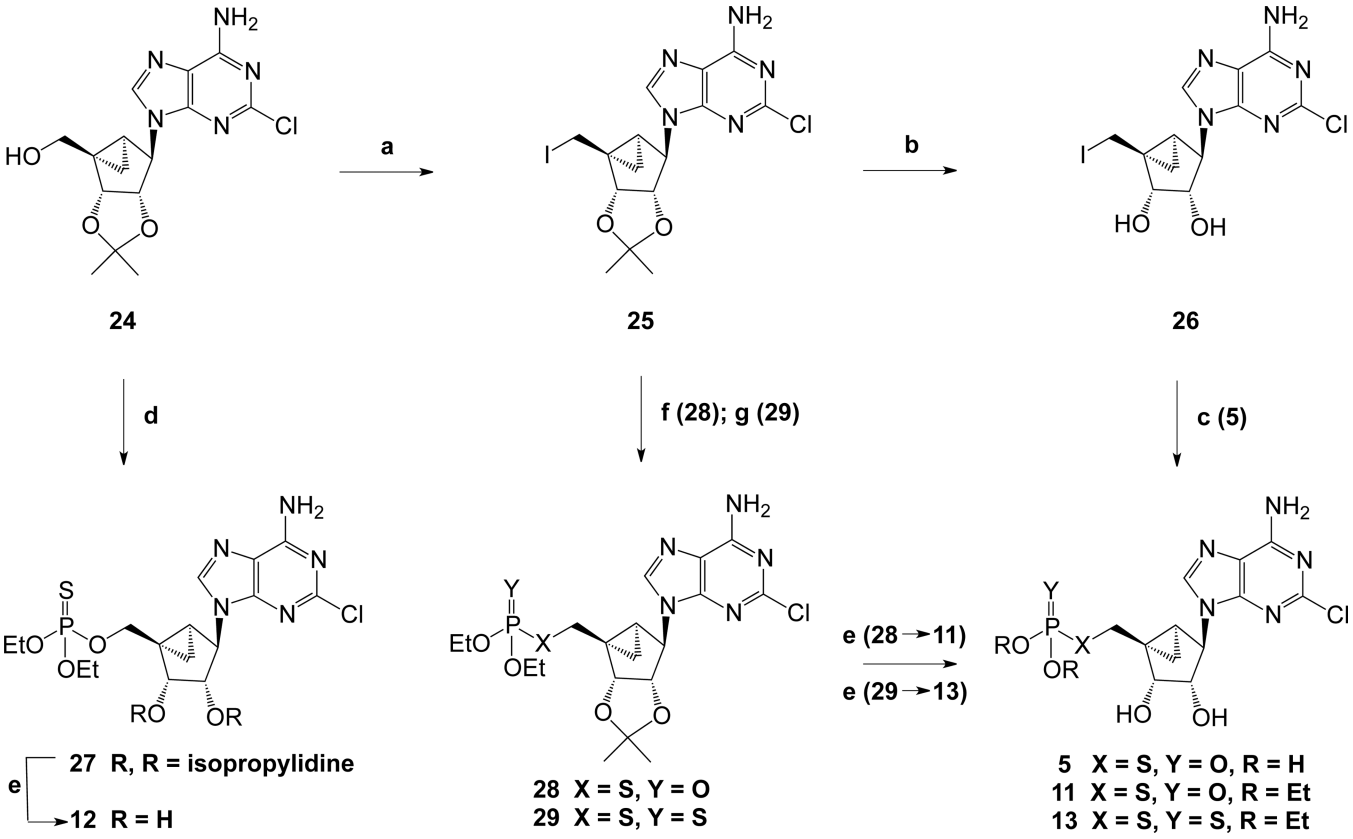
Figure 2. Agonist-like effects of compounds 1 and 3 in adult murine cardiac ventricular myocytes

Ventricular myocytes isolated from 10–12 week old Bl6 wild type mice were studied for contractile response to **1** and **3**. CS was measured under control basal condition, drug exposure and washout. Representative CS tracings were shown for both agents (A). Means and standard errors for **1** in 13 myocytes (B) and for **3** in 8 myocytes (C) were shown. Both agents caused a modest increase in C (P<0.05).



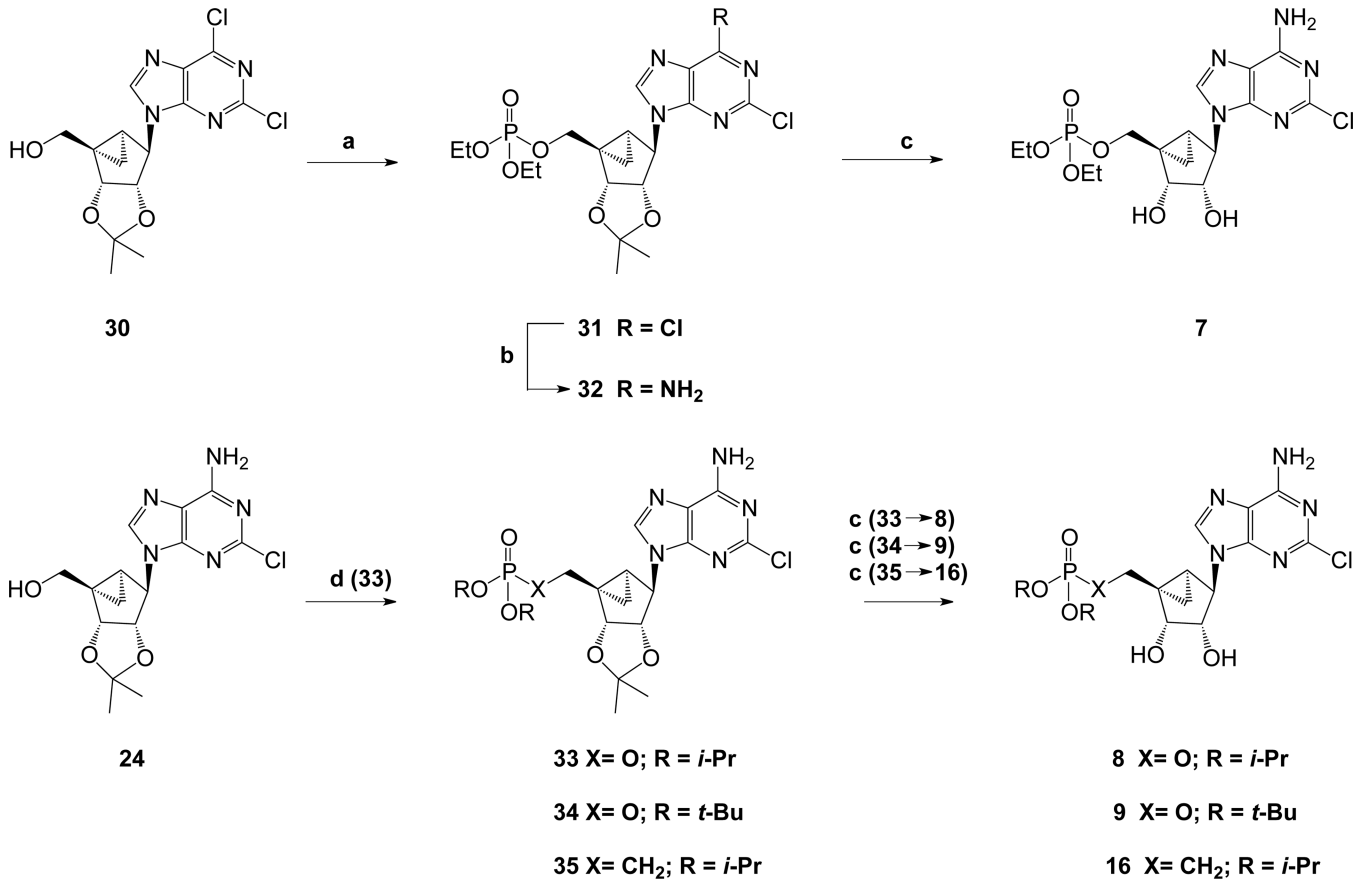
Scheme 1. Reagents and Conditions

a) 2M NH_3 in *i*-PrOH, 70 °C, 68%; b) $LiBD_4$, anhydrous THF, reflux, 72%; c) i) Di-*t*-butyl*N,N*'-diethylphosphoramidite, anhydrous THF, tetrazole; ii) *m*-chloroperbenzoic acid, 76%; d) diethylchlorophosphate, anhydrous pyridine, rt, 65%; e) Dowex-50 resin, MeOH:H_2O (1:1, v/v) 70 °C **(4)**, 55 °C **(10, 22)**, 32% **(4)**, 55% (**10**), 77% (**22**); f) $LiAlD_4$, anhydrous THF, 0 °C to rt, 51%, g) diethylchlorophosphate, anhydrous pyridine, rt, 55%.



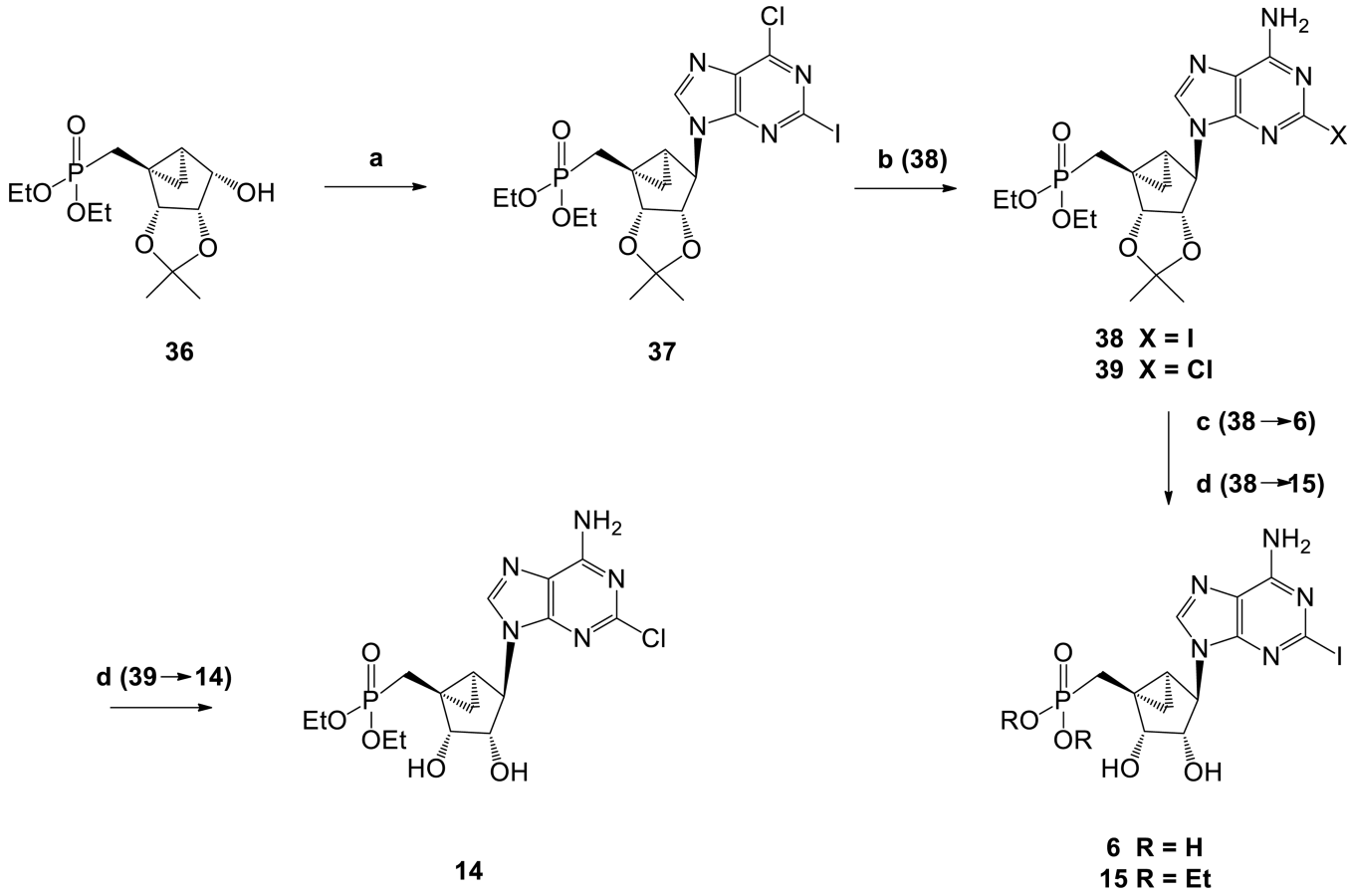
Scheme 2. Reagents and Conditions

a) Triphenylphosphine, I_2, imidazole, anhydrous THF, 74%, b) 10% Aq. trifluoroacetic acid, THF, 65 °C, 60%; c) Trisodium thiophosphate, H_2O, 57%; d) *O,O′*-diethyl chlorothiophosphate, anhydrous pyridine, rt, 13%; e) Dowex-50 resin, MeOH:H_2O (1:1, v/v) 55 °C, 12% (**12**), 48% (**11**), 59% (**13**); f) *O,O*-Diethyl thiophosphate potassium salt, anhydrous CH_3CN, Et_3N, rt, 52%; g) *O,O′*-Diethyl dithiophosphate, anhydrous CH_3CN, Et_3N, rt, 49%;



Scheme 3. Reagents and Conditions

a) Diethylchlorophosphate, anhydrous pyridine, rt, 69%; b) 2M NH_3 in *i*-PrOH, 70 °C, 65%; c) Dowex-50 resin, MeOH:H_2O (1:1, v/v), 55°C (rt for **9**), 59% **(7)**, 43% **(8)**, 9% **(9)**, 49% **(16)**; d) Diisopropylchlorophosphate, anhydrous pyridine, rt, 49%.



Scheme 4. Reagents and Conditions

a) Triphenylphosphine, 6-chloro-2-iodopurine, diisopropyl azodicarboxylate, anhydrous THF, 70%; b) 2M NH_3 in *i*-PrOH, 70 °C, 64%; c) Iodotrimethylsilane, anhydrous CH_2Cl_2, 49%; d) Dowex-50 resin, MeOH:H_2O (1:1, v/v), 55°C, 40% **(14)**, 49% **(15)**.

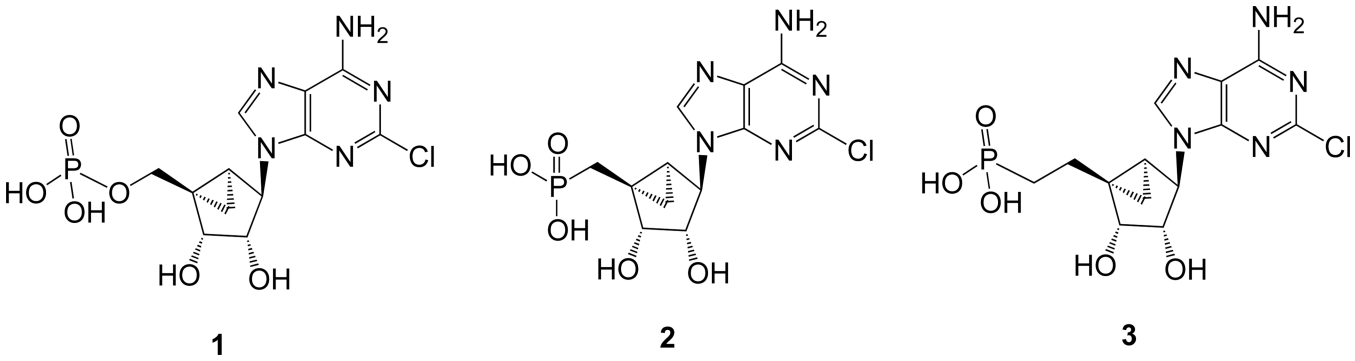
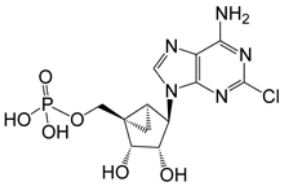
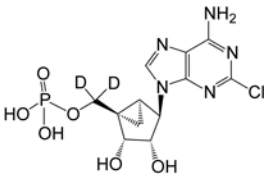
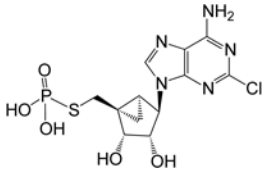
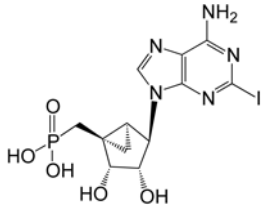
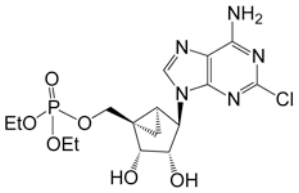
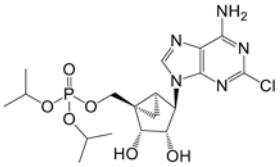
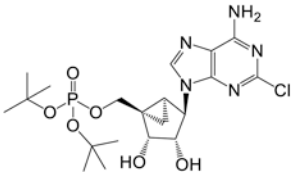
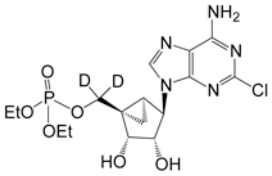
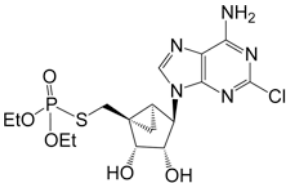
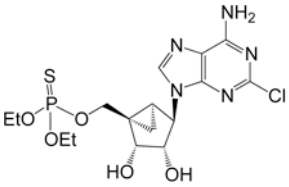
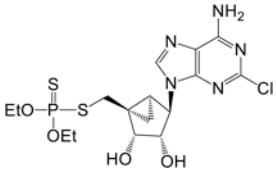
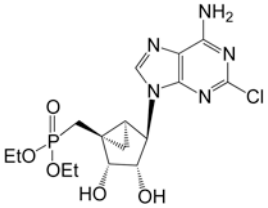


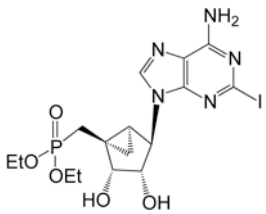
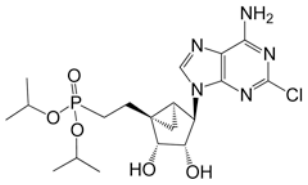
Chart 1.
Representative adenine nucleotide derivatives that display cardioprotective activity by activating a P2X ion channel. Both the phosphate derivative **1** and the homologous phosphonate analogues **2** and **3** served as the lead compounds for the present set of ester derivatives.

Table 1

Novel phosphate and phosphonate analogues: structure and effects on *in vivo* heart function after subcutaneous infusion for 28 days as determined by echocardiography-derived FS in the LAD ligation model of ischemic heart failure in mice.

Compound or condition	Structure	FS in %[a]	n =	Corresponds to compound:
Vehicle control	-	17.33 ± 1.36	10	-
P2X4R transgenic mouse	-	23.69 ± 1.74 *	9	-
Charged nucleotides				
1		22.14 ± 0.68 *	20	-
4		23.06 ± 1.14 *	18	**1** (deuterated)
5		ND		**1** (thio)
6		ND		**1** (phosphonate, 2-iodo)
Masked nucleotides				Ester derivative of:
7		24.71 ± 1.42 *	8	**1**

Compound or condition	Structure	FS in %[a]	n =	Corresponds to compound:
8		25.77 ± 1.10 *	8	**1**
9		24.07 ± 1.64 *	11	**1**
10		21.48 ± 1.38	8	**4**
11		24.31 ± 1.45 *	13	**5**
12		ND		**1** (thio)
13		24.76 ± 1.32 *	12	**5** (dithio)
14		ND		**2**

Compound or condition	Structure	FS in %[a]	n =	Corresponds to compound:
15		23.05 ± 1.45 *	11	**6**
16		ND		**3**

[a] at 10 μM, expressed as mean ± SEM.

* $P<0.05$ vs. vehicle for all compounds tested, except compound **10**, which displayed $P<0.1$.

ND – not determined.

Table 2

Structure and effects on *ex vivo* heart function of novel phosphate and phosphonate analogues after subcutaneous infusion for 28 days, as determined by isolated working heart preparation in the LAD ligation model of ischemic heart failure in mice. Structures are shown in Table 1.

Compound	+dP/dt (mmHg/sec)[a]	n =
Vehicle control	6156 ± 356	21
Charged nucleotides		
1	6508 ± 96*	18
5	6553 ± 435*	11
6	6581 ± 721*	8
Masked nucleotides		
7	6711± 726*	9
9	6681± 528*	9
14	5946± 337	9

[a] at 10 μM, expressed as mean ± SEM.

* $P<0.05$ vs. vehicle for all compounds except **14**.

ND – not determined.