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Synthesis and Structure-Activity Relationships of Pyridoxal-6-arylazo-5'-phosphate and Phosphonate Derivatives as P2 Receptor Antagonists

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Abstract

Novel analogs of the P2 receptor antagonist pyridoxal-5'-phosphate-6-phenylazo-2',4'-disulfonate (PPADS) were synthesized. Modifications were made through functional group substitution on the sulfophenyl ring and at the phosphate moiety through the inclusion of phosphonates, demonstrating that a phosphate linkage is not required for P2 receptor antagonism. Substituted 6-phenylazo and 6-naphthylazo derivatives were also evaluated. Among the 6-phenylazo derivatives, 5'-methyl, ethyl, propyl, vinyl, and allyl phosphonates were included. The compounds were tested as antagonists at turkey erythrocyte and guinea-pig taenia coli P2Y₁ receptors, in guinea-pig vas deferens and bladder P2X₁ receptors, and in ion flux experiments by using recombinant rat P2X₂ receptors expressed in *Xenopus* oocytes. Competitive binding assay at human P2X₁ receptors in differentiated HL-60 cell membranes was carried out by using [³⁵S]ATP- γ -S. A 2'-chloro-5'-sulfo analog of PPADS (C₁₄H₁₂O₉N₃CIPNa), a vinyl phosphonate derivative (C₁₅H₁₂O₁₁N₃PS₂Na₃), and a naphthylazo derivative (C₁₈H₁₄O₁₂N₃PS₂Na₂), were particularly potent in binding to human P2X₁ receptors. The potencies of phosphate derivatives at P2Y₁ receptors were generally similar to PPADS itself, except for the *p*-carboxyphenylazo phosphate derivative C₁₅H₁₃O₈N₃PNa and its *m*-chloro analog C₁₅H₁₂O₈N₃CIPNa, which were selective for P2X vs. P2Y₁ receptors. C₁₅H₁₂O₈N₃CIPNa was very potent at rat P2X₂ receptors with an IC₅₀ value of 0.82 μ M. Among the phosphonate derivatives, [4-formyl-3-hydroxy-2-methyl-6-(2-chloro-5-sulfonylphenylazo)-pyrid-5-yl]methylphosphonic acid (C₁₄H₁₂O₈N₃CIPNa) showed high potency at P2Y₁ receptors with an IC₅₀ of 7.23 μ M. The corresponding 2,5-disulfonylphenyl derivative was nearly inactive at turkey erythrocyte P2Y₁ receptors, whereas at recombinant P2X₂ receptors had an IC₅₀ value of 1.1 μ M. An ethyl phosphonate derivative (C₁₅H₁₅O₁₁N₃PS₂Na₃), whereas inactive at turkey erythrocyte P2Y₁ receptors, was particularly potent at recombinant P2X₂ receptors.

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Keywords

ATP; nucleotides; ion channels; phospholipase C; smooth muscle; guinea pig; turkey erythrocytes

INTRODUCTION

Extracellular adenine nucleosides and nucleotides, acting through P1 and P2 receptors [North and Barnard, 1997; Burnstock, 1996], are widely accepted as neuro-modulators and neurotransmitters in the central and peripheral nervous systems. Two families of P2 receptors, ligand-gated ion channels (P2X subtype) and G protein-coupled receptors (P2Y subtype), have been recently classified [Abbracchio and Burnstock, 1994]. Numerous subtypes of ATP receptors have been cloned, and it is now clear that uracil nucleotides [Communi and Boeynaems, 1997] are also active or even the preferred ligands at some of the P2Y subtypes. P2X₁₋₇ and P2Y_{1,2,4,6} designations have been unambiguously assigned to mammalian nucleotide receptors [Burnstock and King, 1996; Fredholm et al., 1997], although there is still uncertainty about the correspondence of these cloned sequences to pharmacologic phenotypes. Other novel mammalian P2Y subtypes, numbering up to P2Y₁₁, have recently been reported [Communi et al., 1997].

The most significant evidence for ATP acting as a cotransmitter was reported for the sympathetic neurotransmission system in which norepinephrine and ATP are coreleased [Burnstock, 1993]. In vas deferens, isolated blood vessels, skeletal muscle, intestine, kidney and skin, norepinephrine and ATP cause synergistic constriction by means of α_1 -adrenoceptors and P2X receptors, respectively. In rabbit coronary vessels, guinea-pig taenia coli, rat aorta, and rat mesenteric artery, the predominant effect of those transmitters is relaxation by means of β -adrenoceptors and P2Y receptors. Besides sympathetic neurotransmission, P2 receptors are also suggested to function in parasympathetic, sensory-motor NANC inhibitory, and somatic neuromuscular neurotransmission. In platelets, an as yet uncloned P_{2T} receptor [Hourani and Hall, 1996; Greco, 1997] has been shown to be activated mainly by ADP to cause platelet aggregation and is blocked by ATP. More recently, a hypothesis was reported that P2X₃ receptors mediate nociception by means of the dorsal root ganglia, whereas activation of P1 receptors seems to result in anti-nociception [Burnstock and Wood, 1996]. The therapeutic potential [Williams, 1996; Williams and Bhagwat, 1996] and physiologic role of P2 receptors also have been reviewed in various biological systems, including brain [Inoue et al., 1996], central nervous [Gibb and Halliday, 1996], sensory nervous [Thorne and Housley, 1996], cardiovascular [Rongen et al., 1997], immune [Di Virgilio et al., 1996], and renal microvascular [Incho, 1996] systems.

Progress in the field of P2 receptors has been impeded because of the lack of useful ligands, especially P2 receptor selective antagonists and radioligands [Jacobson et al., 1997]. A synthetic polyanionic substance, pyridoxal-5'-phosphate-6-phenylazo-2,4-disulfonate (PPADS), was shown to be a P2 receptor antagonist [Lambrecht et al., 1996]. In smooth muscle assays, it antagonized P2X receptors in rabbit vas deferens [Lambrecht et al., 1992], urinary bladder [Ziganshin et al., 1993], isolated blood vessels [Ziganshin et al., 1994], guinea-pig isolated vas deferens [McLaren et al., 1994], and rat perfused mesenteric arterial bed [Windscheif et al., 1994]. PPADS was initially reported to show moderate selectivity for P2X vs. P2Y receptors [Windscheif et al., 1995a]; however, it was later shown to be more potent than anticipated at P2Y₁ receptors [Schachter et al., 1996]. At the P2Y₁ subtype, PPADS was a competitive antagonist exhibiting an equilibrium inhibition constant (K_i) value of approximately 1 μ M. PPADS had low affinity at the adenylate cyclase-coupled P2Y receptor in rat C6 glioma cells [Boyer et al., 1994], at the P_{2U} receptor in bovine aortic

endothelial cells [Brown et al., 1995], and at P_{2T} receptors in human platelets [Windscheif et al., 1995b].

In the present study, the structure-activity relationships (SARs) of a series of pyridoxal-6-aryloxy-5-phosphate derivatives and corresponding phosphonate congeners are reported (Fig. 1). Both radioligand binding and pharmacologic assays have been conducted with the aim of developing more potent and selective antagonists for P₂ receptor subtypes.

MATERIALS AND METHODS

Pyridoxal-5-phosphate and the reagents for azo coupling reactions were purchased from Aldrich (St. Louis, MO). The aniline derivatives were obtained from Aldrich, Fluka (St. Louis, MO), Lancaster (Windham, NH), Pfaltz & Bauer (Waterbury, CT), and Eastman Kodak (Rochester, NY).

Synthesis

Proton nuclear magnetic resonance spectroscopy was performed on a Varian GEMINI-300 spectrometer and spectra were taken in CDCl₃ or D₂O. The chemical shifts are expressed as parts per million downfield from tetramethylsilane or as relative parts per million from HOD peaks (4.78 ppm). ³¹P-NMR spectra were recorded at room temperature by use of Varian XL-300 spectrometer (121.42 MHz); orthophosphoric acid (85%) was used as an external standard. High-resolution fast atom bombardment mass was performed with JEOL SX102 spectrometer using 6-kV Xe atoms. The phosphate and phosphonate derivatives were desorbed from glycerol or magic bullet matrix.

All pyridoxal phosphate and phosphonate derivatives showed more than 95% purity in high pressure liquid chromatography (HPLC) systems. The determinations of purity were performed with a Hewlett-Packard 1090 HPLC system using an OD-5-60 C18 analytical column (250 mm × 4.6 mm, Separation Methods Technologies, Inc., Newark, DE) in two different linear gradient solvent systems. One solvent system (A) was 0.1 M triethylammonium acetate buffer: CH₃CN = 95:5 to 40:60 for 20 min with a flow rate of 1 ml/min. The other solvent system (B) was 5 mM tetrabutylammonium phosphate buffer:CH₃CN 80:20 to 40:60, in 20 min with a flow rate of 1 ml/min. Peaks were detected by ultraviolet absorption with a diode array detector.

General Procedure of Azo Coupling for the Synthesis of PPADS Analogs

To a stirred solution of each aminoaryl compound (0.4 mmol) in 10 ml of water was added 0.4 ml (0.4 mmol) of 1 N HCl and 28 mg (0.4 mmol) of solid NaNO₂ at 0°C. This solution was stirred for 5 min and the pH was adjusted to ~10 with 1 N NaOH (about 0.2 ml). To the mixture was added dropwise a solution of pyridoxal-5-phosphate (100 mg, 0.4 mmol) or a phosphonate (0.4 mmol) previously dissolved in aqueous NaOH (pH ~10). The pH was adjusted to ~9, and the yellow color changed to red. After stirring for 30 min at 0°C, the progress of reaction was monitored by HPLC using solvent system A. The product peaks appeared at retention times of 8–10 min with an ultraviolet absorption maximum at ~410 nm.

The mixture was frozen and lyophilized, leaving a solid. The crude product was dissolved in a minimal volume of water and purified by ion-exchange column chromatography by using Amberlite CG-50 resin (H⁺ form, weakly acidic) and eluting with water (flow rate 0.5 ml/min). The red fraction showing a single peak in HPLC was collected and lyophilized to give the desired compounds (yield, 50–60%).

Starting with the intermediates 19, 22, 26, 28, and pyridoxal- α^5 -phosphate (1) and following the general procedure, we have synthesized compounds 2, 4, 7–16, 20, 21, 23–25, 27, 29, 30, and 32–34 (Table 1).

6-Phenylazo-pyridoxal- α^5 -phosphate (sodium salt) (2)— ^1H NMR (D_2O) δ 2.45 (3H, s, CH_3), 5.67 (2H, d, $J = 5.5$ Hz, CH_2), 7.49 (3H, m, aromatic H), 7.78 (2H, m, aromatic-H), 10.33 (1H, s, CHO).

Pyridoxal- α^5 -phosphate-6-phenylazo-3'-sulfonate (sodium salt) (4)— ^1H -NMR (D_2O) δ 2.53 (3H, s, CH_3), 5.82 (2H, d, $J = 5.9$ Hz, CH_2), 7.72 (1H, m, Ph), 7.96 (1H, d, $J = 7.0$ Hz, Ph), 8.12 (1H, d, $J = 7.0$ Hz, Ph), 8.31 (1H, s, Ph), 10.52 (1H, s, CHO).

Pyridoxal- α^5 -phosphate-6-phenylazo-2'-methoxy-5'-sulfonate (sodium salt) (8)— ^1H -NMR (D_2O) δ 2.48 (3H, s, CH_3), 4.09 (3H, s, OCH_3), 5.81 (2H, d, $J = 4.9$ Hz, CH_2), 7.37 (1H, d, $J = 8.8$ Hz, Ph), 7.94 (1H, d, $J = 8.8$ Hz, Ph), 8.14 (1H, s, Ph), 10.50 (1H, s, CHO).

Pyridoxal- α^5 -phosphate-6-phenylazo-3'-carboxylic acid (sodium salt) (9)— ^1H -NMR (D_2O) δ 2.45 (3H, s, CH_3), 5.73 (2H, d, $J = 5.9$ Hz, CH_2), 7.63 (1H, t, $J = 6.8$ Hz, Ph), 8.03 (2H, m, Ph), 8.24 (1H, s, Ph), 10.45 (1H, s, CHO).

Pyridoxal- α^5 -phosphate-6-phenylazo-4'-carboxylic acid (sodium salt) (10)— ^1H -NMR (D_2O) δ 2.51 (3H, s, CH_3), 5.80 (2H, d, $J = 5.9$ Hz, CH_2), 8.00 (4H, bs, Ph), 10.51 (1H, s, CHO).

Pyridoxal- α^5 -phosphate-6-phenylazo-3'-chloro-2'-carboxylic acid (sodium salt) (11)— ^1H -NMR (D_2O) δ 2.57 (3H, s, CH_3), 5.81 (2H, d, $J = 6.8$ Hz, CH_2), 7.52 (1H, t, $J = 7.8$ Hz, Ph), 7.65 (1H, d, $J = 7.8$ Hz, Ph), 7.92 (1H, d, $J = 7.8$ Hz, Ph), 10.58 (1H, s, CHO).

6-(2'-chloro-phenylazo)-pyridoxal- α^5 -phosphate (sodium salt) (12)— ^1H -NMR (D_2O) δ 2.51 (3H, s, CH_3), 5.74 (2H, d, $J = 6.0$ Hz, CH_2), 7.43 (2H, m, Ph), 7.58 (1H, d, $J = 7.8$ Hz, Ph), 7.74 (1H, d, $J = 7.8$ Hz, Ph), 10.45 (1H, s, CHO).

Pyridoxal- α^5 -phosphate-6-phenylazo-2'-chloro-5'-sulfonate (sodium salt) (13)— ^1H -NMR (D_2O) δ 2.48 (3H, s, CH_3), 5.81 (2H, d, $J = 5.7$ Hz, CH_2), 7.75 (1H, d, $J = 7.8$ Hz, Ph), 7.84 (1H, d, $J = 7.8$ Hz, Ph), 8.13 (1H, s, Ph), 10.49 (1H, s, CHO).

Pyridoxal- α^5 -phosphate-6-phenylazo-2'-chloro-5'-carboxylic acid (sodium salt) (14)— ^1H -NMR (D_2O) δ 2.53 (3H, s, CH_3), 5.83 (2H, d, $J = 6.8$ Hz, CH_2), 7.70 (1H, d, $J = 8.7$ Hz, Ph), 7.99 (1H, d, $J = 8.7$ Hz, Ph), 8.18 (1H, s, Ph), 10.55 (1H, s, CHO).

Pyridoxal- α^5 -phosphate-6-phenylazo-3'-chloro-4'-carboxylic acid (sodium salt) (15)— ^1H -NMR (D_2O) δ 2.49 (3H, s, CH_3), 5.78 (2H, d, $J = 5.9$ Hz, CH_2), 7.57 (1H, d, $J = 7.8$ Hz, Ph), 7.93 (1H, d, $J = 7.8$ Hz, Ph), 8.03 (1H, s, Ph), 10.50 (1H, s, CHO).

6-(2'-Chloro-5'-nitro-phenylazo)-pyridoxal- α^5 -phosphate (sodium salt) (16)— ^1H -NMR (D_2O) δ 2.54 (3H, s, CH_3), 5.86 (2H, d, $J = 6.0$ Hz, CH_2), 7.86 (1H, d, $J = 7.8$ Hz, Ph), 8.31 (1H, d, $J = 7.8$ Hz, Ph), 8.51 (1H, s, Ph), 10.58 (1H, s, CHO).

Dimethyl (3, α^4 -O-Isopropylidene-3-hydroxy-4-hydroxymethyl-2-methyl-pyrid-5-yl)methylphosphonate (37)—To a solution of 5.11 g (0.0244 mol) of 35 in 100 ml of benzene, was added 1.92 ml (0.0264 mol) of thionyl chloride. The mixture was refluxed for

10 min with stirring and cooled to room temperature. The white solid was collected through filtration, washed with anhydrous acetone, and dried under vacuum to afford **36** (6.1 g, 95%): mass spectrum (MS) (CI, NH₃) 228(M⁺+1), 245 (M+NH₄⁺); ¹H-NMR (CDCl₃) δ 1.64 (6H, s, 2×CH₃), 2.79 (3H, s, CH₃), 4.65 (2H, s, CH₂), 5.12 (2H, s, CH₂), 8.31 (1H, s, H6). The solution of **36** in 50 ml of trimethylphosphite was heated at 80°C for 48 h and evaporated under vacuum. The residue was purified by flash silica gel column chromatography with CHCl₃:MeOH = 60:1 to afford **37** as a colorless oil (4.76 g, 61%): MS (CI, NH₃) 302(M⁺ + 1), 319(M+NH₄⁺); high resolution mass spectroscopy (HRMS) (EI) calcd. 301.1079, found 301.1064; ¹H-NMR (CDCl₃) δ 1.55 (6H, s, 2 × CH₃), 2.39 (3H, s, CH₃), 3.00 (2H, d, *J* = 21.5 Hz, CH₂), 3.68 (3H, s, CH₃), 3.72 (3H, s, CH₃), 4.93 (2H, s, CH₂), 7.92 (1H, s, H6).

Dimethyl (3-hydroxy-4-hydroxymethyl-2-methylpyrid-5-yl)methylphosphonate (38)—A solution of 4.5 g (0.0149 mol) of **37** in 30 ml of 10% formic acid was refluxed for 2 h. After cooling, the mixture was partitioned between CHCl₃ and saturated NaHCO₃, and the aqueous layer was extracted three times. The combined CHCl₃ layer was dried over anhydrous Na₂SO₄, evaporated, and purified by flash silica gel column chromatography with CHCl₃:MeOH = 20:1 to afford **38** as a white solid (2.62 g, 67%): mp = 139–141°C; MS (CI, NH₃) 262(M⁺ + 1); HRMS (EI) calcd. 261.0766, found 261.0752; ¹H-NMR (CDCl₃) δ 2.48 (3H, s, CH₃), 3.14 (2H, d, *J* = 20.5 Hz, CH₂), 3.65 (3H, s, CH₃), 3.69 (3H, s, CH₃), 4.86 (2H, s, CH₂), 7.85 (1H, s, H6).

Dimethyl (4-formyl-3-hydroxy-2-methyl-pyrid-5-yl)methylphosphonate (39)—To a solution of 1.05 g (4.02 mmol) of **38** in 80 ml of anhydrous CHCl₃ was added powdered activated MnO₂ with vigorous stirring for 2 h at room temperature under N₂ atmosphere. The mixture was filtered through a Celite bed, and the filtrate was evaporated under vacuum to afford **39** as a yellow oil (1.01 g, 97%): HRMS (EI) calcd. 259.0609, found 259.0594; ¹H-NMR (CDCl₃) δ 2.52 (3H, s, CH₃), 3.39, 3.46 (2H, 2s, CH₂), 3.70 (3H, s, CH₃), 3.74 (3H, s, CH₃), 8.00 (1H, s, H6), 10.37 (1H, s, CHO), 11.59 (1H, bs, OH).

4-Formyl-3-hydroxy-2-methyl-pyrid-5-yl)methylphosphonic acid (19)—To a stirred solution of 0.8 g (3.09 mmol) of **39** in 30 ml of anhydrous CH₃CN was added dropwise 1.68 ml (12.3 mmol) of trimethylsilyl bromide. The mixture was stirred overnight and evaporated under vacuum. The residue was mixed with 10 ml of water and followed by dropwise addition of 6.5 ml of 0.5 M NH₄HCO₃ solution with stirring until the mixture became a clear solution. The solution was washed with ether and applied to an Amberlite CG-50 (H⁺ form) ion exchange column followed by elution with water. The pure fractions determined by HPLC were collected and lyophilized to afford **19** as a yellow solid (0.55 g, 77%): mp = 310°C (dec.); ¹H-NMR (D₂O) δ 2.46 (3H, s, CH₃), 3.36 (2H, d, *J* = 19.5 Hz, CH₂), 7.77 (1H, s, H6), 10.23 (1H, s, CHO); ³¹P-NMR (D₂O) δ 17.12 (t, *J* = 19.4 Hz, phosphonic acid).

5-Formyl-3,α⁴-O-isopropylidene-3-hydroxy-4-hydroxymethyl-2-methyl-pyridine (40)—To a solution of 5.0 g (0.0239 mol) of **35** in 100 ml of anhydrous CH₂Cl₂ was added powdered pyridinium dichromate (PDC) with vigorous stirring. After overnight stirring, the mixture was filtered through a Celite bed. The filtrate was evaporated under vacuum and purified by flash silica gel column chromatography with CHCl₃:MeOH = 80:1 to afford **40** as a colorless oil (3.81 g, 77%): MS (CI, NH₃) 208(M⁺ + 1), 225 (M+NH₄⁺); ¹H-NMR (CDCl₃) δ 1.57 (6H, s, 2 × CH₃), 2.51 (3H, s, CH₃), 5.18 (2H, s, CH₂), 8.48 (1H, s, H6), 10.05 (1H, s, CHO).

Ethyl 2-(3,α⁴-O-isopropylidene-3-hydroxy-4-hydroxymethyl-2-methyl-pyrid-5-yl)propenoate (41)—To a suspension of 1.6 g (0.04 mol) of 60% sodium hydride (prewashed twice with hexanes) in 200 ml of anhydrous tetrahydrofuran (THF) was added dropwise 8.01 ml (0.04 mol) of triethylphosphonoacetate. The mixture became a clear solution with evolution of H₂ gas. After 5 min of stirring, 7.56 g (0.0364 mol) of *40* in 2 ml of anhydrous THF was added and the solution stirred for 30 min. The mixture was partitioned between CH₂Cl₂ and concentrated aqueous NaCl, and the organic layer dried under anhydrous Na₂SO₄, and evaporated under vacuum. The product was crystallized from hexanes to afford *41* as a white solid (9.65 g, 96%): mp = 81–83°C. Analysis: calcd. C 64.96, H 6.91, N 5.05; found C 65.06, H 6.94, N 5.01; MS (CI, NH₃) 278(M⁺ + 1), 295(M + NH₄⁺); ¹H-NMR (CDCl₃) δ 1.34 (3H, t, *J* = 8.8 Hz, CH₃), 1.57 (6H, s, 2 × CH₃), 2.44 (3H, s, CH₃), 4.28 (2H, q, *J* = 8.8 Hz, CH₂), 4.93 (2H, s, CH₂), 6.37 (1H, d, *J* = 16.6 Hz, CH), 7.54 (1H, d, *J* = 16.6 Hz, CH), 8.27 (1H, s, H-6).

3-(3,α⁴-O-Isopropylidene-3-hydroxy-4-hydroxymethyl-2-methyl-pyrid-5-yl)-1-propenol (42a)—To a stirred solution of 9.35 g (0.0337 mol) of *41* in 120 ml of anhydrous THF was added 165 ml (0.165 mol) of 1.0 M diisobutyl aluminum hydride (DIBAL-H) solution in THF at 0°C. The mixture was stirred for 2 h at 0°C, an additional 2 h at room temperature, and quenched by 0.1 ml of MeOH. The mixture was poured into ice-cold 0.1 N HCl, the pH was adjusted to 7–8, and the product was extracted with 100 ml of EtOAc three times. The combined EtOAc layer was dried over anhydrous Na₂SO₄, evaporated under vacuum, and purified by flash silica gel column chromatography with CHCl₃:MeOH = 100:1 to afford *42a* as a white solid (5.3 g, 67%): mp = 120–122°C; HRMS (EI) calcd. 235.1214, found 235.1208; ¹H-NMR (CDCl₃) δ 1.54 (6H, s, 2 × CH₃), 2.39 (3H, s, CH₃), 4.33 (2H, d, *J* = 4.9 Hz, CH₂), 4.81 (2H, s, CH₂), 6.27 (1H, dt, *J* = 4.9, 15.6 Hz, CH), 6.42 (1H, d, *J* = 15.6 Hz, CH), 8.07 (1H, s, H6).

3-(3,α⁴-O-Isopropylidene-3-hydroxy-4-hydroxymethyl-2-methyl-pyrid-5-yl)-1-propenylchloride hydro-chloride salt (42b)—5.0 g (0.0212 mol) of *42a* reacted by using the same method as for *36* to afford *42b* as a white solid (4.1 g, 67%): mp = 187°C (dec.); HRMS (EI) calcd. 253.0870, found 253.0878; MS (CI, NH₃) 254(M⁺ + 1), 271(M + NH₄⁺); ¹H-NMR (CDCl₃) δ 1.63 (6H, s, 2 × CH₃), 2.78 (3H, s, CH₃), 4.26 (2H, d, *J* = 4.9 Hz, CH₂), 4.98 (2H, s, CH₂), 6.46–6.58 (2H, m, 2 × CH), 8.247 (1H, s, H6).

Dimethyl 3-(3,α⁴-O-isopropylidene-3-hydroxy-4-hydroxymethyl-2-methyl-pyrid-5-yl)-1-propenylphosphonate (42c)—0.14 g (0.482 mmol) of *42b* reacted by using the same method as for *37* except for the reaction temperature (120°C) to afford *42c* as a colorless oil (0.07 g, 45%): HRMS (EI) calcd. 327.1235, found 327.1241; ¹H NMR (CDCl₃) δ 1.55 (6H, s, 2 × CH₃), 2.39 (3H, s, CH₃), 2.79 (2H, dd, *J* = 8.8, 22.5 Hz, CH₂), 3.77 (3H, s, CH₃), 3.81 (3H, s, CH₃), 4.83 (2H, s, CH₂), 6.02–6.12 (1H, m, CH), 6.36 (1H, dd, *J* = 4.9, 15.6 Hz, CH), 8.10 (1H, s, H6).

Dimethyl 3-(3-hydroxy-4-hydroxymethyl-2-methyl-pyrid-5-yl)-1-propenylphosphonate (43)—1.5 g (4.58 mmol) of *42b* reacted by using the same method as for *38* to afford *43* as a colorless oil (0.97 g, 74%): HRMS (EI) calcd. 287.0922, found 287.0924; ¹H-NMR (CDCl₃) δ 2.39 (3H, s, CH₃), 2.74 (2H, dd, *J* = 7.8, 22.5 Hz, CH₂), 3.73 (3H, s, CH₃), 3.77 (3H, s, CH₃), 4.95 (2H, s, CH₂), 5.79–5.92 (1H, m, CH), 6.46 (1H, dd, *J* = 4.9, 15.6 Hz, CH), 7.83 (1H, s, H6).

3-(4-Formyl-3-hydroxy-2-methyl-pyrid-5-yl)-1-propenylphosphonic acid (31)—0.2 g (0.696 mmol) of *43* reacted by using the same method as for *38* to *19* to afford *31* as a yellow solid (0.12 g, two steps in 67%): mp = 118°C (dec.); ¹H-NMR (D₂O) δ 2.39 (3H, s,

CH₃), 2.62, 2.68 (2H, 2m, CH₂), 6.14–6.27 (1H, m, CH), 6.58, 6.88 (1H, 2m, CH), 7.85 (1H, s, H₆), 10.26 (1H, s, CHO); ³¹P-NMR (D₂O) δ 20.15 (dq, *J* = 20.1, 4.9 Hz, phosphonic acid).

Dimethyl 3-(3-hydroxy-4-hydroxymethyl-2-methyl-pyrid-5-yl)

propylphosphonate (44)—To a solution of 0.36 g (1.25 mmol) of 43 in CH₂Cl₂ was added 0.36 g of Pd/C and stirred under H₂ atmosphere (1 atm) at room temperature for 5 h. The mixture was filtered through Celite bed. The filtrate was evaporated under vacuum and purified by flash silica gel column chromatography with CHCl₃:MeOH = 20:1 to afford 44 as a colorless oil (0.34 g, 93.8%): HRMS (EI) calcd. 289.1079, found 289.1073; ¹H-NMR (CDCl₃) δ 1.77–1.87 (4H, m, 2 × CH₂), 2.56 (3H, s, CH₃), 2.71 (2H, t, *J* = 6.8, CH₂), 3.73 (3H, s, OCH₃), 3.76 (3H, s, OCH₃), 5.03 (2H, s, CH₂), 7.75 (1H, s, H₆), 9.07 (2H, bs, 2 × OH).

3-(4-Formyl-3-hydroxy-2-methyl-pyrid-5-yl)-propylphosphonic acid (28)—0.23 g (0.8 mmol) of 44 reacted by using the same method as for 38 to 19 to afford 28 as a yellow solid (0.15 g, two steps in 72%): mp = 128°C (dec.); ¹H-NMR (D₂O + NaOD) δ 1.28–1.37 (2H, m, CH₂), 1.58–1.83 (2H, m, CH₂), 2.21, (3H, s, CH₃), 2.61–2.83 (2H, m, CH₂), 7.23(1H, s, H₆), 10.28 (1H, s, CHO); ³¹P-NMR (D₂O + NaOD) δ 22.65–23.01 (m, phosphonic acid).

2-(4-Formyl-3-hydroxy-2-methyl-pyrid-5-yl)ethylphosphonic acid (22)—The title compound was synthesized by the reported method [Hullar, 1969] with minor modification of reagents. ¹H-NMR (D₂O + NaOD) δ 1.47–1.58 (2H, m, CH₂), 2.22, (3H, s, CH₃), 2.75–2.98 (2H, m, CH₂), 7.29(1H, s, H₆), 10.30 (1H, s, CHO); ³¹P-NMR (D₂O + NaOD) δ 21.41–21.71 (m, phosphonic acid).

2-(4-Formyl-3-hydroxy-2-methyl-pyrid-5-yl)-ethenylphosphonic acid (26)—The title compound was synthesized by the reported method [Hullar, 1969] with minor modification of reagents. ¹H-NMR (D₂O) δ 2.47 (3H, s, CH₃), 6.41 (1H, dd, *J* = 17.6, 20.5 Hz, CH), 7.61 (1H, dd, *J* = 17.6, 20.5 Hz, CH), 7.89 (1H, s, H₆), 10.39 (1H, s, CHO); ³¹P-NMR (D₂O) δ 10.77–11.08 (m, phosphonic acid).

[(4-Formyl-3-hydroxy-2-methyl-6-(2',5'-disulfonylphenylazo))-pyrid-5-yl]methylphosphonate (sodium salt) (20)—¹H-NMR (D₂O) δ 2.42 (3H, s, CH₃), 3.98, 4.05 (2H, 2s, CH₂), 7.82 (1H, d, *J* = 7.8 Hz, Ph), 8.04 (1H, d, *J* = 7.8 Hz, Ph), 8.16 (1H, s, Ph), 10.22 (1H, s, CHO).

[(4-Formyl-3-hydroxy-2-methyl-6-(2'-chloro-5'-sulfonylphenylazo))-pyrid-5-yl]methylphosphonate (sodium salt) (21)—¹H-NMR (D₂O) δ 2.41, (3H, s, CH₃), 3.92, 3.99 (2H, s, CH₂), 7.63 (1H, d, *J* = 8.8 Hz, Ph), 7.73 (1H, d, *J* = 8.8 Hz, Ph), 8.03 (1H, s, Ph), 10.25 (1H, s, CHO).

2-[(4-Formyl-3-hydroxy-2-methyl-6-(2',5'-disulfonylphenylazo))-pyrid-5-yl]ethylphosphonate (sodium salt) (23)—¹H-NMR (D₂O) δ 1.88–1.91 (2H, m, CH₂), 2.40, (3H, s, CH₃), 3.51–3.59 (2H, m, CH₂), 7.76(1H, d, *J* = 7.8 Hz, Ph), 8.01 (1H, d, *J* = 7.8 Hz, Ph), 8.20 (1H, s, Ph), 10.25 (1H, s, CHO).

2-[(4-Formyl-3-hydroxy-2-methyl-6-(2'-chloro-5'-sulfonylphenylazo))-pyrid-5-yl]ethylphosphonate (sodium salt) (24)—¹H-NMR (D₂O) δ 1.86–1.97 (2H, m, CH₂), 2.40, (3H, s, CH₃), 3.59–3.67 (2H, m, CH₂), 7.63 (1H, d, *J* = 8.7 Hz, Ph), 7.74 (1H, d, *J* = 8.7 Hz, Ph), 8.00 (1H, s, Ph), 10.41 (1H, s, CHO).

2-[(4-Formyl-3-hydroxy-2-methyl-6-(4'-carboxyphenylazo))-pyrid-5-yl]ethylphosphonate (sodium salt) (25)—¹H-NMR (D₂O) δ 1.83–1.95 (2H, bm, CH₂), 2.42, (3H, s, CH₃), 3.45–3.62 (2H, bm, CH₂), 7.83 (4H, dd, *J* = 38.1, 6.8 Hz, Ph), 10.35 (1H, s, CHO).

2-[(4-Formyl-3-hydroxy-2-methyl-6-(2',5'-disulfonylphenylazo))-pyrid-5-yl]ethenylphosphonate (sodium salt) (27)—The title compound was purified by crystallization from EtOH/H₂O, due to the instability during the ion-exchange chromatography. ¹H-NMR (D₂O) δ 2.35 (3H, s, CH₃), 6.05 (1H, dd, *J* = 17.6, 19.5 Hz, CH), 7.78 (1H, dd, *J* = 17.6, 19.5 Hz, CH), 7.85 (1H, d, *J* = 8.7 Hz, Ph), 8.08 (1H, d, *J* = 8.7 Hz, Ph), 8.31 (1H, s, Ph), 10.08 (1H, s, CHO).

3-[(4-Formyl-3-hydroxy-2-methyl-6-(2',5'-disulfonylphenylazo))-pyrid-5-yl]propylphosphonate (sodium salt) (29)—¹H-NMR (D₂O) δ 1.77–1.83 (4H, m, 2 × CH₂), 2.41, (3H, s, CH₃), 3.32–3.39 (2H, m, CH₂), 7.75 (1H, d, *J* = 7.8 Hz, Ph), 7.99 (1H, d, *J* = 7.8 Hz, Ph), 8.16 (1H, s, Ph), 10.21 (1H, s, CHO).

3-[(4-Formyl-3-hydroxy-2-methyl-6-(2'-chloro-5'-disulfonylphenylazo))-pyrid-5-yl]propylphosphonate (sodium salt) (30)—¹H-NMR (D₂O) δ 1.63–1.86 (4H, m, 2 × CH₂), 2.42, (3H, s, CH₃), 3.43–3.47 (2H, m, CH₂), 7.62 (1H, d, *J* = 7.8 Hz, Ph), 7.74 (1H, d, *J* = 7.8 Hz, Ph), 7.94 (1H, s, Ph), 10.37 (1H, s, CHO).

Pyridoxal-α⁵-phosphate-6-(2'-naphthylazo-5',7'-disulfonate) (sodium salt) (32)—¹H-NMR (D₂O) δ 2.48, (3H, s, CH₃), 5.74, (2H, bs, CH₂), 8.29 (1H, d, *J* = 7.8 Hz, Naph), 8.41 (1H, s, Naph), 8.57 (2H, bs, Naph), 8.70 (1H, d, *J* = 9.8 Hz, Naph), 10.39 (1H, s, CHO).

Pyridoxal-α⁵-phosphate-6-(2'-naphthylazo-6',8'-disulfonate) (sodium salt) (33)—¹H-NMR (D₂O) δ 2.36, (3H, s, CH₃), 5.71, (2H, d, *J* = 6.6, CH₂), 7.97 (2H, pt, Naph), 8.35 (2H, pt, Naph), 9.05 (1H, bs, Naph), 10.39 (1H, s, CHO).

Pyridoxal-α⁵-phosphate-6-(2'-naphthylazo-3',6',8'-trisulfonate) (sodium salt) (34)—¹H-NMR (D₂O) δ 2.58, (3H, s, CH₃), 5.87, (2H, d, *J* = 6.8 Hz, CH₂), 8.53 (1H, s, Naph), 8.70 (1H, s, Naph), 8.79 (2H, s, Naph), 10.57 (1H, s, CHO).

Pharmacology

Binding assay in differentiated HL-60 cell membranes—Human HL-60 cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in PRMI 1640 supplemented with 10% fetal bovine serum. To differentiate HL-60 cells, 1 × 10⁶ cells/ml were treated with phorbol myristate acetate (60 ng/ml) for 72 h. The differentiated cells were washed twice with 10 ml of 10 mM Tris, 5 mM ethylenediaminetetraacetic acid buffer, pH 8.25 at 5°C, and then scraped into 5 ml of the same buffer. The cells were homogenized, the suspension was centrifuged at 35,000 × *g* for 20 min and the resulting pellet was resuspended in 50 mM Tris buffer, pH 8.25 at 5°C, containing 1 mM ethylenediaminetetraacetic acid. Membranes were frozen at –80°C before assay. The binding of 0.2 nM [³⁵S]ATPγS to HL-60 cell membranes was determined as described [Buell et al., 1996] in a final assay volume of 0.25 ml. Incubations for 3 h at 4°C were terminated by vacuum filtration. Non-specific binding was defined by using 10 μM ATPγS.

Antagonism of contractile responses of the guinea-pig urinary bladder and vas deferens and relaxant responses of the guinea-pig taenia coli—Male white guinea-pigs (250–400 g) were killed by a blow to the head and exsanguination. The urinary bladder, vasa deferentia, and ventricular taenia coli were removed and strips of smooth

muscle, approximately 2×10 mm, were prepared and suspended vertically in 10 ml organ baths for isometric recording of mechanical activity. An initial load of 1 g was applied to the strips which then were allowed to equilibrate for at least 60 min. Electrical field stimulation (EFS) was applied by means of two platinum-wire rings 2.5 mm in diameter, 10 mm apart, through which the strip was threaded. The modified Krebs solution used in these experiments had the following composition (mM): NaCl 133, KCl 4.7, NaHCO₃ 16.4, MgSO₄ 0.6, NaH₂PO₄ 0.8, CaCl₂ 2.5 and glucose 7.7, gassed with 95% O₂/5% CO₂ (pH 7.3–7.4) and maintained at $37 \pm 1^\circ\text{C}$. Contractions were recorded with a Linton FSG-01 force-displacement transducer, acquired by Biopac MP100WSW Data Acquisition System and displayed on a PC screen. EFS was provided by a Grass S9 stimulator and applied at a given frequency with a pulse width of 0.5 ms and supramaximal voltage. Adenosine-5'-triphosphate disodium salt (ATP), phentolamine, atropine sulphate and carbamylcholine chloride (carbachol) were obtained from Sigma (St. Louis, MO).

Atropine (0.3 μM) and phentolamine (1 μM) were present in a Krebs solution throughout experiments on urinary bladder and vas deferens. Tissue was stimulated with EFS (0.5–64 Hz) until a maximal contraction was reached and the tone declined by approximately 30%. Intervals of 1 min were maintained between stimulations. All contractile responses were calculated as a percentage of the response evoked by KCl at a concentration of 240 mM, which was added at the end of the experiment.

In experiments on taenia coli, relaxant responses to ATP (1, 10, and 100 μM) and to EFS (0.5–8 Hz) were measured by using carbachol (25 nM) -precontracted tissue. Intervals of 10 min were maintained between two subsequent precontractions by carbachol for washout. Relaxant responses were calculated as a percentage of maximal relaxation possible.

Responses to EFS and ATP were examined in preparations before and after incubation with a given compound at a concentration of 10 and 30 μM for at least 30 min. Time-control preparations were maintained in parallel and established that there was no significant change in response to EFS or ATP throughout experiments.

Each concentration of compound was tested three times on tissues from three different animals ($n = 3$). Means were compared by the paired Student's t-test. A probability of $=0.05$ was considered as significant. Data are presented as mean (M) \pm s.e.m.

Antagonist activity at recombinant P2X₂ receptors—*Xenopus* oocytes were harvested and prepared as previously described [King et al., 1997]. Defolliculated oocytes were injected cytosolically with rat P2X₂ cRNA (30 nL, 1 $\mu\text{g}/\text{ml}$) [Brake et al., 1994] incubated for 24 h at 18°C in Barth's solution and kept for up to 12 days at 4°C until used in electrophysiologic experiments.

ATP-activated membrane currents ($V_h = -90$ mV) were recorded from cRNA-injected oocytes by using the twin-electrode voltage-clamp technique (Axoclamp 2B amplifier). Voltage recording (1–2 M Ω tip resistance) and current-recording microelectrodes (5 M Ω tip resistance) were filled with 0.6 M K₂SO₄ and 3.0 M KCl, respectively. Oocytes were held in an electrophysiologic chamber and superfused with Ringer's solution (5 mM/min, at 18°C) containing (mM) NaCl, 110; KCl, 2.5; HEPES, 5; CaCl₂, 1.8, adjusted to pH 7.5.

ATP (10 μM , approximately the EC₇₀ value) was superfused over the oocytes for 120 s then washed out for a period of 5 min. For inhibition curves, data were normalized to the current evoked by ATP at pH 7.5. Test substances were added for 5 min before ATP exposure; all compounds were tested for reversibility of their effects. The concentration required to inhibit the ATP response by 50% (IC₅₀) was taken from Hill plots constructed by using the formula

$\log(I/I_{\max} - 1)$, where I is the current evoked by ATP in the presence of an antagonist. Data are presented as mean \pm s.e.m. ($n = 4$) for data from different batches of oocytes.

Phospholipase C assay—P2Y₁ receptor-promoted stimulation of inositol phosphate formation by adenine nucleotide analogs was measured in turkey erythrocyte membranes as previously described [Harden et al., 1988; Boyer et al., 1989]. The $K_{0.5}$ values were averaged from three to eight independently determined concentration-effect curves for each compound. Briefly, 1 ml of washed turkey erythrocytes was incubated in inositol-free medium (DMEM; Gibco, Gaithersburg, MD) with 0.5 mCi 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. Erythrocyte ghosts were prepared by rapid lysis in hypotonic buffer (5 mM sodium phosphate, pH 7.4, 5 mM MgCl₂, 1 mM EGTA) as previously described [Boyer et al., 1989]. Phospholipase C activity was measured in 25 μ l of [³H]inositol-labeled ghosts (~175 μ g of protein, 200–500,000 cpm/assay) in a medium containing 424 μ M CaCl₂, 0.91 mM MgSO₄, 2 mM EGTA, 115 mM KCl, 5 mM KH₂PO₄, and 10 mM HEPES, pH 7.0. Assays (200- μ l final volume) contained 1 μ M GTP γ S and the indicated concentrations of nucleotide analogs. Ghosts were incubated at 30°C for 5 min, and total [³H]inositol phosphates were quantitated by anion exchange chromatography as previously described [Harden et al., 1988; Boyer et al., 1989].

Agonist potencies were calculated by using a four-parameter logistic equation and the GraphPad software package (GraphPad, San Diego, CA). Antagonist IC₅₀ values (mean \pm standard error) represent the concentration needed to inhibit by 50% the effect elicited by 10 nM 2-methylthioadenosine-5'-diphosphate. All concentration-effect curves were repeated in at least three separate experiments carried out with different membrane preparations by using duplicate or triplicate assays.

RESULTS

Synthesis

The 6-arylazopyridoxal-5-phosphate analogs that were prepared and characterized (Table 1) could be divided into three categories. Among substituted-phenylazo derivatives and their precursors (Table 2) there are both 5-phosphate (compounds 1–18) and 5-phosphonate (compounds 19–31) analogs, and several naphthylazo pyridoxal-5-phosphate derivatives (compounds 32–34) have been included. The phosphate analogs were modified through substitution of the phenyl ring with sulfo, chloro, nitro, and carboxylate groups. The phosphonate analogs all contain 5'-sulfo and either chloro- or sulfo-groups at the 2'-position. Several compounds (compounds 1–3, 5, 6, 17, and 18) previously reported by Lambrecht [1996] are included in this study for comparison.

The synthetic procedures for the various 3-hydroxy-2-methyl-pyridine-5-alkyl or 5-olefinic phosphonic acid derivatives are shown in Scheme 1. The starting isopropylidene derivative, 35, was synthesized from pyridoxine according to the previous report [Yang et al., 1991]. The transformation of hydroxymethyl group of 35 to the corresponding chloromethyl group of 36 [Tomita et al., 1966], followed by an Arbuzov reaction using trimethylphosphite, afforded the phosphonate compound, 37 with an overall yield of 61%. After the hydrolytic cleavage of isopropylidene group of 37 with 10% formic acid, oxidation of 4-hydroxymethyl group of 38 with activated manganese (IV) oxide provided the 4-formyl compound 39 in high yield. A mild deprotection of the dimethyl ester of the phosphonate was accomplished by an ester exchange reaction by using trimethylsilyl bromide and followed by hydrolysis of the silyl esters. After purification, by using ion exchange chromatography (Amberlite CG-50, H⁺ form), 19 was isolated in moderate yield.

The precursor for chain extension between the pyridine ring and the phosphonic acid group was compound *40*, which was obtained by oxidation of *35* by using PDC in CH₂Cl₂. 5-*trans*-Vinyl and 5-ethyl phosphonate derivatives, *26* and *22*, were synthesized by the reported procedures [Hullar, 1969] with minor modification of reagents. 5-Allyl and 5-propyl phosphonic acid derivatives were prepared through a Wittig reaction of the aldehyde group [Tomita et al., 1966] in *40* leading to the *trans*-vinyl carboxylic acid ester, *41*. Selective reduction of the ester in *41* with DIBAL-H afforded the alcohol *42a*, which was converted to phosphonate *31* by using the same procedures as for the preparation of *19* from *35*. A saturated propyl phosphonate, *28*, was prepared through catalytic reduction of the dimethyl phosphonate intermediate, *43*, followed by oxidation and deprotection.

The phosphonic acid derivatives, *19*, *22*, *26*, and *28* were subjected to the azo coupling reaction (Scheme 2) with two different aromatic amine moieties, aniline-2,5-disulfonic acid and aniline-2-chloro-5-sulfonic acid. The azo-linked products obtained were compounds *20*, *21*, *23–25*, *27*, *29*, and *30* (Table 2). The chemical characterization and HPLC retention times in two solvent systems of these compounds are reported in Table 1.

Radioligand Binding Studies at Human P2X₁ Receptors

Assays at P2X receptors consisted of both radioligand binding and biological assays. The binding assay involved inhibition of specific binding of [³⁵S]ATP- γ -S to membranes of HL-60 cells. The P2X₁ receptor is the principal subtype of P2 receptors expressed in differentiated HL-60 cells, and agonist binding selectivities determined with HL-60 membranes were shown by Buell et al. [1996] to correspond to the affinity of agonists at the human P2X₁ receptor. We verified that the order of potency in inhibition of radioligand binding by known receptor agonists and antagonists was approximately as expected from the published potencies at P2X₁ receptors. For example, the following IC₅₀ values (all nM) were measured: ATP, 5.51 \pm 0.37; ATP- γ -S, 3.88 \pm 1.14; 2-Cl-ATP, 6.55 \pm 1.60; L- β , γ -me-ATP, 24,800 \pm 5400; suramin, 730 \pm 171.

The IC₅₀ values for most of the PPADS derivatives in the P2X₁ receptor binding assay were greater than 100 μ M (Table 3). Even PPADS, *6*, displayed only 18% inhibition at 100 μ M, which was somewhat weaker than expected. IsoPPADS, *7*, was somewhat more potent than PPADS in inhibition of binding. Several derivatives displayed relatively favorable affinity in the binding assay. For example, a phenylazo phosphonate derivative, *27*, and a naphthylazo phosphate derivative, *32*, exhibited IC₅₀ values of 10 and 15 μ M, respectively. Both of these compounds contained two sulfonate groups on the aryl rings. Compound *2*, an unsubstituted phenylazo phosphate derivative, was of intermediate affinity with an IC₅₀ value of 45 μ M.

Smooth Muscle Assays at Guinea-pig P2X₁ Receptors—Functional assays consisted of classic smooth muscle systems, specifically antagonism of electric field-stimulated contraction (P2X system) of vas deferens and guinea-pig urinary bladder. Most of the phosphate and a few phosphonate derivatives displayed antagonism to some degree in the smooth muscle assays of guinea-pig P2X₁ receptors in the urinary bladder [Ziganshin et al., 1993] and vas deferens [McLaren et al., 1994]. The three most potent analogs in the human P2X₁ receptor binding assay, i.e., *2*, *27*, and *32*, showed considerable potency of varying degrees in these smooth muscle assays. The compounds that consistently showed the greatest degree of antagonism in both bladder and vas deferens were: *8*, *10*, *13*, *20*, and *34*. Curiously, compounds *12*, *16*, *21*, and *28–30* antagonized electric field-induced contraction in the vas deferens but not in the bladder. Compound *10*, a phosphate derivative that contained a carboxyl group at the 4-position of the phenylazo ring, displayed a particularly favorable degree of antagonism in both bladder (Fig. 2A) and vas deferens (Fig.

2B). The *m*-chloro-*p*-carboxyphenylazo derivative *15* inhibited contraction in the bladder, whereas in the vas deferens inhibition occurred at = 16 Hz but not at 8 Hz stimulation.

Smooth Muscle Assay at Guinea-pig P2Y₁ Receptors and Biochemical Assay at Turkey Erythrocyte P2Y₁ Receptors

Antagonism at P2Y₁ receptors was established by using a smooth muscle system, i.e. guinea-pig taenia coli, in which antagonism of either agonist-induced or electric field-induced relaxation was measured [Windscheif et al., 1995a]. The phenylazo derivative phosphate derivative *2* and the ethylphosphonate analog of isoPPADS *23* had measurable K_B values in antagonizing ATP-induced relaxation of 6.3 and 12.5 μM, respectively. Other compounds that consistently showed the greatest degree of antagonism in the guinea-pig taenia coli were *8*, *9*, *11*, *19*, *29*, and *34*. The ethyl phosphonate derivative *25* was essentially inactive at guinea-pig P2Y₁ receptors and was also inactive as an antagonist at P2Y₂ receptors of C6 glioma cells (data not shown) [Boyer et al., 1994]. The compounds were further evaluated at the phospholipase C-coupled P2Y₁ receptor of turkey erythrocytes. At both guinea-pig and turkey erythrocyte P2Y₁ receptors, most of the phosphate derivatives, except for compounds *10* and *15*, showed activity similar to PPADS, *6*. Compounds *10* and *15* were either inactive or very weak at both taenia coli (Fig. 3) and turkey erythrocyte P2Y₁ receptors. Potency of antagonism at turkey erythrocyte P2Y₁ receptors among compounds with IC₅₀ values of <10 μM decreased in the order *12*, *21*, *14*, *34*>*8*, *13*, *24*, *16*, *33*, *32*, *6*. Phosphonate derivatives, except for compounds *21* and *24*, were inactive or very weak at turkey erythrocyte P2Y₁ receptors. Compound *21*, although relatively potent at turkey erythrocyte P2Y₁ receptors (IC₅₀ value of 7.23 μM), was inactive in the guinea-pig taenia coli. Among the phosphonate congeners, the homologous 2-chloro-5-sulfonyl-phenylazo phosphonate derivatives *21*, *24*, and *30* displayed a trend of decreasing potency at turkey erythrocyte P2Y₁ receptors, depending on the chain length between the pyridine ring and phosphorous atom (Table 3). Nevertheless, a measurable potency (IC₅₀ value of 20.2 μM) was observed even for the propyl phosphonate *30*.

Curiously, the propyl phosphonate derivative *30* and the naphthylazo derivative *33* seemed to enhance the relaxation of the taenia coli in response to ATP but not to electrical stimulation. This phenomenon may conceivably occur through potential inhibition of enzymes which degrade ATP, such as nucleotidases, by the analogs. Numerous derivatives that act as ATP antagonists are also known to inhibit ecto-nucleotidases [Jacobson et al., 1997].

Although pyridoxal-5-phosphate *1* itself has P2-antagonist properties [Trezise et al., 1994], the presence of the 6-aryloxy moiety (with or without substitution) clearly enhanced potency at P2 receptors. The other non-azo-linked pyridoxal-5-phosphate/phosphonate precursors *19*, *22*, *26*, and *28* were all essentially inactive at turkey erythrocyte P2Y₁ receptors.

Recombinant Rat P2X₂ Receptor Ion Channel Assay—Compounds were tested in a functional assay of recombinant rat P2X₂ receptors expressed in *Xenopus* oocytes, in which ATP-induced currents were inhibited (Fig. 4) [King et al., 1997]. Of the novel compounds synthesized in our study, compound *15* was the most potent at rat P2X₂ receptors, with an IC₅₀ value of 0.82 μM. Thus, *15* was intermediate in potency between isoPPADS, *7*, and PPADS, *6*, which had IC₅₀ values of 0.40 and 1.6 μM, respectively. Other compounds that displayed roughly the same potency as PPADS at rat P2X₂ receptors were the unsubstituted phenylazo derivative *2*, the *m*-carboxyphenylazo derivative *9*, the methylphosphonate analog of isoPPADS *20*, and the corresponding ethylphosphonate *23*, and the *p*-carboxyphenylazo ethylphosphonate derivative *25*. The order of potency at rat P2X₂ receptors evident in Figure 4 was *7* > *15* > *20*, *2* > *23*, *6* > *25*, *9*. Thus, the particular

combination of phosphonate and substituted phenylazo units has indications of displaying relatively high potency at the P2X₂ receptor subtype.

PPADS has been shown to inhibit P2X receptors with slow reversibility [Windscheif et al., 1995a; Humphrey et al., 1995]. The effects of some derivatives (*2*, *7*, *15*, *20*, *23*, and *25*) at the rat P2X₂ receptor were readily reversible, whereas others (*6*, *9*, *10*, *22*, and *29*) were only slowly reversible (data not shown).

DISCUSSION

In contrast to the initial reports, it seems that PPADS is not selective for P2X receptors. As reconfirmed in this study, PPADS also has considerable affinity for P2Y receptors [Schachter et al., 1996; Boyer et al., 1994; Brown et al., 1995]. In the present study, PPADS and isoPPADS and numerous analogs thereof, were compared in a variety of P2X and P2Y receptor assays. We have shown that at rat P2X₂ and turkey erythrocyte P2Y₁ receptors, isoPPADS is more potent as an antagonist than PPADS. Recently we have shown that isoPPADS is more potent than PPADS also at recombinant rat P2X₁ receptors [Jacobson et al., 1998]. Thus, the 2',5'-disulfonate substitution of the phenylazo ring is a more favorable lead structure for ligand development than the 2',4'-isomer.

In the present study, we have greatly expanded the range of PPADS and isoPPADS analogs for the study of SARs and selectivity at various P2 receptor subtypes. Based on mutagenesis results, the 4-aldehyde moiety of PPADS is proposed to aid in binding of the antagonist through the formation of a Schiff base with the ε-amino group of a specific lysine residue on the large extracellular domain of P2X₁ and P2X₂ receptors [Humphrey et al., 1995]. All of the present analogs have retained the aldehyde group. The potency of PPADS as a P2X antagonist has been reported to be not highly dependent on substituents in the 6-phenylazo ring, except for electron-withdrawing substituents, such as a nitro group, which decreases the potency [Lambrecht et al., 1996]. We have confirmed that the unsubstituted phenylazo derivative *2* acts as a P2 receptor antagonist and found further substitution patterns on the arylazo ring that serve to modulate the potency and selectivity of the derivatives.

A major finding of the present study is that the phosphate group of PPADS may be replaced with a phosphonate resulting in preservation of P2 receptor antagonist properties. The 5-methylphosphate group of PPADS was previously shown to be required for its activity as a P2 receptor antagonist [Trezise et al., 1994], and the present study reveals that a phosphonate substitution is also acceptable for P2 receptor antagonism. The phosphonate group may be linked to the pyridine ring at the 5-position through a hydrocarbon chain consisting of methyl, ethyl, propyl, vinyl or allyl groups. Among these analogs, the saturated chain-linked phosphonate compound *23* showed significant selectivities for P2X₂ receptors vs. P2X₁ or P2Y₁ receptors, whereas in the case of vinyl phosphonate analogs the potency at P2X₁ receptors was restored. These results indicate that altering the electron density or electrostatic potential surrounding the phosphate group in PPADS may be a means of resolving selectivity among the subtypes.

Compounds *10* and *15*, both of which contained a carboxyl group at the 4-position of the phenylazo ring, displayed particularly good selectivity for P2X compared with P2Y₁ receptors (Figs. 2, 3). Of the novel compounds synthesized in our study, compound *15*, a 3'-chloro-4'-carboxylic acid derivative, was the most potent at P2X₂ receptors. Compound *15* was essentially inactive at guinea-pig and turkey P2Y₁ receptors and at 30 μM only moderately antagonized guinea-pig bladder P2X₁ receptors (see below). Thus, compound *15* is of further interest as a selective pharmacologic probe of P2X₂ receptors. Compound *10*, a 4'-carboxylic acid derivative, and compound *20*, a methylphosphonate derivative, were not

very selective within P2X subtypes examined but were highly selective when compared with P2Y₁ receptors. Also, compounds *23* and *25* were relatively potent at rat P2X₂ receptors, whereas *23* was relatively weak at both P2X₁ and P2Y₁ receptors (Table 3). Considerable differences between the taenia coli and turkey P2Y receptors in antagonist potency were evident (Table 3), suggesting the presence of different subtypes. Similarly among agonists, α,β -me-ATP is active in the taenia coli [Burnstock et al, 1994] but not at recombinant P2Y₁ receptors.

The development of potent and selective antagonists of P2 receptors remains a challenge. Among nucleotide antagonists, a P2Y₁ selective antagonist *N*⁶-methyl-2'-deoxyadenosine-3',5'-bisphosphate (MRS 2179) having a K_i value of approximately 100 nM was recently reported [Camaioni et al., 1998; Boyer et al., 1998]. Non-nucleotide heterocyclic P2 receptor antagonists (PPADS, Reactive Blue 2, suramin, etc.) have not shown subtype selectivity before the present study [Jacobson et al., 1997]. In the present study, we have identified several novel derivatives of PPADS, that display encouraging selectivity, including the *p*-carboxyphenylazo phosphate derivative *10* (MRS 2159) and its *m*-chloro analog *15* (MRS 2160), which were selective for P2X vs. P2Y₁ receptors. Among the phosphonate derivatives, [4-formyl-3-hydroxy-2-methyl-6-(2-chloro-5-sulfonylphenylazo)-pyrid-5-yl]methylphosphonic acid, *21* (MRS 2192), showed high potency at P2Y₁ receptors with an IC₅₀ of 7.23 μ M. The corresponding 2,5-disulfonylphenyl derivative *20* (MRS 2191) was nearly inactive at turkey erythrocyte P2Y₁ receptors, whereas at recombinant P2X₂ receptors had an IC₅₀ value of 1.1 μ M. Thus a single ring substitution, sulfo instead of chloro, has a major effect on the selectivity of these methylphosphonates as P2Y receptor antagonists. An ethyl phosphonate derivative *23* (MRS 2142) was selective for recombinant P2X₂ vs. either P2X₁ or turkey erythrocyte P2Y₁ receptors.

The present study demonstrates that it is possible to achieve selectivity for P2 receptor subtypes by using a relatively nonselective lead structure, PPADS. It will be necessary to study the present set of PPADS analogs at other P2 receptor subtypes and compare potencies within the same species to establish the complete pharmacologic profiles as antagonists. PPADS itself does not potently antagonize P2Y₂, P2Y₄, or P2Y₆ receptors. Also, at P2X receptors, PPADS is much more potent at P2X₁-P2X₃ and P2X₅ receptors than at P2X₄, P2X₆, and P2X₇ receptors. Thus, by optimizing the pharmacologic properties of PPADS analogs, it may be possible to achieve high affinity and selectivity for subtypes within P2X and P2Y receptor families.

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Abbreviations

ATP	adenosine 5'-triphosphate
DIBAL-H	diisobutyl aluminum hydride
EFS	electrical field stimulation

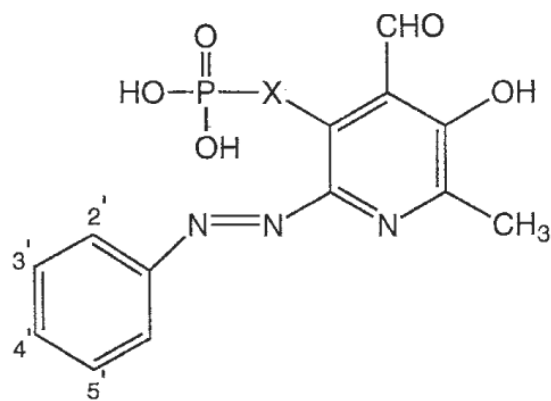
HPLC	high pressure liquid chromatography
HRMS	high resolution mass spectroscopy
K_i	equilibrium inhibition constant
me-ATP	adenosine-5'-methylenetriphosphate, (α,β) or (β,γ) isomers
2-MeSATP	2-methylthioadenosine-5'-triphosphate
MRS 2179	N ⁶ -methyl-2'-deoxyadenosine 3',5-bisphosphate
MS	mass spectrum
PDC	pyridinium dichromate
PPADS	pyridoxal-5'-phosphate-6-phenylazo-2,4-disulfonate
SAR	structure-activity relationship
THF	tetrahydrofuran

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$X = -\text{CH}_2\text{O}-$	phosphates
$= -(\text{CH}_2)_n-, n = 1-3$	alkyl phosphonates
$= -\text{CH}=\text{CH}-(\text{CH}_2)_n-, n = 0-1$	alkenyl phosphonates

Fig. 1. Structure of pyridoxal-6-phenylazo-5'-phosphate or phosphonate derivatives prepared in the present study.

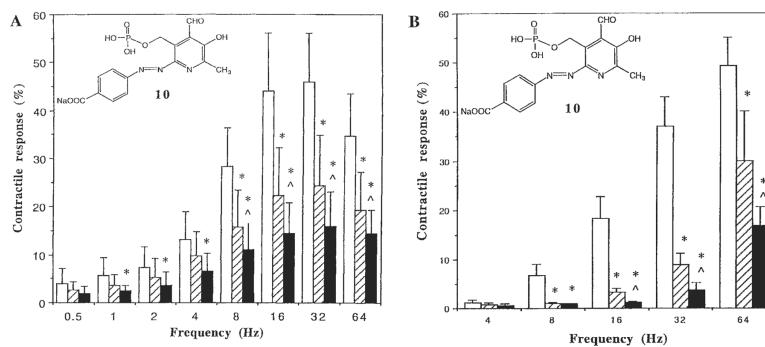


Fig. 2. P2X₁ receptor-mediated contractile responses of control (white bars) and compound *10* (10 μM, slash bars; 30 μM, black bars) calculated as percentage of the response evoked by KCl at a concentration of 240 mM, which was added at the end of experiments. (A) Guinea-pig urinary bladder and (B) guinea-pig vas deferens were stimulated with electrical field stimulation (0.5–64 Hz) in a Krebs solution containing atropine (0.3 μM) and phentolamine (1 μM) until a maximal contraction was reached and the tone declined by approximately a third. Intervals of 1 min were always kept between two subsequent stimulations. *, $P < 0.05$ from control; ^, $P < 0.05$ from 10 μM (n = 3).

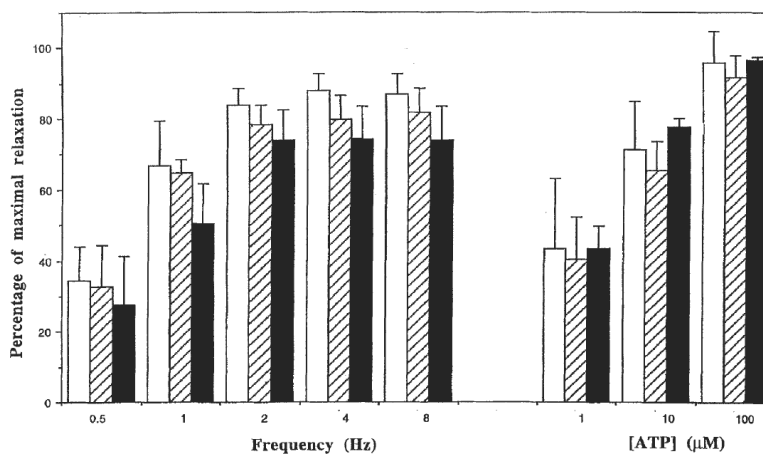


Fig. 3. P2Y₁ receptor-mediated relaxant responses of control (white bars) and compound 10 (10 μM, slash bars; 30 μM, black bars) calculated as a percentage of maximal relaxation possible: guinea-pig taenia coli relaxant responses to ATP (1, 10, and 100 μM) and to electrical field stimulation (0.5–8 Hz) were tested on carbachol-precontracted tissue (25 nM). Intervals of 10 min were kept between two subsequent precontractions by carbachol for washout.

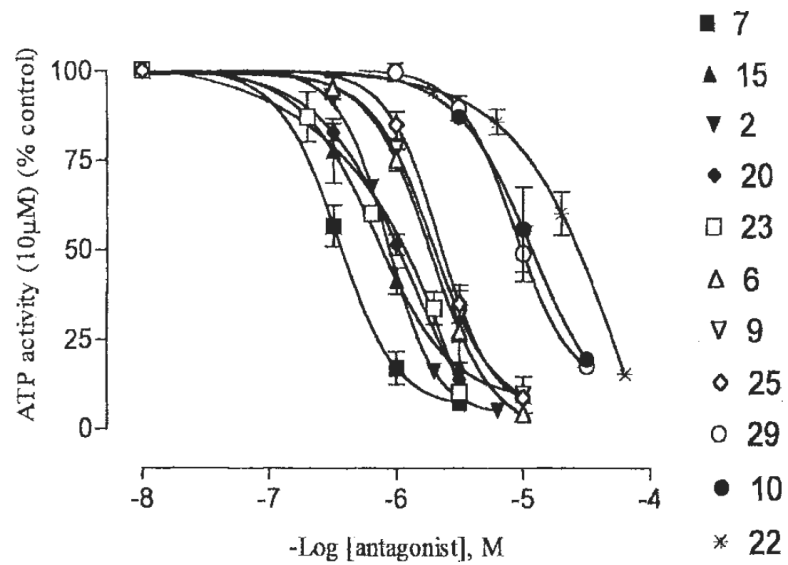
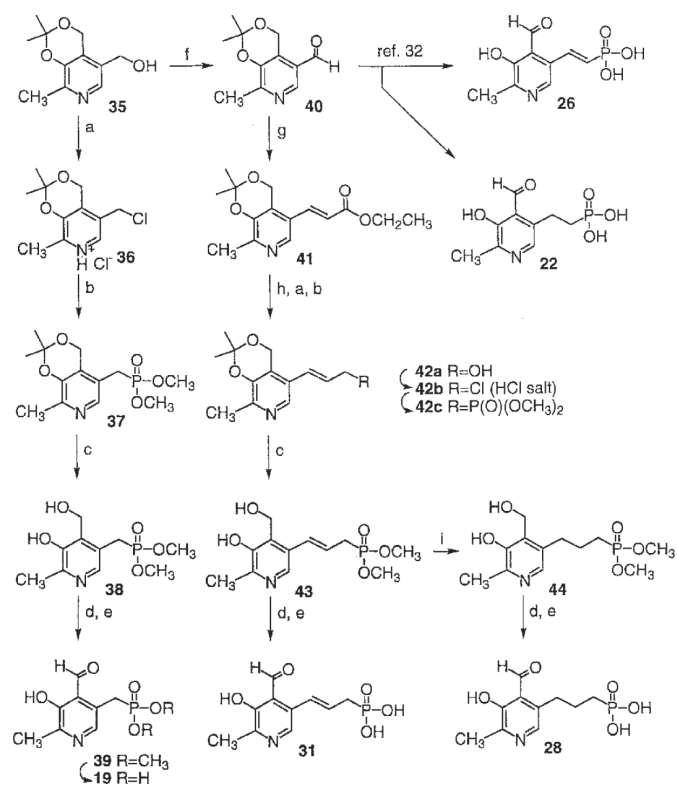
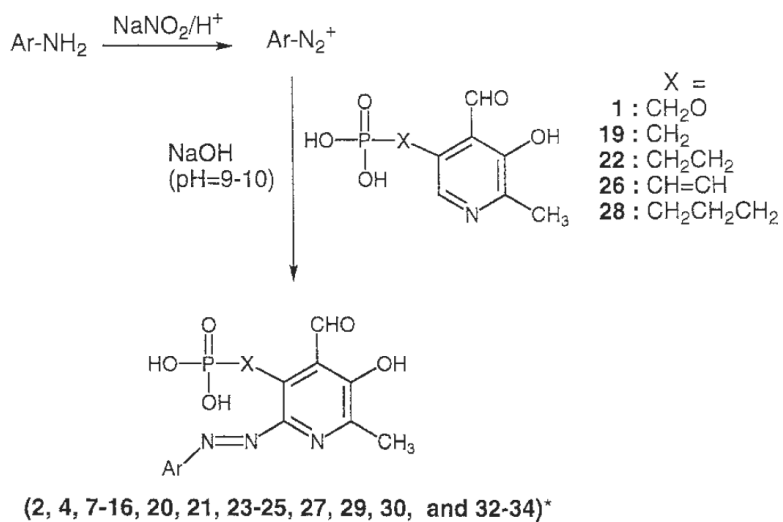


Fig. 4. Effects of various analogs as indicated on current induced by activation by 10 μ M ATP (approximate EC₇₀ value) of recombinant rat P2X₂ receptors, expressed in *Xenopus* oocytes, by using the twin-electrode voltage-clamping technique. The medium consisted of Ca²⁺ Ringer's buffer at pH 7.50.

**Scheme 1.**

a: SOCl_2 in benzene, reflux, 10 min. **b:** $\text{P}(\text{OCH}_3)_3$, reflux, 48 h. **c:** 10% formic acid reflux, 2 h. **d:** Activated MnO_2 in CHCl_3 , 2 h. **e:** TMS-Br in CH_3CN , 24 h. **f:** PDC in CH_2Cl_2 , 24 h. **g:** $\text{EtOCOCH}_2\text{P}(\text{O})(\text{OEt})_2$, NaH in THF, 30 min. **h:** DIBAL-H in THF at 0°C , 2 h. **i:** 1 atmosphere H_2 , Pd/C in CH_2Cl_2 , 5 h.

**Scheme 2.**

Ar, substituted phenyl or naphthyl. The precise structures are presented in Table 2.

TABLE 1

MS and HPLC Characterization of PPADS Analogues Synthesized^a

Compound ^b	Formula	FAB (M-H ⁺)		HPLC (rate, min)	
		Calculated	Found	System A	System B
2	C ₁₄ H ₁₃ O ₆ N ₃ PNa ₂	396.0337	396.0345	10.15	13.64
4	C ₁₄ H ₁₃ O ₆ N ₃ PSNa	451.9929	451.9935	8.84	14.01
8	C ₁₅ H ₁₅ O ₁₀ N ₃ PSNa	482.0035	482.0015	8.80	14.33
9	C ₁₅ H ₁₃ O ₈ N ₃ P	394.0440	394.0407	9.14	14.06
10	C ₁₅ H ₁₃ O ₈ N ₃ PNa	416.0260	416.0251	7.90	13.94
11	C ₁₅ H ₁₂ O ₈ N ₃ CIPNa	449.9870	449.9869	8.56	14.18
12	C ₁₄ H ₁₃ O ₆ N ₃ CIP	384.0152	384.0155	11.93	14.93
13	C ₁₄ H ₁₂ O ₆ N ₃ CIPNa	485.9540	485.9560	9.40	15.21
14	C ₁₅ H ₁₂ O ₈ N ₃ CIPNa	428.0050	428.0049	10.09	15.33
15	C ₁₅ H ₁₂ O ₈ N ₃ CIPNa	450.9948	450.9965	7.94	14.07
16	C ₁₄ H ₁₂ O ₈ N ₄ CIP	429.0003	428.9997	12.07	15.11
19	C ₈ H ₁₀ O ₅ NP	230.0218	230.0217	7.63	8.70
20	C ₁₄ H ₁₁ O ₁₁ N ₃ PS ₂ Na ₃	559.9187	559.9183	8.22	15.22
21	C ₁₄ H ₁₂ O ₈ N ₃ CIPNa	469.9591	469.9583	10.80	14.64
22	C ₉ H ₁₂ O ₅ NP	244.0375	244.0380	7.54	9.76
23	C ₁₅ H ₁₅ O ₁₁ N ₃ PS ₂ Na ₃	575.9500	575.9501	8.13	15.62
24	C ₁₅ H ₁₄ O ₈ CIPNa	483.9747	483.9756	10.32	15.00
25	C ₁₆ H ₁₅ O ₇ N ₃ P	392.0648	392.0645	8.49	12.41
26	C ₉ H ₁₀ O ₅ NP	242.0218	242.0220	8.11	9.36
27	C ₁₅ H ₁₂ O ₁₁ N ₃ PS ₂ Na ₃	571.9187	571.9256	7.13	13.58
28	C ₁₀ H ₁₄ O ₅ NP	258.0531	258.0544	8.72	9.39
29	C ₁₆ H ₁₇ O ₁₁ N ₃ PS ₂ Na	543.9862	543.9827	9.19	15.38
30	C ₁₆ H ₁₆ O ₈ N ₃ CIPNa	497.9904	497.9890	11.20	15.18
31	C ₁₀ H ₁₂ O ₅ NP	256.0371	256.0375	9.25	10.05
32	C ₁₈ H ₁₄ O ₁₂ N ₃ PS ₂ Na ₂	581.9654	581.9670	8.37	15.82

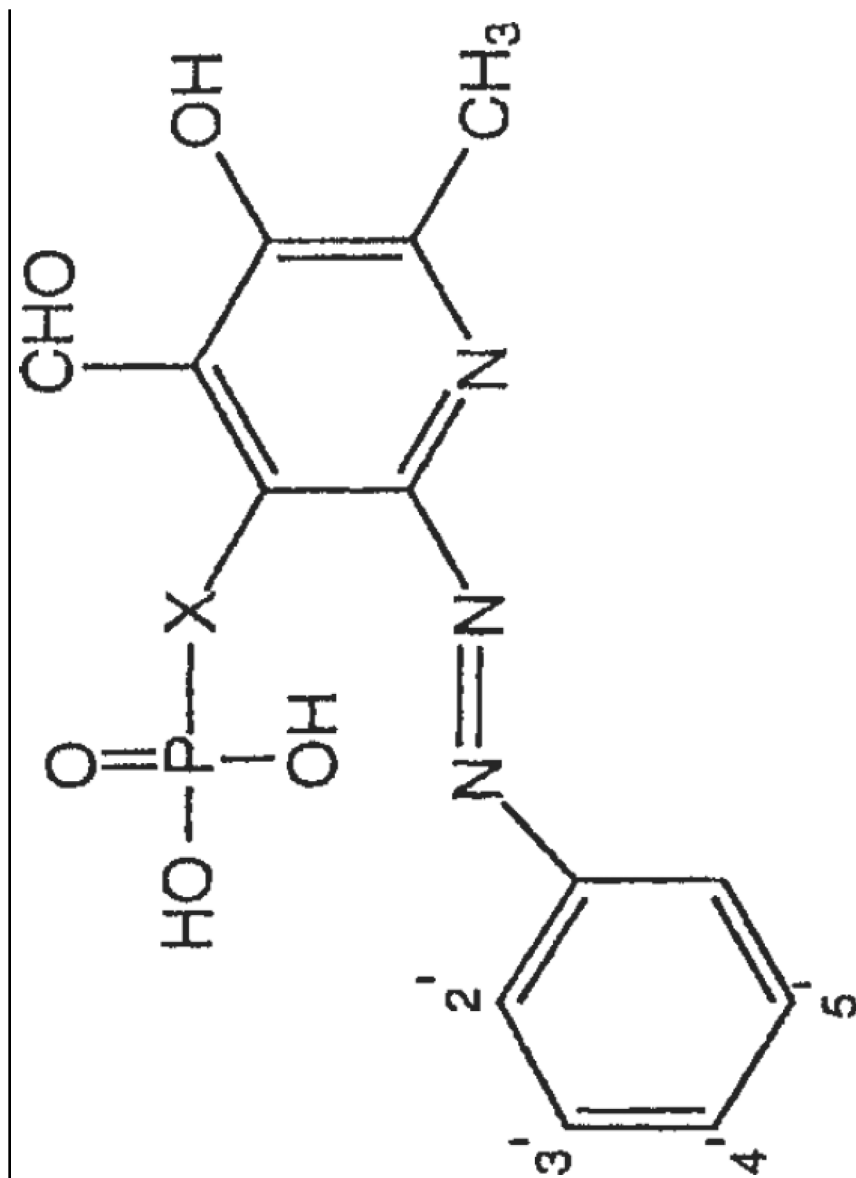
Compound ^b	Formula	FAB (M-H ⁺)		HPLC (rate, min)	
		Calculated	Found	System A	System B
33	C ₁₈ H ₁₄ O ₁₂ N ₃ PS ₂ Na ₂	581.9654	581.9647	8.29	15.78
34	C ₁₈ H ₁₄ O ₁₅ N ₃ PS ₂ Na ₂	683.9042	683.9044	7.50	16.74

^aMS, mass spectrum; HPLC, high pressure liquid chromatography; PPADS, pyridoxal-6'-phosphate-b-phenylazo-2,4-disulfonate; FAB, fast atom bombardment. System A was 0.1 M triethylammonium acetate buffer:CH₃CN = 95:5 to 40:60 for 20 min with flow rate 1 ml/min. System B was 5 mM tetrabutylammonium phosphate buffer:CH₃CN 80:20 to 40:60, in 20 min with flow rate 1 ml/min.

^bStructures are shown in Table 2 and Schemes 1 and 2.

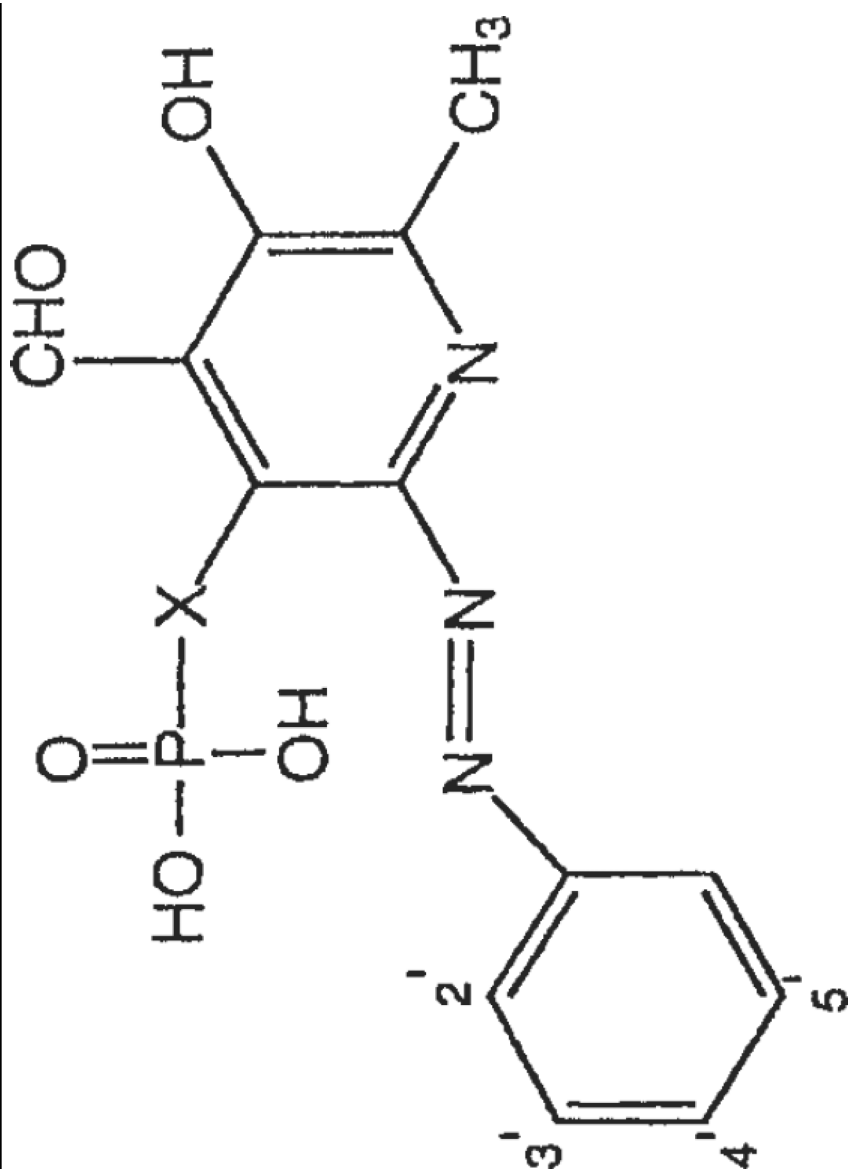
TABLE 2

Structures of Pyridoxal-6-azoaryl-5-phosphate Derivatives Examined as Antagonists at P2 Receptors



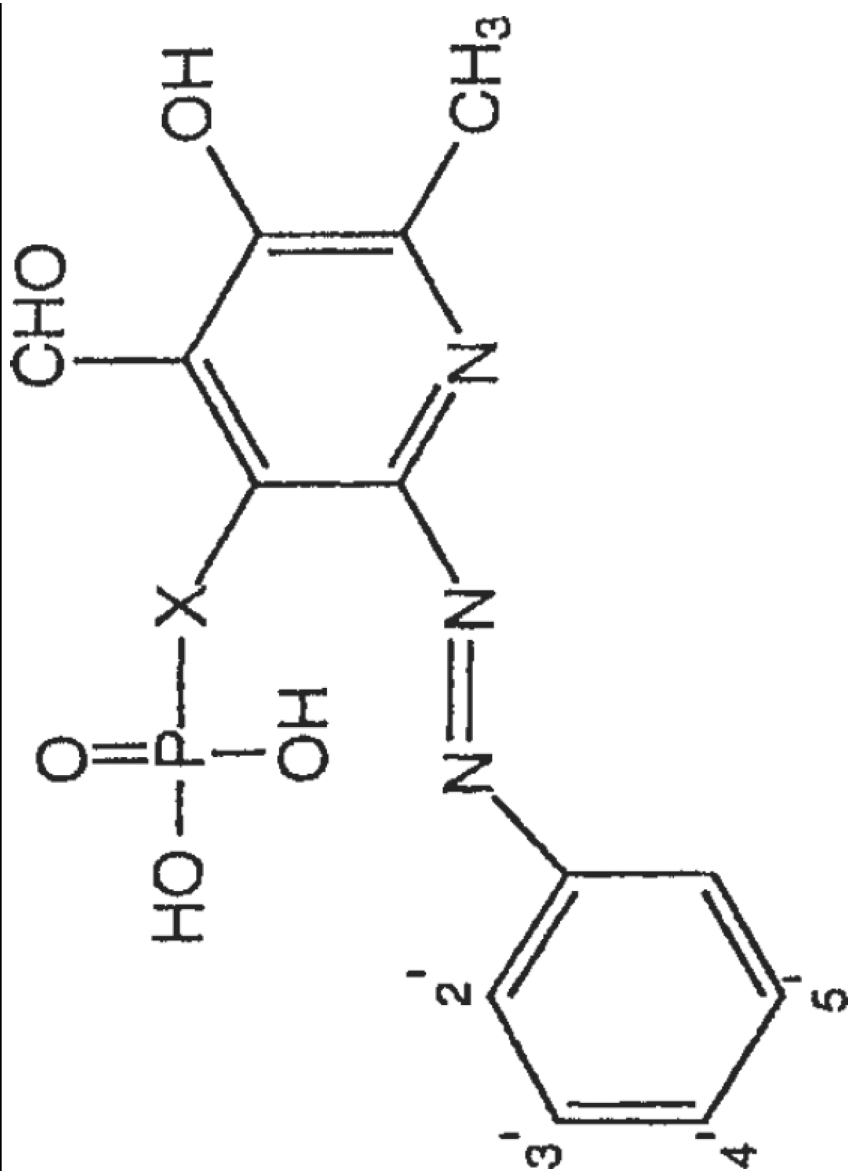
(2-18, 20, 21, 23-25, 27, 29,30)

Compound ^a	2'	3'	4'	5'	X
2	H	H	H	H	CH ₂ O
3	SO ₃ H	H	H	H	CH ₂ O



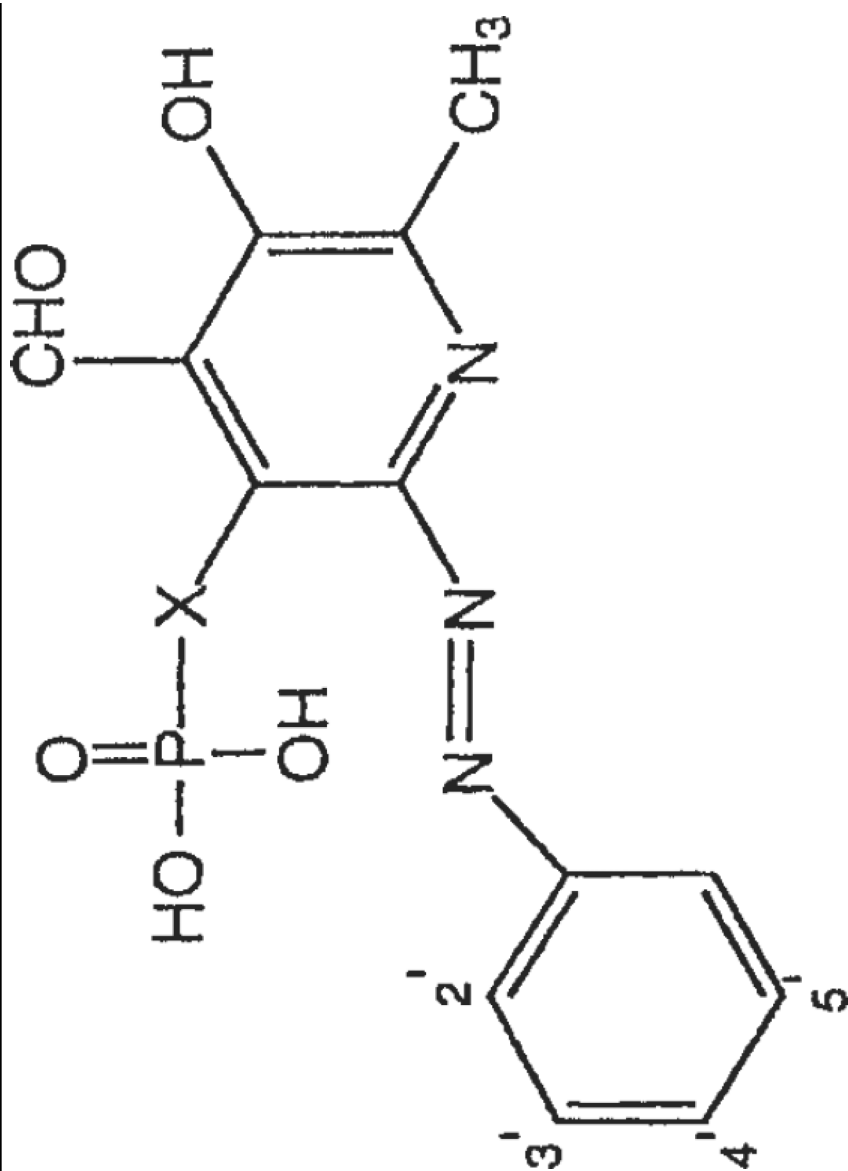
(2-18, 20, 21, 23-25, 27, 29,30)

Compound ^a	2'	3'	4'	5'	X
4	H	SO ₃ H	H	H	CH ₂ O
5	H	H	SO ₃ H	H	CH ₂ O
6 (PPADS)	SO ₃ H	H	SO ₃ H	H	CH ₂ O
7 (IsoPPADS)	SO ₃ H	H	H	SO ₃ H	CH ₂ O



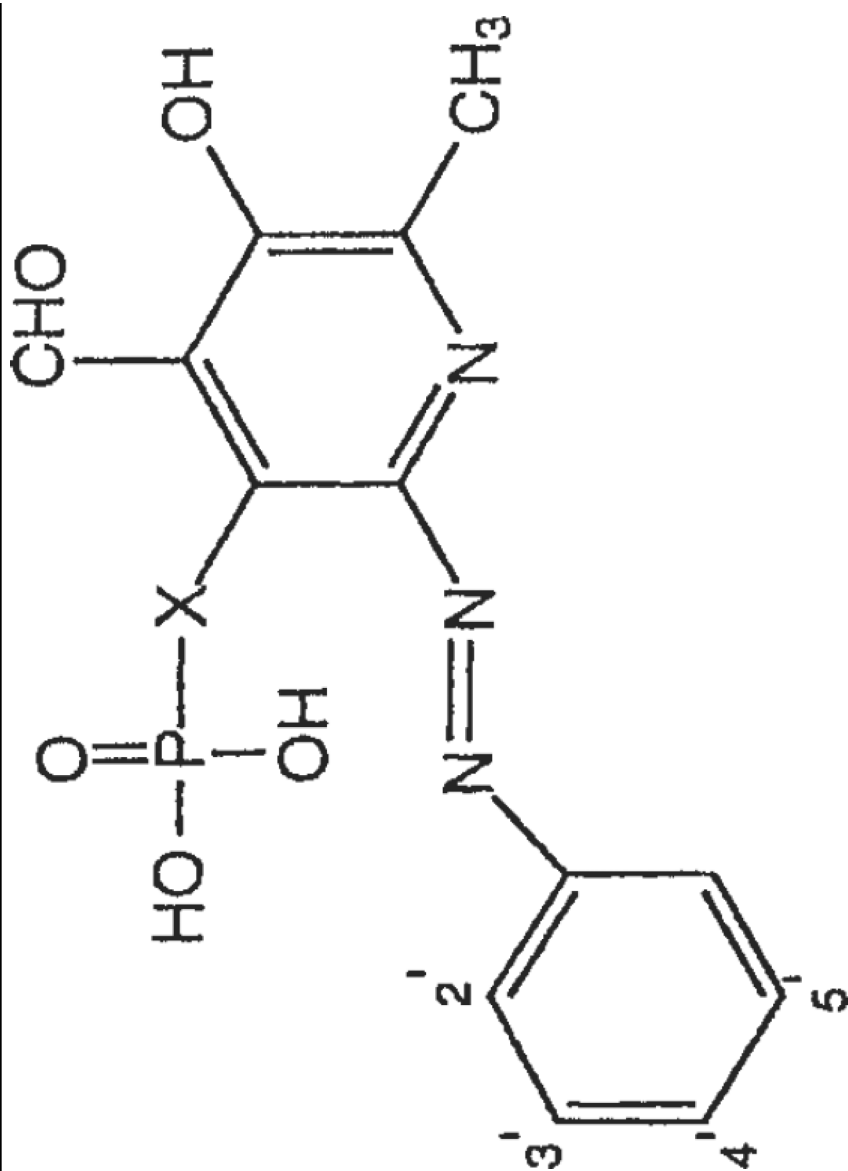
(2-18, 20, 21, 23-25, 27, 29,30)

Compound ^a	2'	3'	4'	5'	X
8	OCH ₃	H	H	SO ₃ H	CH ₂ O
9	H	COOH	H	H	CH ₂ O
10	H	H	COOH	H	CH ₂ O
11	COOH	Cl	H	H	CH ₂ O



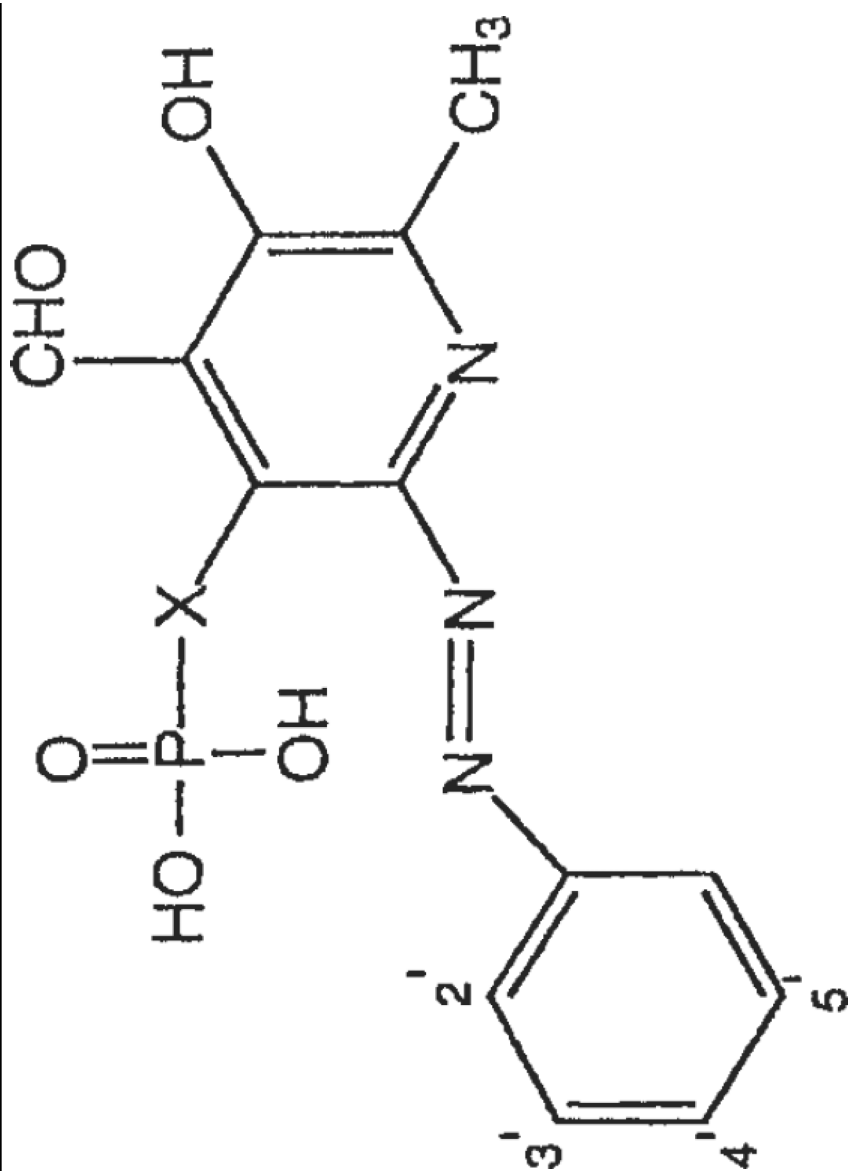
(2-18, 20, 21, 23-25, 27, 29,30)

Compound ^a	2'	3'	4'	5'	X
12	Cl	H	H	H	CH ₂ O
13	Cl	H	H	SO ₃ H	CH ₂ O
14	Cl	H	H	COOH	CH ₂ O
15	H	Cl	COOH	H	CH ₂ O



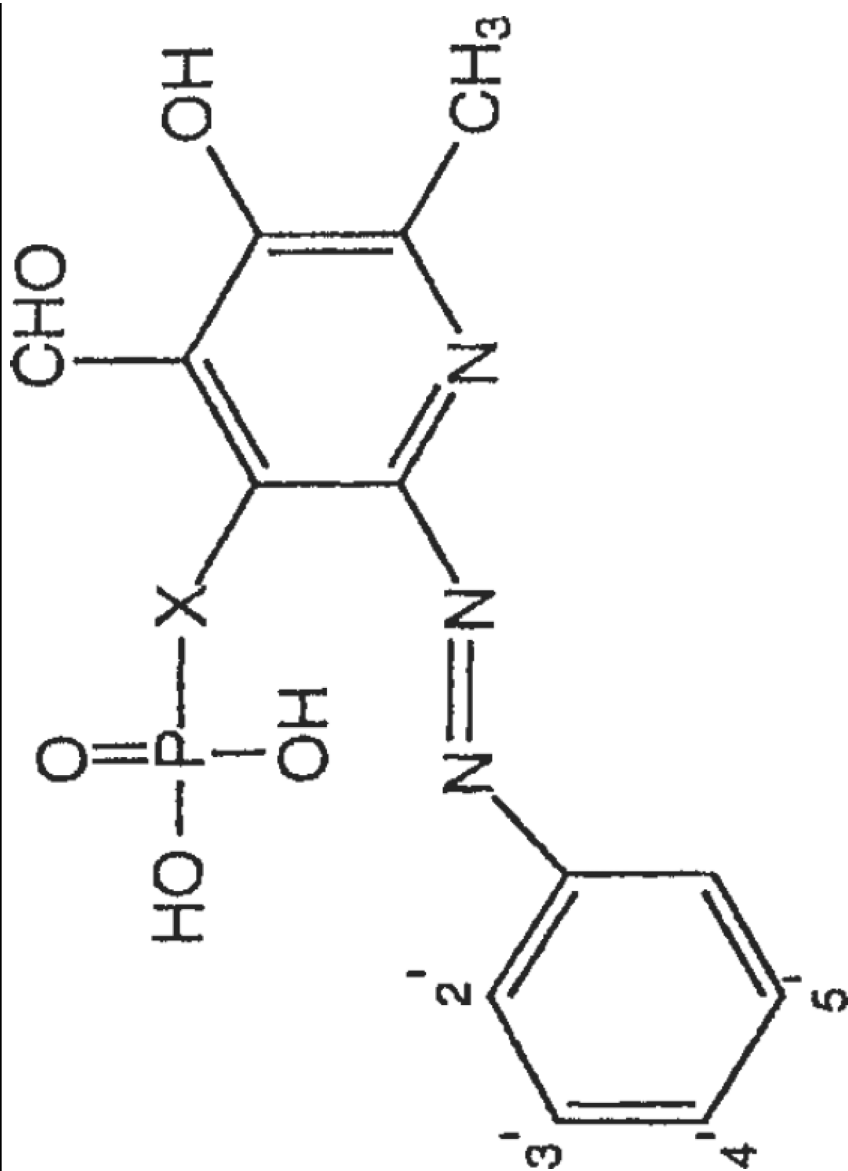
(2-18, 20, 21, 23-25, 27, 29,30)

Compound ^a	2'	3'	4'	5'	X
16	Cl	H	H	NO ₂	CH ₂ O
17	NO ₂	H	H	NO ₂	CH ₂ O
18	H	H	NO ₂	H	CH ₂ O
20	SO ₃ H	H	H	SO ₃ H	CH ₂



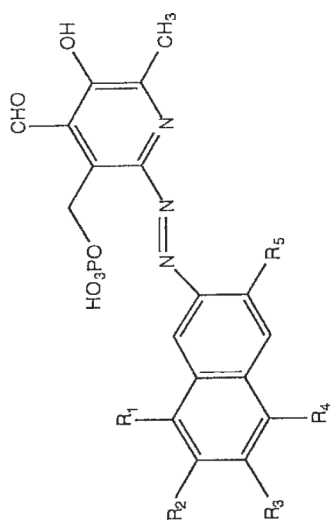
(2-18, 20, 21, 23-25, 27, 29,30)

Compound ^a	2'	3'	4'	5'	X
21	Cl	H	H	SO ₃ H	CH ₂
23	SO ₃ H	H	H	SO ₃ H	CH ₂ CH ₂
24	Cl	H	H	SO ₃ H	CH ₂ CH ₂
25	H	H	COOH	H	CH ₂ CH ₂



(2-18, 20, 21, 23-25, 27, 29, 30)

Compound ^a	2'	3'	4'	5'	X
27	SO ₃ H	H	H	SO ₃ H	CH=CH
29	SO ₃ H	H	H	SO ₃ H	CH ₂ CH ₂ CH ₂
30	Cl	H	H	SO ₃ H	CH ₂ CH ₂ CH ₂



(32–34)

	R₁	R₂	R₃	R₄	R₅
32	H	SO ₃ H	H	SO ₃ H	H
33	SO ₃ H	H	SO ₃ H	H	H
34	H	SO ₃ H	H	SO ₃ H	SO ₃ H

^aCompounds 1 (X=CH₂O), 19 (X=CH₂), 22 (X=CH₂CH₂), 26 (X=CH=CH), and 28 (X=CH₂CH₂CH₂), and 31 (X=CH=CHCH₂) are non-azo-linked precursors (see Schemes 1 and 2). Compounds 1, 2, 3, 5, 6, 17, and 18 were reported by Lambrecht et al., 1996.

TABLE 3

Pharmacologic Activities of Pyridoxal-6-azoaryl-5-phosphate and Phosphonate Derivatives at P2 Receptors (Structures in Table 2 and Schemes 1 and 2)

Compound	P2X ₁			P2X ₂		P2Y ₁		Turkey erythrocyte Inhibition of PLC IC ₅₀ (μ M) ^e
	Human HL-60 cells Inhibition of binding % at 100 μ M ^a	Guinea pig urinary bladder Inhibition of contraction % of 30 μ M ^b	Guinea pig vas deferens Inhibition of contraction % of 30 μ M ^b	Recombinant rat, in oocytes Inhibition of ion flux IC ₅₀ (μ M) ^c	Guinea Pig taenia coli Inhibition of relaxation by 2 Hz stim., % at 30 μ M ^d	Guinea pig taenia coli Inhibition of relaxation by K ₃ (μ M), or % at 30 μ M ^d		
2	55 ± 1		31	1.2 ± 0.2		6.3	22.5	
4	25 ± 1	43 ± 20	91 ± 2		63 ± 11	n.a.	20.2 ± 0.5	
6	18 ± 1			1.6 ± 0.1			16.6 ± 2.5	
7	31 ± 1			0.398 ± 0.129		1.1–3.7 ^h	21.4 ± 9.0	
8	14 ± 6	57 ± 10	95 ± 1		58 ± 19	38 ± 19%	11.8 ± 3.9	
9	10 ± 7	36 ± 8	74 ± 13	2.6 ± 0.0	70 ± 3	43 ± 27%	25.1 ± 6.3	
10	25 ± 6	61 ± 19	87 ± 2	11.9 ± 1.4	n.a.	n.a.	>100	
11	16 ± 5	30 ± 11	56 ± 8		41 ± 17	49 ± 12%	32.5 ± 7.5	
12	52 ± 1	n.a. ^f	86 ± 6		46 ± 8	n.a.	6.94 ± 1.75	
13	53 ± 5	71 ± 7	91 ± 3		35 ± 6	44 ± 19%	12.7 ± 5.6	
14	42 ± 2	51 ± 7	76 ± 4		63 ± 3	n.a.	8.23 ± 1.30	
15	11 ± 2	59 ± 14	n.a. ^g	0.819 ± 0.279	n.a.	n.a.	>100	
16	64 ± 5	n.a. ^f	85 ± 5		47 ± 9	n.a.	13.8 ± 2.2	
19	22 ± 1	34 ± 6	60 ± 12		61 ± 12	64 ± 8%	>100	
20	43 ± 1	67 ± 3	85 ± 6	1.1 ± 0.0	n.a.	n.a.	>100	
21	28 ± 1	n.a.	88 ± 5		n.a.	n.a.	7.23 ± 2.10	
22	7 ± 2	22 ± 15	78 ± 11	33.0 ± 11.0		63	n.a.	
23	25 ± 3	40 ± 16	84 ± 10	1.5 ± 0.1		12.5	n.a.	
24	17 ± 0						13.1 ± 1.2	
25		53 ± 18	97 ± 1	2.4 ± 0.3	30 ± 10	n.a.	>100	
26	15 ± 1						>100	
27	81 ± 1						>100	
28	28 ± 1	n.a. ^f	46 ± 15		n.a.	n.a.	n.a.	
29	22 ± 3	n.a.	83 ± 5	10.7 ± 1.0	54 ± 11	56 ± 8%	>100	

Compound	P2X ₁			P2X ₂		P2Y ₁	
	Human HL-60 cells Inhibition of binding % at 100 μM ^e	Guinea pig urinary bladder Inhibition of contraction % of 30 μM ^b	Guinea pig vas deferens Inhibition of contraction % of 30 μM ^b	Recombinant rat, in oocytes Inhibition of ion flux IC ₅₀ (μM) ^c	Guinea Pig taenia coli Inhibition of relaxation by 2 Hz stim., % at 30 μM ^d	Guinea pig taenia coli Inhibition of relaxation by K _B (μM), or % at 30 μM ^d	Turkey erythrocyte Inhibition of PLC IC ₅₀ (μM) ^e
30	18 ± 1	n.a.	89 ± 3	n.a.	n.a.	Enhancement ^f	20.2 ± 3.4
31	11 ± 2	n.a.	n.a.	n.a.	n.a.	n.a.	>100
32	71 ± 2	41 ± 20	86 ± 1		31 ± 20	n.a.	15.6 ± 3.4
33	30 ± 10	34 ± 9	75 ± 13		25 ± 20	Enhancement ^f	14.7 ± 3.2
34	26 ± 1	55 ± 16	80 ± 3		36 ± 13	75 ± 4%	9.31 ± 3.64

^aInhibition of specific binding of [³⁵S]ATPγS (mean ± s.e.m., n = 3). The IC₅₀ values determined for compounds 2, 7, 27, and 32 were 45, 90, 10, and 15 μM, respectively.

^bInhibition of contractile response (mean ± s.e.m., n = 3) in isolated smooth muscle to electrical field stimulations 8 Hz, unless noted). For compounds reported by Lambrecht et al. [1996], IC₅₀ values (μM) of twitch contractions at P2X receptors in rabbit isolated vas deferens with 0.5 Hz electrical field stimulation were 1 (34.6), 2 (3.1), 3 (2.6), 5 (3.1), 6 (1.4), 17 (3.97), and 18 (5.14), n.a., inactive.

^cInhibition of ion current (mean ± s.e.m., n = 3) induced by ATP (10 μM) at recombinant P2X₂ receptors expressed in *Xenopus* oocytes.

^dP2Y receptors in carbachol-precontracted guinea-pig taenia coli, inhibition of relaxation (mean ± s.e.m., n = 3) induced by electrical field stimulations (2 Hz) or by application of ATP (1 μM), unless noted, n.a., inactive.

^eInhibition of 10 mM 2-MeSADP-stimulated phospholipase C in turkey erythrocyte membranes (mean ± s.e.m. for n = 3), labeled using [³H]inositol. n.a., inactive.

^f~40% inhibition at 16 Hz.

^g~90% inhibition at 16 Hz.

^hApparent K_d value (μM) in guinea-pig taenia coli against ADPβS [Bültmann et al., 1996].

ⁱEnhanced relaxation observed upon application of compounds 30 (93.0 ± 4.2% vs. 44.3 ± 11.0% for 10 μM ATP control) and 33 (52.7 ± 9.2% vs. 32.7 ± 13.3% for 1 μM ATP control).