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(21) International Application Number: PCT/US97/19922 (22) International Filing Date: 23 October 1997 (23.10.97) (30) Priority Data: 60/029,855 30 October 1996 (30.10.96) US (71) Applicant (for all designated States except US): THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL [US/US]; 308 Bynum Hall, Campus Box 4105, Chapel Hill, NC 27599-4105 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): BOYER, José, L. [MX/US]; 103 Autumn Lane, Chapel Hill, NC 27516 (US). HARDEN, T., Kendall [US/US]; 191 Lair Court, Chapel Hill, NC 27514 (US). JACOBSON, Kenneth, A. [US/US]; Apartment 504, 1111 University Boulevard W., Silver Spring, MD (US). CAMAIONI, Emdio [US/US]; 11317 Palisades Court, Kensington, MD 20895 (US). (74) Agents: BISWAS, Sorojini, J. et al.; Myers Bigel Sibley & Sajovec, LLP, P.O. Box 37428, Raleigh, NC 27627 (US).		(81) Designated States: AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, GB, GE, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: P2Y RECEPTOR ANTAGONISTS (57) Abstract Novel P2Y receptor antagonists derived from ATP and UTP are described. P2Y receptor antagonists with competitive antagonist activity at the P2Y receptor are described in particular, as are P2Y receptor antagonists that bind selectively to the P2Y ₁ receptor. Also described herein are methods of detecting a P2Y receptor in a biological sample.		

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P2Y RECEPTOR ANTAGONISTS

Statement of Federal Support

This invention was made with Government support under grant numbers GU38213, GM29536, and HL54889 from the United States Public Health Service of the National Institutes of Health. The Government has certain rights to this invention.

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Related Applications

This application claims the benefit of U.S. Provisional Application No. 60/029,855, filed October 30, 1996.

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Field of the Invention

This invention relates to compounds that are antagonists of P2Y purinergic receptors, and more specifically to antagonists that have competitive antagonist activity at P2Y purinergic receptors.

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Background of the Invention

Extracellular adenine and uridine nucleotides regulate a broad range of physiological responses through an increasingly diverse set of G-protein-coupled and ligand-gated ion channel receptors. See, G. Burnstock, *Prog. Biochem. Pharmacol.* **16**, 141-154 (1980); G. R. Dubyak, G. R., and C. El-Moatassim, *Am. J. Physiol.* **265**, C577-C606 (1993); T. K. Harden, et al., *Ann. Rev. Pharmacol. Toxicol.* **35**, 541-579 (1995). Delineation of multiple receptor subtypes initially evolved from tissue- and agonist-specific physiological responses to nucleotide analogs. However, the complexity of the molecular species that respond to adenine and uridine nucleotides has been emphasized recently with reports of the cloning of approximately a dozen different P2 receptor

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genes. See T. K. Harden et al., *Ann. Rev. Pharmacol. Toxicol.* **35**, 541-579 (1995).; B. B. Fredholm, *Pharmacol. Rev.* **46**, 143-156 (1994). Following both pharmacological and genetic characterization of P2 receptors, ATP is now widely accepted as an

extracellular signaling molecule released from nerves and many other tissues. B.B. Fredholm et al., *Pharmacol. Rev.*, **46**, 143-156 (1994). Adenine nucleotides activate both ligand-gated ion channels of the P2X superfamily and G-protein coupled receptors of the P2Y superfamily. M.P. Abbracchio et al., *Pharmacol. Therap.* **64**, 445-475 (1994). In addition, uridine nucleotides act as agonists at certain subtypes of P2Y receptors. S.E. O'Connor et al., *Trends. Pharmacol. Sci.* **12**, 137-141 (1991); J.M. Boeynaems et al., in *P2 Purinoreceptors: localization, function and transduction mechanisms*, (John Wiley & Sons, Chichester, England)(Ciba Foundation Symposium 198) at 266-277 (1996).

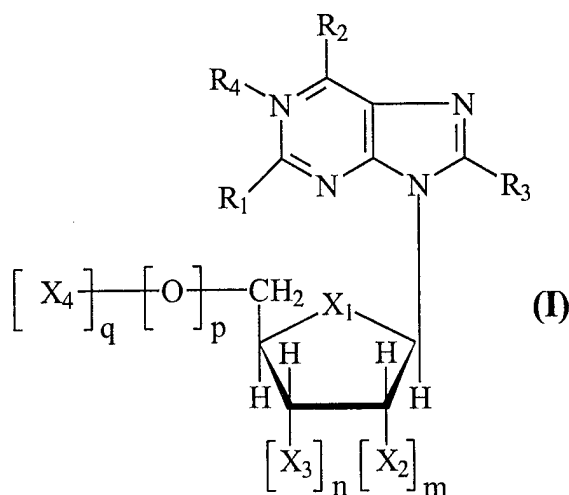
Since extracellular ATP is known to be responsible for the regulation of a large number of physiological functions, specific competitive receptors for receptors activated by nucleotides have potentially valuable therapeutic applications. Although progress has been made in identifying adenine nucleotide analogs that exhibit selectivities among P2 receptors, the availability of antagonists of these receptors is limited. Two compounds, reactive blue 2 and suramin, have been available as general P2 receptor antagonists. Recently, a pyridoxal phosphate analog, PPADS, has been reported to exhibit selectivity as a competitive antagonist of certain P2 receptors. G. Lambrecht, et al., *Eur. J. Pharmacol.* **217**, 217-219 (1992)); A.U. Ziganshin, et al., *Br. J. Pharmacol.* **110**, 1491-1495 (1993). The compound 2-propylthio- β,γ -difluoromethylene-D-ATP has also been reported to be a relatively high affinity antagonist of the ADP receptor on platelets. R. G. Humphries, et al., *Trends Pharmacol. Sci.* **16**, 179-181 (1995); R. G. Humphries, et al., *Br. J. Pharmacol.* **113**, 1057-1063 (1994). However, the majority of these molecules are disadvantageously non-selective in that they interact with a broad range of proteins that are unrelated to adenine nucleotide-regulated receptor signaling.

Summary of the Invention

The present invention is based on the initial discovery of 2'- and 3'-phosphate derivatives of ATP and UTP that were found to be partial agonists (and consequently, competitive antagonists) of P2Y receptors. In particular, adenosine 3',5'- and 2',5'-

bisphosphates were found to act as competitive antagonists at the P2Y₁ receptor. It has now been discovered that a subset of this group of analogs has potent, competitive antagonistic activity without any partial agonist activity at the human P2Y₁ receptor. In general, these compounds can be described as 2'- and 3'-deoxyadenosine bisphosphate and 2'- and 3'-deoxyuridine bisphosphate analogs containing various structural modifications at, for example, the 2-, 6-, and 8-positions of the adenine rings, on the ribose moiety, and on the phosphate groups. Significantly, the compounds of the present invention exhibit higher apparent affinities of interaction with the P2Y₁ receptor than does ATP, and exhibit absolute selectivity for binding to the P2Y₁ receptor over four other G-protein-linked P₂ receptor subtypes.

Accordingly, a first aspect of the invention is a novel compound of Formula I:



wherein:

m is 1, 2, or 3, with 1 being preferred;

n is 1, 2, or 3, with 1 being preferred;

p is 0 or 1, with 1 being preferred;

q is 0, 1, 2, or 3, with the proviso that when p is zero, q is not zero;

R₁ is selected from the group consisting of H, lower alkyl, halo, alkoxy, and alkylthio, with H being preferred;

R₂ is selected from the group consisting of hydroxy, halo, alkylthio, lower alkyl, substituted lower alkyl, and -NR'R'', wherein R' and R'' are each independently selected from the group consisting of H, lower alkyl, aroyl, alkoxy, substituted lower alkyl, alkoxy, and alkoxyalkyl;

R_3 is selected from the group consisting of H, halo, and lower alkyl, with H being preferred;

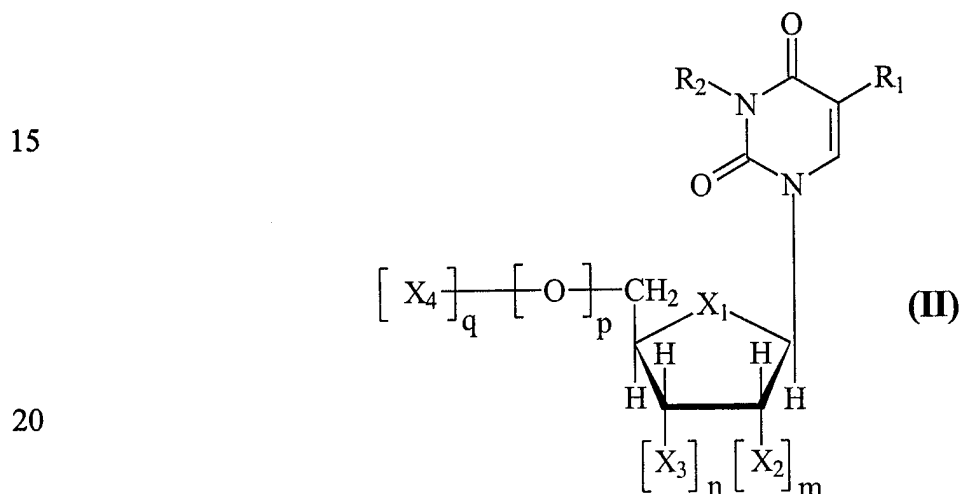
R_4 is selected from the group consisting of H and lower alkyl, with H preferred;

5 X_1 is selected from the group consisting of O, S, N, and CH_2 ; with O being preferred; and

X_2 , X_3 and X_4 are each independently selected from the group consisting of H, hydroxy, amino, lower alkyl, halo, alkoxy, phosphate, thiophosphate, carboxylate, and nitro, or X_2 and X_3 together are cyclophosphate, with phosphate
10 being preferred;

together with the pharmaceutically acceptable salts thereof.

A second aspect of the invention is a novel compound of Formula II:



wherein:

m is 1, 2, or 3; with 1 being preferred;

n is 1, 2, or 3; with 1 being preferred;

25 p is 0 or 1; with 1 being preferred;

q is 0, 1, 2, or 3, with the proviso that when p is zero, q is not zero;

R_1 is selected from the group consisting of H, lower alkyl, and halo; with H being preferred;

30 R_2 is selected from the group consisting of H, and lower alkyl; with H being preferred;

X_1 is selected from the group consisting of O, S, N, and CH_2 ; with O being preferred; and

X_2 , X_3 and X_4 are each independently selected from the group consisting of H, hydroxy, amino, lower alkyl, halo, alkoxy, phosphate, thiophosphate, carboxylate, and nitro, or X_2 and X_3 together are cyclophosphate, with phosphate being preferred;

5 together with the pharmaceutically acceptable salts thereof.

A third aspect of the present invention is a method of detecting a P2Y receptor in a biological sample suspected of containing a P2Y receptor, comprising contacting said biological sample with a compound of the present invention that binds selectively to a P2Y receptor, and then detecting the presence or absence of binding of the P2Y
10 receptor-binding compound to a receptor in the biological sample, the presence of binding indicating the presence of a P2Y receptor.

Additional aspects of the present invention include methods of treating disorders that respond to treatment with compounds that interfere with the signaling proteins that interact with extracellular adenine or uridine (including ATP-induced vasoconstriction, bladder disease, prostate disease, hyperthyroidism, hyperinsulinemia, and disorders
15 characterized by excessive production of adeno-cortical hormones), the methods comprising administering to a subject in need of such treatment a compound of the present invention as provided above, in an amount sufficient to treat the disorder.

A final aspect of the present invention is a pharmaceutical composition useful in the treatment of a disorder characterized by being responsive to treatment with compounds that interfere with the signaling proteins that are utilized by extracellular adenine or uridine nucleotides, the pharmaceutical composition comprising, in a pharmaceutically acceptable carrier, a compound of the present invention or a pharmaceutically acceptable salt thereof, in an amount effective to combat the disorder.
20

25 The foregoing and other objects and aspects of the present invention are explained in detail in the drawings herein and the specification set forth below.

Brief Description of the Drawings

30 In the figures, data shown are the mean of either duplicate or triplicate assays, and the results are representative of those obtained in experiments repeated at least three times.

FIG 1. is a graphical illustration of the effect of sulfate-substituted adenine nucleotide analogs on inositol lipid hydrolysis by turkey erythrocyte membranes. The capacity of the indicated concentrations of 2MeSATP (filled circles), adenosine 3'-phosphate, 5'-phosphosulphate (open circles), ADP (open diamonds), and adenosine 5'-phosphosulfate (filled diamonds) to stimulate the hydrolysis of inositol lipids by turkey erythrocyte membranes was determined as described below in Example 3.

FIG. 2 is a graphical illustration of the effect of adenosine 3'-phosphate, 5'-phosphosulphate on P2Y receptor-mediated activation of phospholipase C in turkey erythrocytes. [³H]Inositol labeled turkey erythrocyte membranes were incubated with the indicated concentrations of adenosine 3'-phosphate, 5'-phosphosulphate in the absence (filled circles), or in the presence of 1 (open circles), 3 (filled diamonds), 10 (open diamonds), 30 (filled triangles), 100 (open triangles), 300 (filled squares), and 1000 (open squares) nM 2MeSATP.

FIG. 3 illustrates competitive inhibition of 2MeSATP-mediated activation of P2Y receptors by adenosine 3'-phosphate, 5'-phosphosulphate and adenosine 3',5' *bis* phosphate. [³H]Inositol labeled turkey erythrocyte membranes were incubated with the indicated concentrations of 2MeSATP in the absence (filled circles), or in the presence of 0.1 (open circles), 0.3 (filled triangles), 1 (open triangles), 3 (filled squares), 10 (open squares), 30 (filled circles), 100 (open circles), 300 (filled diamonds), and 1000 (open diamonds) μ M adenosine 3'-phosphate, 5'-phosphosulphate (Panel A) or adenosine 3',5'-*bis*phosphate (Panel C). Inositol phosphate accumulation induced by adenosine 3'-phosphate, 5'-phosphosulphate or or adenosine 3',5'-*bis*phosphate alone was subtracted from the accumulation in the presence of the indicated concentrations of 2MeSATP. Schild regression analysis of data shown in A and C are shown in panels B and D, respectively.

FIG. 4 illustrates the effect of or adenosine 3',5'-*bis*phosphate on the activation of phospholipase C by β -adrenergic receptors in turkey erythrocyte membranes. The capacity of the indicated concentrations of or adenosine 3',5'-*bis*phosphate to stimulate the accumulation of inositol phosphates in the absence (open diamonds) or in the presence (closed diamonds) of 10 μ M isoproterenol was determined as described below in Example 8.

FIG. 5 illustrates that adenosine 3',5'-bisphosphate is a competitive antagonist without agonistic activity at the human P2Y₁ receptor stably expressed in 1321N1 human astrocytoma cells. In Panel A, cells were incubated with the indicated concentrations of adenosine 3',5'-bisphosphate in the absence (filled circles) or in the presence of 10 (filled diamonds), 30 (filled triangles), 100 (open triangles), 300 (filled squares), and 1000 nM 2MeSATP (open squares). In Panel B, the concentration-dependence of 2MeSATP for the activation of phospholipase C in 1321N1 cells was determined in the absence (filled circles), or in the presence of 0.3 (open diamonds), 1 (filled triangles), 3 (open triangles), 10 (filled squares), 30 (open squares), 100 (filled diamonds), and 300 μ M (open circles) adenosine 3',5'-bisphosphate. Panel C illustrates Schild regression analysis of data shown in Panel B. Slope value was not significantly different from unity.

FIG. 6 illustrates the lack of agonist or antagonist effects of adenosine 3',5'-bisphosphate on the adenylyl cyclase-coupled P2Y receptor of C6 cells. The capacity of indicated concentrations of 2MeSATP to inhibit isoproterenol-stimulated cyclic AMP accumulation (Panel A), or the capacity of adenosine 3',5'-bisphosphate to affect the response of 10 μ M isoproterenol (open diamonds), or 10 μ M isoproterenol + 1 nM 2MeSATP (filled diamonds)-induced accumulation of cyclic AMP (Panel B) was studied as described in Example 10.

FIG. 7 illustrates the effects of deoxyadenosine bisphosphate derivatives on phospholipase C in turkey erythrocyte membranes. Both concentration-dependent stimulation of inositol phosphate formation and its inhibition by compounds 2'-Deoxyadenosine-3',5'-bisphosphate (compound **4**)(triangles) and 3'-Deoxyadenosine-2',5'-bisphosphate (compound **17**) (circles) were observed. Membranes from [³H]inositol-labelled erythrocytes were incubated for 5 min at 30°C in the presence of the indicated concentrations of **4** and **17**, either alone (open symbols) or in combination (solid symbols) with 10 nM 2-MeSATP.

FIG. 8 illustrates the effects of N⁶-alkyl analogues of adenosine and 2'-deoxyadenosine bisphosphate derivatives on agonist-stimulated phospholipase C in turkey erythrocyte membranes. Membranes from [³H]inositol-labelled erythrocytes were incubated for 5 min at 30°C in the presence of 10 nM 2-MeSATP and the indicated

concentrations of the compounds 2'-Deoxy-N⁶-methyladenosine 3',5'-bisphosphate (Compound 9, squares), 2'-Deoxy-N⁶-ethyladenosine-3',5'-bisphosphate (Compound 10, triangles), 2'-Deoxy-N⁶-propyladenosine-3',5'-bisphosphate (Compound 11, diamonds), 2'-Deoxy-N⁶-dimethyladenosine-3',5'-bisphosphate (Compound 13, asterisks), and 2'-Deoxy-N⁶-aminohexyladenosine-3',5'-bisphosphate (Compound 23, circles).

FIG. 9 is a graphical illustration showing the log dose response curves of the P2Y₁ agonist 2MeSATP in the presence of a control (circles), or 0.1 μM (diamonds), 0.3 μM (triangles), 1 μM (squares), 3 μM (asterisks), 10 μM (crosses) and 30 μM (X-es) of the compound 2'-Deoxy-N⁶-methyladenosine 3',5'-bisphosphate (Compound 9). The parallel shifting of the agonist log dose response curve to the right illustrates the competitive antagonist activity of Compound 9. Data is shown in terms of [³H] Inositol Phosphates (percentage of maximum) as a function of the log Molar concentration of 2MeSATP.

FIG. 10 is a Schild regression of the data presented in **FIG. 9**.

In **FIG. 10**, the calculation of the ratio of the agonist (2MeSATP) concentration that elicits equal responses in the absence and presence of antagonist (Compound 9) at increasing concentrations (termed the dose ratio) is plotted according to the relationship $\log(\text{dose ratio}-1)$ vs. $\log$ concentration (in Molar) of Compound 9. In that the slope of the line produced by the Schild regression is approximately equal to 1 (slope = 0.912), the compound 9 is indicated to be a competitive antagonist of the P2Y₁ receptor.

FIG. 11 is a graphical illustration the selective binding and specificity of Compound 9 to the P2Y₁ receptor. Inositol phosphate accumulation in 1321N1 human astrocytoma cells expressing the cloned, human P2Y₁ (far left), P2Y₂ (second from left), P2Y₄ (second from right) or P2Y₆ (far right) receptor was measured in the absence (open bars) or presence (filled bars) of Compound 9 disclosed herein. While the presence of Compound 9 had no effect on the accumulation of inositol phosphates in cells expressing P2Y₂ and P2Y₆, and had the slight effect of increasing the amount of inositol phosphates detected in cells expressing the P2Y₄ receptor, the presence of Compound 9 completely inhibited the generation of inositol phosphates in cells expressing P2Y₁. This result indicates that Compound 9 binds selectively to the P2Y₁ receptor, and

further that the compound is a potent antagonist of the P2Y₁ receptor.

Detailed Description of the Invention

The present invention will now be described more fully hereinafter, in which
5 preferred embodiments of the invention are shown. This invention may, however, be
embodied in different forms and should not be construed as limited to the embodiments
set forth herein. Rather, these embodiments are provided so that this disclosure may
fully convey the scope of the invention to those skilled in the art.

Purinergic receptors are referred to herein according to the guidelines of the
10 IUPHAR Nomenclature Committee, which has provided general recommendations that
G protein-coupled nucleotide receptors be designated as P2Y receptors, and that
receptors in a subfamily be denoted by numbers that reflect the chronological order in
which the sequences of functional receptors have become available to the public
domain. See, B. B. Fredholm, et al., *Pharmacol. Rev.* **46**, 143-156 (1994). However,
15 the receptor cloned by Chang et al. (*J. Biol. Chem.* **270**, 26152-26158 (1995)) has been
referred to as the P2Y₆ receptor, even though it was the third functional P2Y receptor for
which a sequence was published. This receptor is referred to herein as the P2Y₆
receptor. The P2Y₂ receptor has been previously referred to in the literature as the P2U
purinergic receptor.

20 As used herein, the term "lower alkyl" is to be broadly interpreted and includes,
but is not limited to, C1 to C4 linear, branched, saturated, unsaturated, cyclic, and
acyclic alkyls. Halogenated alkyls, (e.g., fluoroalkyls, chloroalkyls) are also
encompassed by this definition. The term "alkoxy" is defined herein to be a residue
represented by the formula -OR, with R representing, for example, lower alkyl as
25 defined above. Halogenated alkoxy groups, such as fluoroalkoxides, are also included
within this definition. The term "amino" as used herein refers to the group NR'R",
wherein R' and R" are independently selected from H or lower alkyl as defined above,
i.e., -NH₂, -NHCH₃, -N(CH₃)₂, etc. The term "alkylthio" as used herein refers to a
residue of the formula -SR, where R represents, for example, lower alkyl as defined
30 above. The term "halo" refers to a residue selected from the group consisting of -F, -
Cl, -Br and -I. Terms not defined specifically herein are to be given the meaning
normally understood in the art.

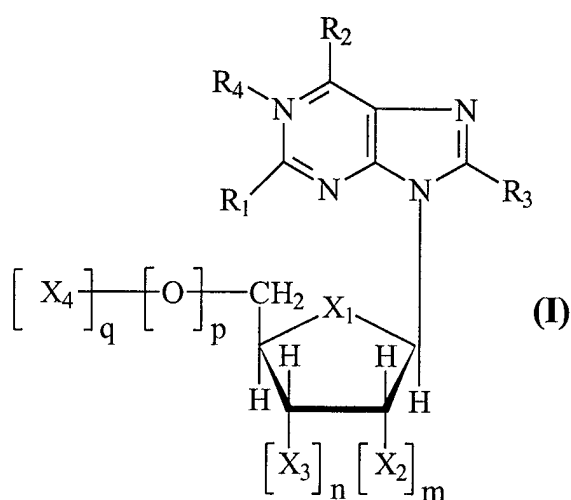
As used herein, the term "agonist" is used to refer to a compound that binds to a physiological receptor and mimics the effects of the endogenous regulatory compound. Compounds that bind to physiological receptors and also inhibit or interfere with the binding of the endogenous agonist (i.e., by competing for the agonist binding site) are generally referred to herein as "antagonists." If the inhibition caused by the antagonist can be overcome by increasing the concentration of the agonist, ultimately achieving the same maximal effect, then the antagonist is referred to herein as a "competitive antagonist."

The present invention relates to novel compounds, and to methods of using both novel compounds and compounds that are known. Compounds that are illustrative of the compounds of the present invention (*hereinafter* the "active compounds") include those compounds according to Formula I and Formula II, and the pharmaceutically acceptable salts thereof, as provided above in the Summary of the Invention. The novel compounds of the present invention are those which are defined according to Formula I above, with the proviso that when R_1 , R_3 , and R_4 are each H, and m , n , and p are each 1, and q is 1, 2, or 3, and X_1 is O, and X_4 is phosphate, and X_2 and X_3 are each either hydroxy, H or phosphate or together are cyclophosphate, R_2 is not $-NH_2$. Novel compounds of the present invention also include compounds of Formula II as set forth above, with the proviso that when R_1 and R_2 are H, and p , m , and n are each 1, and q is 1, 2 or 3, and X_1 is O, X_2 and X_3 are not H, X_2 and X_3 are not $-OH$, and X_2 and X_3 together are not cyclophosphate.

Preferred compounds of the invention are those compounds of Formula I and Formula II that are deoxy at the 2' and 3' positions (R_2 and R_3 in Formulae I and II) of the compound. Other preferred compounds of the present invention are the substituted N^6 analogue compounds of Formula I, wherein (in reference to Formula I), R_2 is $-R'R''$, R' is H and R'' is lower alkyl.

Compounds that are particularly preferred are the deoxyadenosine-based compounds set out and identified by compound number in **Table 1**, below. In the general structure illustrated in **Table 1**, X_1 is O.

Table 1. Chemical structures of novel adenine nucleotides synthesized as potential P2Y₁ receptor antagonists.

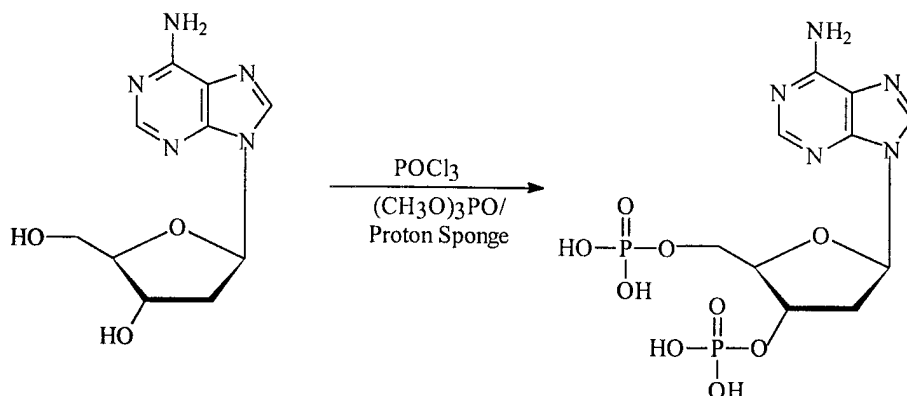


No	R ₄	R ₁	R ₂	R ₃	X ₂	X ₃	X ₄
4	H	H	NH ₂	H	H	PO ₄ H ₂	PO ₄ H ₂
5	CH ₃	H	NH ₂	H	H	PO ₄ H ₂	PO ₄ H ₂
6	H	Cl	NH ₂	H	H	PO ₄ H ₂	PO ₄ H ₂
7	H	SCH ₃	NH ₂	H	H	PO ₄ H ₂	PO ₄ H ₂
8	H	H	NH ₂	Br	H	PO ₄ H ₂	PO ₄ H ₂
9	H	H	NHCH ₃	H	H	PO ₄ H ₂	PO ₄ H ₂
10	H	H	NHCH ₂ CH ₃	H	H	PO ₄ H ₂	PO ₄ H ₂
11	H	H	NH(CH ₂) ₂ CH	H	H	PO ₄ H ₂	PO ₄ H ₂
12	H	H	NHCOC ₆ H ₆	H	H	PO ₄ H ₂	PO ₄ H ₂
13	H	H	N(CH ₃) ₂	H	H	PO ₄ H ₂	PO ₄ H ₂
14	H	H	Cl	H	H	PO ₄ H ₂	PO ₄ H ₂
15	H	H	OH	H	H	PO ₄ H ₂	PO ₄ H ₂
16	H	H	SCH ₃	H	H	PO ₄ H ₂	PO ₄ H ₂
17	H	H	NH ₂	H	PO ₄ H	H	PO ₄ H ₂
18	H	H	NHCH ₃	H	PO ₄ H	H	PO ₄ H ₂
19	H	H	NH	H	PO ₄ H	H	Cl
20	H	H	NH ₂	H	OCH ₃	PO ₄ H ₂	PO ₄ H ₂
21	H	H	NH ₂	H	H	PSO ₃ H ₂	PSO ₃ H ₂

Active compounds of the present invention also include the adenosine and uridine 2'- and 3'- sulphonate derivatives of the compounds of Formula I and Formula II provided above, and the adenosine and uridine 2'- and 3'- borate derivatives of the compounds of Formula I provided above.

Compounds that are known, but which are nonetheless useful in the practice of the methods of the present invention are commercially available, or may be made according to techniques known to one skilled in the art. The novel compounds of the present invention may also be made in accordance with known procedures, or variations thereof which will be apparent to those skilled in the art. *See generally*, J. L. Boyer, et al., *Br. J. Pharmacol.* **116**, 2611-2616 (1995); J. L. Boyer, et al., *J. Biol. Chem.* **264**, 884-890 (1989); B. Fischer, et al., *J. Med. Chem.* **36**, 3937-3946 (1993). For example, to synthesize the compounds in **Table 1** above (compounds **4-22**), the appropriate starting nucleosides may first purchased or synthesized, and then either phosphorylated or thiophosphorylated. Several intermediates, such as 2'-deoxy-2-methylthioadenosine, 8-bromo-2'-deoxyadenosine, N⁶-ethyl and N⁶-propyl 2'-deoxyadenosines, and 3'-deoxy-N⁶-methyladenosine, may be prepared by the skilled artisan by adapting published procedures. *See, e.g.*, L.F. Christensen et al., *J. Med. Chem.* **15**, 735-739 (1972); M. Ikehara et al., in *Nucleic Acid Chemistry*; (L.B. Townsend and R.S. Tipson, eds., John Wiley and Sons: New York, 1978)(Vol U, at 837-841); E.M. van der Wenden et al., *J. Med. Chem.* **38**, 4000-4006 (1995); V. Nair, *Synthesis* 670-672 (1982).

The phosphorylation of 2'- and 3'-deoxyadenosine and their analogues may be achieved with a single step reaction using phosphorous oxychloride (or thiophosphoryl chloride), trimethyl phosphate, and Proton Sponge[®] in an ice bath, as illustrated in Scheme I:



The reaction will typically completed after one hour and may quenched by the addition of buffer (e.g., triethylammonium bicarbonate). The resulting mixture may be lyophilized, according to the wishes and general knowledge of the skilled artisan. Purification of the compounds of the present invention may be performed using, for example, a Sephadex ion-exchange column with a linear gradient of water/ammonium bicarbonate (0.01 to 0.5 M). The chemical structures of the phosphorylated nucleosides of the present invention may be verified using ^1H -NMR and ^{31}P -NMR techniques, as well as high resolution mass spectroscopy, which techniques are known to those skilled in the art. Using ^1H -NMR it is possible to monitor the chemical shift of ribose protons at 5'- and 2'- (or 3'-) position, and to distinguish them before and after phosphorylation. The presence of *bis*phosphorylation is indicated by two phosphate signals in the ^{31}P -NMR spectra.

The active compounds of the present invention may be prepared, utilized, and/or administered by themselves or in the form of their pharmaceutically acceptable salts. Such pharmaceutically acceptable salts include, for example, alkali metal salts such as sodium or potassium salts, alkaline earth metal salt, or ammonium or tetraalkyl ammonium salts represented by the formula NX_4^+ (wherein X is a C_{1-4} alkyl group). Pharmaceutically acceptable salts are defined herein as those salts that retain the desired biological activity of the parent compound but do not impart undesired toxicological effects.

The active compounds of the present invention are useful in delineating the physiological roles of extracellular adenine and uridine nucleotides and P2 receptors in target tissues. Accordingly, a method of the present invention is a method of detecting the presence or absence of a P2Y receptor in a biological sample suspected of containing a P2Y receptor. Such methods comprise contacting a biological sample suspected of containing the a P2Y receptor to an active compound of the present invention, which active compound is capable of specifically binding to a P2Y receptor, under conditions which permit the binding of a P2Y receptor to an active compound of the present invention; and then detecting the presence or absence of the binding. Biological samples taken from human or animal subjects for use in this method are generally biological fluids such as serum, blood plasma, or ascites fluid. In the alternative, the

sample taken from the subject can be a tissue sample (e.g., biopsy tissue; scrapings; etc.). Additionally, a biological sample may comprise a cell culture containing cells that may or may not express a P2Y receptor. Any suitable assay format known to one skilled in the art can be used to carry out the detection of the binding of the active compound to the P2Y receptor, one example being the turkey erythrocyte/phospholipase C assay known in the art and set forth below in Example 3. Those skilled in the art will be familiar with numerous specific assay formats and variations thereof which may be useful for carrying out the method disclosed herein. Active compounds useful in the method of the present invention include the novel compounds set forth above, but also include known compounds of Formula I and Formula II above, such as adenosine 3',5'-bisphosphate and adenosine 2',5'-bisphosphate. These compounds, although known, have heretofore not been recognized as being able to specifically bind to, for example, the human P2Y₁ receptor. It is contemplated that active compounds according to Formula I and as disclosed herein are particularly useful in detecting the absence or presence of the P2Y₁ receptor in a biological sample, as specific binding to the P2Y₁ receptor by these compounds is illustrated below in the Examples section. Additionally, active compounds according to Formula II and as disclosed herein are particularly useful in detecting the presence or absence of P2Y receptors known to be activated by extracellular uridine nucleotides, e.g., the P2Y₂ receptor and the P2Y₄ receptor.

Active compounds of the present invention may additionally be used as therapeutic agents that interfere with the signaling proteins used by extracellular adenine and uridine nucleotides. Accordingly, compounds of the present invention are useful in methods of treating disorders characterized as being responsive to treatment with compounds that interfere with the interaction between extracellular adenine and uridine nucleotides and signaling proteins utilized by these nucleotides. These disorders include, but are not limited to, ATP-induced vasoconstriction, hyperthyroidism, hyperinsulinemia, and disorders associated with excessive production of adeno-cortical hormones. In that P2Y receptors have been shown to be highly expressed in bladder and prostate muscle, the present invention is also useful in the treatment of disorders of the bladder (e.g., incontinence) and prostate (e.g., prostatic inflammation, prostatic hyperplasia). In a therapeutic method of the present invention, an active compound as described herein is administered to a subject suffering from a disorder that it

characterized as being responsive to treatment with compounds that interfere with the interaction between extracellular adenine and uridine nucleotides and the signaling proteins utilized by these nucleotides, in a therapeutically effective amount. A therapeutically effective amount is defined herein as an amount of the active compound sufficient to inhibit binding between a P2Y receptor and an extracellular adenine nucleotide or extracellular uridine nucleotide.

The present invention is concerned primarily with the treatment of human subjects. However, the present invention may also be employed for the treatment of other mammalian subjects, such as dogs and cats, for veterinary purposes.

The dosage of a compound of the present invention, or the pharmaceutically acceptable salt thereof, will vary depending on the condition being treated and the state of the subject, but generally may be an amount sufficient to achieve dissolved concentrations of active compound in the blood of the subject or at the receptor site of from about 10^{-9} to about 10^{-4} moles/liter. Depending upon the solubility of the particular formulation of active compound administered, the daily dose may be divided among one or several unit dose administrations. The dose of active agent will vary according to the condition being treated and the dose at which adverse pharmacological effects occur. One skilled in the art will take such factors into account when determining dosage.

Pharmaceutical compositions of the present invention include those suitable for parenteral (including subcutaneous, intradermal, intramuscular, intravenous and intraarticular), oral, inhalation, topical (including buccal, sublingual, dermal and intraocular) and transdermal administration. The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art. The most suitable route of administration in any given case may depend upon the nature and severity of the condition being treated, and the particular active compound which is being used. The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art.

In the manufacture of a medicament according to the invention (a "formulation"), active agents or the physiologically acceptable salts thereof (the "active compound") are typically admixed with, *inter alia*, an acceptable carrier. The carrier must, of course, be acceptable in the sense of being compatible with any other ingredients in the formulation and must not be deleterious to the patient. The carrier

may be a solid or a liquid, or both, and is preferably formulated with the compound as a unit-dose formulation, for example, a tablet. One or more active compounds may be incorporated in the formulations of the invention (e.g., the formulation may contain one or more additional anti-tubercular agents as noted above), which formulations may be prepared by any of the well known techniques of pharmacy consisting essentially of admixing the components, optionally including one or more accessory therapeutic ingredients.

Formulations of the present invention suitable for parenteral administration comprise sterile aqueous and non-aqueous injection solutions of the active compound, which preparations are preferably isotonic with the blood of the intended recipient. These preparations may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient. Aqueous and non-aqueous sterile suspensions may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, saline or water-for-injection immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Formulations suitable for oral administration may be presented in discrete units, such as capsules, cachets, lozenges, or tablets, each containing a predetermined amount of the active compound; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water or water-in-oil emulsion. Such formulations may be prepared by any suitable method of pharmacy which includes the step of bringing into association the active compound and a suitable carrier (which may contain one or more accessory ingredients as noted above). In general, the formulations of the invention are prepared by uniformly and intimately admixing the active compound with a liquid or finely divided solid carrier, or both, and then, if necessary, shaping the resulting mixture. For example, a tablet may be prepared by compressing or molding a powder or granules containing the active compound, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing, in a suitable machine, the compound in a free-flowing form, such as a powder or granules

optionally mixed with a binder, lubricant, inert diluent, and/or surface active/dispersing agent(s). Molded tablets may be made by molding, in a suitable machine, the powdered compound moistened with an inert liquid binder. Formulations for oral administration may optionally include enteric coatings known in the art to prevent degradation of the formulation in the stomach and provide release of the drug in the small intestine.

Formulations suitable for buccal (sub-lingual) administration include lozenges comprising the active compound in a flavored base, usually sucrose and acacia or tragacanth; and pastilles comprising the compound in an inert base such as gelatin and glycerin or sucrose and acacia.

Formulations suitable for topical application to the skin preferably take the form of an ointment, cream, lotion, paste, gel, spray, aerosol, or oil. Carriers which may be used include vaseline, lanoline, polyethylene glycols, alcohols, transdermal enhancers, and combinations of two or more thereof.

Formulations suitable for transdermal administration may be presented as discrete patches adapted to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Formulations suitable for transdermal administration may also be delivered by iontophoresis (see, e.g., *Pharmaceutical Research* 3, 318 (1986)) and typically take the form of an optionally buffered aqueous solution of the active compound.

The following Examples are provided to more fully illustrate the present invention and should not be construed as restrictive thereof.

As used in the following Examples, ATP means adenosine 5'-triphosphate, PPADS means pyridoxal phosphate 6-azophenyl 2', 4'-disulphonic acid; MeATP means adenosine-5'-methylenetriphosphate, (α,β) or (β,γ) isomers; 2MeSATP means 2-methylthioadenosine 5'-triphosphate; DEAE means diethylaminoethyl; DMSO means dimethylsulfoxide; FAB means fast atom bombardment (mass spectroscopy); HPLC means high pressure liquid chromatography; HRMS means high resolution mass spectroscopy; PAPS means adenosine-3'-phosphate-5'-phosphosulfate; TBAP means tetrabutylammonium phosphate; TEAA means triethylammonium acetate; TEAB means triethylammonium bicarbonate; and TLC means thin-layer chromatography.

In the following Examples, adenosine 3'-phosphate, 5'-phosphosulphate,

adenosine 3',5'-bisphosphate, adenosine 2',5'-bisphosphate, and (-) isoproterenol (+)-bitartrate, nucleosides, nucleotides, 23 - 26, (bisphosphate analogues), and reagents used for the syntheses described below were obtained from Sigma Chemical Company (St. Louis, MO). 2MeSATP was obtained from Research Biochemicals Inc. (Natick, MA). 2-[³H]myo-inositol (20 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Inositol-free DMEM was obtained from Gibco BRL (Grand Island, NY).

The purity of the compounds synthesized as described in the following Examples was determined by a Hewlett-Packard 1090 HPLC apparatus using SMT OD-5-60 RP-C18 (250 x 4.6 mm; Separation Methods Technologies, Inc., Newark, DE) as an analytical column in two solvent systems, as follows:

System A: linear gradient solvent system: 0.1 M TEAA/CH₃CN from 95/5 to 40/60 in 20 min; flow rate 1 mL/min.

System B: linear gradient solvent system: 5 mM TBAP/CH₃CN from 80:20 to 40/60 in 20 min; flow rate 1 mL/min.

Peaks were detected by UV absorption using a diode array detector. Purification of the synthesized nucleotides was carried out on DEAE-A25 Sephadex columns as described above. All compounds synthesized according to the Examples below exhibited more than 95% purity in HPLC system.

In the Examples below, ¹H-NMR spectra were obtained with a Varian Gemini-300 spectrometer using D₂O as a solvent. 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 FAB (fast atom bombardment) mass spectrometry was performed with JEOL SX102 spectrometer using 6-kV Xe atoms following desorption from a glycerol matrix.

Example 1: Materials and Methods

Cell Culture

C6 rat glioma cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal calf serum in a humidified atmosphere of 95% air and 5% CO₂. Cells were passaged by trypsinization. Experiments were carried out with confluent cell cultures 2-4 days after plating in 12-well clusters as previously described in J. L. Boyer et al., *Br. J. Pharmacol.* **116**, 2611-2616 (1995). 1321N1 human astrocytoma cells stably expressing the human or turkey P2Y₁ receptor, or the human P2Y₂ receptor (P_{2U}-purinergic receptor), or the human P2Y₄-receptor, or the human or rat P2Y₆-receptor, were grown in DMEM supplemented with 5% fetal calf serum and 600 µg/mL G-418.

Example 2: Materials and Methods

Turkey Erythrocyte Labeling

Fresh blood was obtained from female turkeys by venous puncture and collected into a heparinized syringe. Erythrocytes were washed twice by centrifugation and resuspension with sterile DMEM, followed by a final wash in inositol-free DMEM. One mL of washed packed erythrocytes was resuspended in a final volume of 4.2 mL of inositol-free DMEM in the presence of 0.5 mCi [³H]inositol. Cells were incubated in a stirred glass vial for 16-20 h at 37°C in a humidified atmosphere of 95% air, 5 % CO₂ as previously described in J. L. Boyer, et al., *J. Biol. Chem.* **264**, 884-890 (1989).

Example 3

Turkey erythrocyte/Phospholipase C assay

P2Y₁ receptors activate phospholipase C, which consequently results in the generation of inositol phosphates and diacyl glycerol from phosphatidyl inositol(4,5)bisphosphate. This receptor has been extensively studied in turkey erythrocyte membranes, and this system has been applied to identify molecules that have antagonistic (B.Fischer et al., *J. Med. Chem.* **1993**, 36, 3937-3946; J.L. Boyer, *Br. J. Pharmacol.* **1996**, 118, 1959-1964) or competitive antagonistic (J.L. Boyer et al., *Br. J. Pharmacol.* **1994**, 113, 614-620; J. L. Boyer, et al., *Mol. Pharmacol.* **50**, 1323-1329 (1996)) properties toward P2Y₁ receptors. 2-MeSATP has a high potency for stimulation of inositol phosphate accumulation in membranes isolated from [³H]inositol-

labelled turkey erythrocytes. The analogues α,β -MeATP and β,γ -MeATP, which are potent as P2X receptor agonists, show little or no effect at the turkey erythrocyte P2Y₁ receptor.

P2Y₁ receptor-promoted stimulation of inositol phosphate formation by adenine nucleotide analogues described herein was measured in turkey erythrocyte membranes as previously described. T.K. Harden et al., *Biochem. J.* **252**, 583-593 (1988); J.L. Boyer et al., *J Biol Chem* **264**, 884-90 (1989). The K_{0.5} values were averaged from 3-8 independently determined concentration-effect curves for each compound. Erythrocyte ghost membranes were prepared from [³H]inositol-labeled cells by hypotonic lysis in 15 volumes of a buffer containing 5 mM sodium phosphate, pH 7.4, 5 mM MgCl₂, and 1 mM EGTA (lysis buffer). The membranes were washed three times by centrifugation and resuspension with lysis buffer. The final resuspension was in 20 mM Hepes, pH 7.0, to a concentration of 6 mg protein/mL. This preparation was used immediately for assay of phospholipase C. Twenty five µl of labeled membranes (approximately 150 µg protein; 200,000 cpm) was combined in a final volume of 200 µl of a medium containing 0.91 mM MgSO₄, 115 mM KCl, 5 mM potassium phosphate, 0.424 mM CaCl₂, 2 mM EGTA, 10 mM Hepes, pH 7.0 (free Ca⁺⁺ concentration was approximately 1 µM). Since receptor and G-protein-promoted activation of phospholipase C in turkey erythrocyte membranes is strictly dependent on the presence of guanine nucleotides, the non-hydrolyzable analogue of GTP, GTPγS (1 µM) was included in the assay. Membranes were incubated for 5 min at 30°C with the indicated concentrations of adenine nucleotide analogues. Incubations were terminated by addition of 1 mL of ice cold chloroform:methanol (1:2 by volume) followed by 350 µl of chloroform and 350 µl of water. Samples were mixed and centrifuged, and 1 mL of the aqueous upper phase was diluted with 8 mL of water and transferred onto Dowex AG-X8 columns (formate form). Columns were washed with 8 mL of 200 mM ammonium formate, 100 mM formic acid, and the eluant was discarded. Total inositol phosphates (IP₂ through IP₄) were eluted with 5 mL of 1.2 M ammonium formate, 100 mM formic acid, and collected in scintillation vials. [³H]Inositol phosphates were quantitated by scintillation spectroscopy.

Inositol phosphate accumulation in 1321N1 human astrocytoma cells expressing the cloned human P2Y₁, P2Y₂, P2Y₄, or the human or rat P2Y₆ receptors was measured as described previously in E. R. Lazarowski, *Br. J. Pharmacol.* **116**, 1619-1627 (1995).

Example 4

Data analysis

Agonist potencies were calculated using a four parameter logistic equation and the GraphPad software package (GraphPad, San Diego, CA). All concentration effect curves were repeated in at least three separate experiments using duplicate or triplicate assays.

Example 5

Activity of adenosine 3'-phosphate, 5'-phosphosulphate at the Turkey Erythrocyte P2Y receptor

The activity of adenosine 3'-phosphate, 5'-phosphosulphate at the turkey erythrocyte P2Y receptor was examined in order to determine whether sulfate-containing analogues of adenine nucleotides might be potent P2Y receptor agonists that are more resistant to hydrolysis by ectonucleotidases. Relatively small effects of adenosine 3'-phosphate, 5'-phosphosulphate on inositol phosphate formation occurred (**FIG. 1**), although detailed analyses illustrated that this stimulation was both concentration-dependent and apparently saturable with an EC_{50} of $0.83 \pm 0.08 \mu\text{M}$ (**FIG. 1** and **Table 2**). The maximal effect of adenosine 3'-phosphate, 5'-phosphosulphate relative to that of 2MeSATP, ATP, and ADP was somewhat variable, but typically ranged from 10% to 25% of the maximal full agonist effect (**Table 2**).

Table 2. Potency and efficacy of adenine nucleotide analogs on the P2Y receptor of turkey erythrocytes.

Nucleotide	EC ₅₀ (μM)	Relative Efficacy *
2MeSATP	0.013 ± 0.003	-
ADP	6.86 ± 1.51	1
A5PS	221 ± 61	1
adenosine 3'-phosphate, 5'-phosphosulphate	0.83 ± 0.08	0.17 ± 0.03
adenosine 3',5'-bisphosphate	2.23 ± 0.84	0.17 ± 0.05
adenosine 2',5'-bisphosphate	1.65 ± 0.35	0.21 ± 0.06

Each value represents the mean ± SEM of at least four experiments carried out with different membrane preparations.

* Relative efficacies of adenine nucleotides were determined by comparison with that produced by a maximal effective concentration of 2MeSATP in the same experiment.

The capacity of adenosine 3'-phosphate, 5'-phosphosulphate to augment or inhibit the effects of a submaximal concentration of 2MeSATP was also determined. adenosine 3'-phosphate, 5'-phosphosulphate antagonized the stimulatory effect of 10 nM 2MeSATP (**FIG. 2**) over the same concentration range necessary to observe the stimulatory effects of adenosine 3'-phosphate, 5'-phosphosulphate alone. This result suggests that the effect of adenosine 3'-phosphate, 5'-phosphosulphate occurred as a consequence of binding to the P2Y receptor rather than as a result of interaction with another of the components that comprise the receptor-regulated phospholipase C response.

Example 6

Interaction of adenosine 3'-phosphate, 5'-phosphosulphate with the P2Y receptor

To further assess the potential interaction of adenosine 3'-phosphate, 5'-phosphosulphate with the P2Y receptor, the concentration dependence of the effects of adenosine 3'-phosphate, 5'-phosphosulphate was examined over a broad range of

concentrations (1-1000 nM) of 2MeSATP (**FIG. 2**). adenosine 3'-phosphate, 5'-phosphosulphate produced a concentration-dependent antagonism of the effects of 2MeSATP at all concentrations of 2MeSATP above 3 nM (**FIG. 2**). Moreover, adenosine 3'-phosphate, 5'-phosphosulphate caused a concentration dependent parallel rightward shift of the concentration effect curve of 2MeSATP (**FIG. 3A**). Schild analysis (**FIG. 3B**) confirmed that the antagonism was apparently competitive, and the pK_B of adenosine 3'-phosphate, 5'-phosphosulphate was 6.46 ± 0.17 . This apparent potency of adenosine 3'-phosphate, 5'-phosphosulphate is 10-fold greater than that of ATP ($4.23 \pm 1.52 \mu\text{M}$) for stimulation of inositol lipid hydrolysis in the same membranes.

Example 7

Effect of sulfate substitution on antagonist activity

Activities of compounds with structures related to adenosine 3'-phosphate, 5'-phosphosulphate were examined to establish whether sulfate substitution at the 5' position of ATP or substitution at the 3' position is important for conferring antagonist activity. Antagonism appears to be unrelated to 5'-sulfate-substitution since adenosine 3',5'-bisphosphate also was a partial agonist with an EC_{50} for activation of $2.23 \pm 0.84 \mu\text{M}$ and a maximal effect that ranged from 4-35% of the maximal effect observed with 2MeSATP (**Table 2**). adenosine 3',5'-bisphosphate also caused a parallel rightward shift of the 2MeSATP concentration effect curve (**FIG. 3C**), and a calculated pK_B of 5.66 ± 0.21 was determined from Schild regressions with slopes not significantly different from unity (**FIG. 3D**). Thus, adenosine 3',5'-bisphosphate also appears to interact with the P2Y receptor with a potency that is greater than or equal to that of the parent ATP molecule. Effects identical to those of adenosine 3',5'-bisphosphate were observed with the isomer adenosine 2',5'-bisphosphate (**Table 2**).

Partial agonist activity also was observed with other adenosine 3'- and 2'-phosphate analogues such as adenosine 2'-phosphate 5'-phosphosulfate, and adenosine 2'-phosphate 5'-phosphoribose (data not shown). Adenosine 5'-phosphosulfate (**FIG. 1**) and adenosine 5'-diphosphoribose were full agonists (data not shown) indicating that substitution with phosphate in the 2'- or 3'- positions of adenine nucleotides is required

for antagonistic activity. Other 2' or 3' substitutions did not confer antagonistic properties to adenine nucleotides. For example, 3'-amino ATP and 3'-benzoylbenzoyl ATP were full agonists, and 3'-N-benzylamino ATP was a very weak agonist devoid of any antagonist activity (data not shown).

5

Example 8

Selectivity of adenosine 3'-phosphate, 5'-phosphosulphate and adenosine 3',5'-bisphosphate at Phospholipase C-coupled P2Y Receptor: Effects on β -adrenergic receptor

10 The selectivity of the effects of adenosine 3'-phosphate, 5'-phosphosulphate and adenosine 3',5'-bisphosphate was confirmed by examining their effects at the phospholipase C-coupled β -adrenergic receptor natively expressed in turkey erythrocytes. In contrast to the effects of adenosine 3'-phosphate, 5'-phosphosulphate and adenosine 3',5'-bisphosphate on 2MeSATP-stimulated inositol lipid hydrolysis, the
15 agonist effects of these 3'-phosphate-substituted analogues were additive to a maximally effective concentration of the β -adrenergic agonist isoproterenol (**FIG. 4**). These results indicate that 3'-phosphate adenine nucleotide analogues interact specifically with the P2Y receptor of turkey erythrocyte membranes and not with other components of the receptor-phospholipase C cascade.

20

Example 9

Effects of adenosine 3'-phosphate, 5'-phosphosulphate and Adenosine 3',5'-bisphosphate on Cloned P2Y₁ Receptor Expressed in Human Astrocytoma Cells

25 The effects of adenosine 3'-phosphate, 5'-phosphosulphate and adenosine 3',5'-bisphosphate were determined in 1321N1 human astrocytoma cells stably expressing the cloned receptors. Antagonist activities very similar to those observed with turkey erythrocyte membranes were observed with the expressed turkey P2Y₁ receptor (data not shown). In contrast to the results with the turkey erythrocyte P2Y receptor,
30 adenosine 3'-phosphate, 5'-phosphosulphate (data not shown) and adenosine 3',5'-bisphosphate (**FIG. 5**) exhibited no partial agonist activity and were simple competitive antagonists at the human P2Y₁ receptor. The calculated pK_B (6.05 ± 0.01) for adenosine 3',5'-bisphosphate was essentially the same as that observed in the turkey erythrocyte

membrane preparation (FIG. 5).

Example 10

Selectivity of adenosine 3'-phosphate, 5'-phosphosulphate and adenosine 3',5'-bisphosphate for the P₂Y₁ receptor: Effects at other P₂ receptors

The selectivity of adenosine 3'-phosphate, 5'-phosphosulphate and adenosine 3',5'-bisphosphate for the P₂Y₁ receptor was tested by examining their effects at other P₂ receptors. No effect of either compound as agonists or antagonists was observed at the P₂Y receptor of C6 rat glioma cells (FIG. 6). It has been previously shown that this adenylyl cyclase-linked P₂ receptor differs from the phospholipase C-coupled P₂Y₁ receptor in specificity of second messenger signaling and pharmacological selectivity (J. L. Boyer, et al., *Br. J. Pharmacol.* **116**, 2611-2616 (1995)). adenosine 3'-phosphate, 5'-phosphosulphate and adenosine 3',5'-bisphosphate also were neither antagonists nor agonists at the human P₂Y₂ (Table 3), the human P₂Y₄ (data not shown), or the rat P₂Y₆ (data not shown) receptors stably expressed in 1321N1 cells.

Table 3. Effect of adenosine 3'-phosphate, 5'-phosphosulphate and adenosine 3',5'-bisphosphate on the human P₂Y₂ receptor stably expressed in 1321N1 cells.

Nucleotide	Inositol Phosphates (cpm)
Basal	5534 ± 679
UTP	25730 ± 1445
adenosine 3'-phosphate, 5'-phosphosulphate	5129 ± 727
adenosine 3'-phosphate, 5'-phosphosulphate + UTP	34875 ± 7176
adenosine 3',5'-bisphosphate	4553 ± 458
adenosine 3',5'-bisphosphate + UTP	27952 ± 675

Data shown are the mean ± S.E.M. of values from at least three different experiments.

Example 11
Preparation of Deoxyadenosine Compounds 4-22

The general procedure for phosphorylation of nucleosides was as follows:

Nucleoside (0.1 mmol) and Proton Sponge[®] (107 mg, 0.5 mmol) were dried for several hours in high vacuum at room temperature and then suspended in 2 mL of trimethyl phosphate. Phosphorous oxychloride (37 μ L, 0.4 mmol) was added, and the mixture was stirred for 1 h at 0°C. The reaction was monitored by analytical HPLC (eluting with gradient from buffer:CH₃CN = 95:5 to 40:60; buffer: 0.1 M triethylammonium acetate (TEAA); elution time: 20 min; flow rate: 1 mL/min; column: SMT OD-5-60 RP-C18; detector: UV, Emax $\approx$ 260-300 nm). The reaction was quenched by adding 2 mL of triethylammonium bicarbonate buffer and 3 mL of water. The mixture was subsequently frozen and lyophilized. Purification was performed on an ion-exchange column packed with Sephadex DEAE A-25 resin, linear gradient (0.01 to 0.5M) of 0.5M ammonium bicarbonate was applied as the mobile phase, and UV and HPLC were used to monitor the elution. All nucleotides (**4 - 22**) were collected, frozen and lyophilized as the ammonium salts. The synthesis data, including ¹H-NMR and ³¹P-NMR results for compounds **4-22** are summarized in **Table 4**, below. The syntheses of the individual compounds is as follows:

2'-Deoxyadenosine-3',5'-bis(diammonium phosphate) (Compound 4)

Starting from 25 mg (0.099 mmol) of 2'-deoxyadenosine and following the general procedure, 21 mg (0.044 mmol, 44% yield) of the desired compound **4** was obtained.

¹H-NMR (D₂O) δ 2.79, (2H, m, CH₂-2'); 4.09, (2H, m, CH₂-5'); 4.47, (1H, bs, H4'); 5.02, (1H, m, H3'); 6.54, (1H, t, J = 6.9 Hz, H1'); 8.25, (1H, s, H2); 8.48, (1H, s, H8).

³¹P-NMR (D₂O) δ -0.127 (d, J = 18.27 Hz, P-3); 0.628, (pt, P-5').

2'-Deoxy-(N¹-methyl)adenosine-3',5'-bis(ammonium phosphate) (Compound 5)

Starting from 10 mg (0.025 mmol) of 2'-deoxy-N¹-methyladenosine and following the general procedure, 8.2 mg (0.017 mmol, 69% yield) of the desired compound **5** was obtained.

¹H-NMR (D₂O) δ 2.78 and 2.94, (2H, 2m, CH₂-2'); 3.92, (3H, s, N¹-CH₃); 4.08, (2H, bs,

CH₂-5'); 4.46, (1H, bs, H4'); 5.01, (1H, m H3'); 6.59, (1H, t, *J* = 6.8 Hz, H1'); 8.52, (1H, s, H2); 8.64, (1H, s, H8).

³¹P-NMR (D₂O) δ 0.693 (bs, P-3'); 1.176, (bs, P-5').

5 **2-Chloro-2'-deoxyadenosine-3',5'-bis(diammonium phosphate) (Compound 6)**

Starting from 25 mg (0.088 nmol) of 2-chloro-2'-deoxyadenosine and following the general procedure 26 mg (0.051 mmol 58% yield) of the desired compound 6 was obtained.

10 ¹H-NMR (D₂O) δ 2.77, (2H, m CH₂-2'); 4.04, (2H, m CH₂-5'); 4.42, (1H, bs, H4'); 4.94, (1H, m, H3'); 6.41, (1H, t, *J* = 6.9 Hz, H1'); 8.45, (1H, s, H8).

³¹P-NMR (D₂O) δ 1.436 (d, *J* = 18.45 Hz, P-3'); 1.820, (pt, P-5').

2'-Deoxy-2-methylthioadenosine-3',5'-bis(diammonium phosphate) (Compound 7)

15 Starting from 20 mg (0.067 mmol) of 2'-deoxy-2-methylthioadenosine and following the general procedure 17 mg (0.032 mmol 48% yield) of the desired compound 7 was obtained.

¹H-NMR (D₂O) δ 2.51, (3H, s, CH₃-S); 2.75 and 2.90, (2H, 2m, CH₂-2'); 4.07, (2H, m CH₂-5'); 4.42, (1H, bs, H4'); 5.00, (1H, m H3'); 6.44, (1H, t *J* = 6.8 Hz, H1'); 8.27, (1H, s, H8).

20 ³¹P-NMR (D₂O) δ -0.214 (d, *J* = 17.25 Hz, P-3'); 0.553, (pt, P-5').

8-Bromo-2'-deoxyadenosine-3',5'-bis(diammonium phosphate) (Compound 8)

25 Starting from 25 mg (0.076 mmol) of 8-bromo-2'-deoxyadenosine and following the general procedure 14 mg (0.025 mmol 33% yield) of the desired compound 8 was obtained.

¹H-NMR (D₂O) δ 2.65 and 3.44, (2H, 2m, CH₂-2'); 4.15, (2H, m CH₂-5'); 4.34, (1H, m, H4'); 5.11, (1H, m, H3'); 6.55, (1H, t, *J* = 6.8 Hz, H1'); 8.20, (1H, s, H2).

³¹P-NMR (D₂O) δ -0.109 (d, *J* = 18.09 Hz, P-3'); 0.652, (pt, P-5').

30 **2'-Deoxy-N⁶-methyladenosine-3',5'-bis(diammonium phosphate) (Compound 9)**

Starting from 25 mg (0.094 mmol) of 2'-deoxy-N⁶-methyladenosine and following the general procedure 19 mg (0.039 mmol 41% yield) of the desired

compound **9** was obtained.

¹H-NMR (D₂O) δ 2.77, (2H, m CH₂-2'); 2.99, (3H, s, NH-CH₃); 4.07, (2H, bs, CH₂-5'); 4.44, (1H, bs, H4'); 4.97, (1H, m, H3'); 6.40, (1H, t, *J* = 6.9 Hz, H1'); 8.07, (1H, s, H2); 8.32, (1H, s, H8).

5 ³¹P-NMR (D₂O) δ -0.129 (d, *J* = 16.95 Hz, P-3'); 0.623, (pst P-5').

2'-Deoxy-N⁶-ethyladenosine-3',5'-bis(diammonium phosphate) (Compound 10)

Starting from 12 mg (0.043 mmol) of 2'-deoxy-N⁶-ethyladenosine and following the general procedure 8.8 mg (0.017 mmol, 40% yield) of the desired compound **10** was obtained.

10

¹H-NMR (D₂O) δ 1.27, (3H, m CH₂CH₃); 2.77, (2H, m CH₂-2'); 3.57, (2H, m, NHCH₂); 4.00, (2H, bs, CH₂-5'); 4.40, (1H, bs, H4'); 4.89, (1H, m, H3'); 6.49, (1H, pt, H1'); 8.23, (1H, s, H2); 8.50, (1H, s, H8).

³¹P-NMR (D₂O) δ -0.128 (d *J* = 16.59 Hz, P-3'); 0.628, (pt, P-5').

15

2'-Deoxy-N⁶-propyladenosine-3',5'-bis(diammonium phosphate) (Compound 11)

Starting from 13 mg (0.044 mmol) of 2'-deoxy-N⁶-propyladenosine and following the general procedure 9.4 mg (0.018 mmol, 41% yield) of the desired compound **11** was obtained.

20

¹H-NMR (D₂O) δ 0.95, (3H, t, *J* = 6.8 Hz, CH₂CH₃); 1.65, (2H, q, *J* = 6.8, 6.8 Hz, CH₂CH₃); 2.76, (2H, m, CH₂-2'); 3.45, (2H, m, NHCH₂); 4.06, (2H, bs, CH₂-5'); 4.45, (1H, bs, H4'); 4.98, (1H, m, H3'); 6.48, (1H, t, *J* = 6.8 Hz, H1'); 8.17, (1H, s, H2); 8.39, (1H, s, H8).

³¹P-NMR (D₂O) δ -0.03 (d, *J* = 18.09 Hz, P-3'); 0.670, (pt, P-5').

25

N⁶-Benzoyl-2'-deoxyadenosine-3',5'-bis(diammonium phosphate) (Compound 12)

Starting from 25 mg (0.070 mmol) of N⁶-benzoyl-2'-deoxyadenosine and following the general procedure, 22 mg (0.038 mmol, 54% yield) of the desired compound **12** was obtained.

30

¹H-NMR (D₂O) δ 2.85, (2H, m, CH₂-2'); 3.96, (2H, bs, CH₂-5'); 4.43, (1H, bs, H4'); 4.84, (1H, m, H3'); 6.62, (1H, pt H1'); 7.46, (2H, d, *J* = 6.8 Hz, Ph); 7.57, (1H, d, *J* = 6.8 Hz, Ph); 7.88, (2H, d, *J* = 6.8 Hz, Ph); 8.66, (1H, s, H2); 8.81, (1H, s, H8).

³¹P-NMR (D₂O) δ 3.285 (d, *J* = 16.26 Hz, P-3'); 3.765, (pt, P-5').

2'-Deoxy-N⁶-dimethyladenosine-3',5'-bis(diammonium phosphate) (Compound 13)

Starting from 18 mg (0.064 mmol) of 2'-deoxy-N⁶-dimethyladenosine and following the general procedure, 15 mg (0.029 mmol 46% yield) of the desired compound **13** was obtained.

¹H-NMR (D₂O) δ 2.75, (2H, m, CH₂-2'); 3.23, (6H, s, N(CH₃)₂); 4.04, (2H, m, CH₂-5'); 4.42, (1H, bs, H4'); 4.95, (1H, m, H3'); 6.38, (1H, t, *J*=6.8 Hz, H1'); 7.96, (1H, s, H2); 8.28, (1H, s, H8).

³¹P-NMR (D₂O) δ 0.403 (bs, P-3'); 1.017, (bs, P-5').

6-Chloro-2'-deoxypurineriboside-3',5'-bis(diammonium phosphate) (Compound 14)

Starting from 10 mg (0.037 mmol) of 6-chloro-2'-deoxypurineriboside and following the general procedure, 12 mg (0.024 mmol 65% yield) of the desired compound **14** was obtained.

¹H-NMR (D₂O) δ 2.85, (2H, m, CH₂-2'); 4.00, (2H, bs, CH₂-5'); 4.43, (1H, bs, H4'); 4.91, (1H, m, H3'); 6.65, (1H, t, *J*=6.8 Hz, H1'); 8.72, (1H, s, H2); 8.93, (1H, s, H8).

³¹P-NMR. (D₂O) δ 1.954 (d, *J* = 18.09 Hz, P-3'); 2.253, (pt, P-5').

2'-Deoxyinosine-3',5'-bis(diammonium phosphate) (Compound 15)

Starting from 25 mg (0.079 mmol) of 2'-deoxyinosine and following the general procedure, 8.4 mg (0.017 mmol, 22% yield) of the desired compound **15** was obtained.

¹H-NMR (D₂O) δ 2.85, (2H, m CH₂-2'); 4.09, (2H, bs, CH₂-5'); 4.47, (1H, bs, H4'); 5.01, (1H, bs, H3'); 6.55, (1H, t, *J*=6.8 Hz, H1'); 8.22, (1H, s, H2); 8.51, (1H, s, H8).

³¹P-NMR (D₂O) δ -0.234 (d, *J* = 17.25 Hz, P-3'); 0.543, (pt, P-5').

2'-Deoxy-6-methylthiopurineriboside-3',5'-bis(diammoniumphosphate) (Compound 16)

Starting from 18 mg (0.064 mmol) of 2'-deoxy-6-methylthiopurineriboside and following the general procedure, 13 mg (0.025 mmol, 39% yield) of the desired compound **16** was obtained.

¹H-NMR (D₂O) δ 2.66, (3H, s, SCH₃); 2.85, (2H, m, CH₂-2'); 4.03, (2H, d, *J* = 3.9 Hz, CH₂-5'); 4.44, (1H, s, H4'); 4.94, (1H, m, H3'); 6.58, (1H, t, *J* = 6.8 Hz, H1'); 8.60, (1H, s, H2); 8.69, (1H, s, H8).

³¹P-NMR (D₂O) δ 1.588 (d, *J* = 17.76 Hz, P-3'); 1.942, (pt, P-5').

5

**3'-Deoxyadenosine-2',5'-bis(diammonium phosphate) (Compound 17) and
5'-chloro-3'-deoxyadenosine-2'-(diammonium phosphate) (Compound 19)**

Starting from 25 mg (0.099 mmol) of 3'-deoxyadenosine and following the general procedure, 16 mg (0.033 mmols, 34% yield) of desired compound **17** was obtained, and 7.2 mg (0.021 mmol, 21% yield) of 5'-chloro derivative **19** was obtained. as a by-product.

10

17: ¹H-NMR (D₂O) δ 2.42, (2H, m, CH₂-3'); 3.98, (1H, m, CH₂-5'); 4.18, (1H, m, CH₂-5'); 4.75, (1H, bs, H4'); 5.08, (1H, m, H2'); 6.22, (1H, s, H1'); 8.14, (1H, s, H2); 8.41, (1H, s, H8).

15

³¹P-NMR (D₂O) δ 0.337 (d, *J* = 18.12 Hz, P-3'); 1.220, (t, *J* = 11.89 Hz, P-5').

19: ¹H-NMR (D₂O) δ 2.51, (2H, m, CH₂-3'); 3.88, (2H, d_{ABQ}, *J* = 22.5, 12.9, 3.9 Hz, CH₂-5'); 4.84, (1H, bs, H4'); 5.23, (1H, m, H2'); 6.25, (1H, s, H1'); 8.20, (1H, s, H2); 8.33, (1H, s, H8).

³¹P-NMR (D₂O) δ 0.312 (d, *J* = 18.27 Hz, P-3').

20

3'-Deoxy-N⁶-methyladenosine-2',5'-bis(diammonium phosphate) (Compound 18)

Starting from 25 mg (0.094 mmol) of 3'-deoxy-N⁶-methyladenosine and following the general procedure 10.4 mg (0.021 mmol, 22% yield) of the desired compound **18** was obtained.

25

¹H-NMR (D₂O) δ 2.41, (2H, m, CH₂-3'); 3.05, (3H, s, NH-CH₃); 4.01, (1H, m, CH₂-5'); 4.20, (1H, m, CH₂-5'); 4.72, (1H, bs, H4'); 5.07, (1H, m, H2'); 6.22, (1H, s, H1'); 8.20, (1H, s, H2); 8.36, (1H, s, H8).

³¹P-NMR (D₂O) δ -0.098 (d, *J* = 19.60 Hz, P-3'); 0.900, (pt, P-5').

30

2'-Deoxy-2'-O-methyladenosine-3',5'-bis(diammonium phosphate) (Compound 20)

Starting from 10 mg (0.036 mmol) of 2'-deoxy-2'-O-methyladenosine and following the general procedure, 3 mg (0.006 mmol 17% yield) of the desired

compound **20** was obtained.

¹H-NMR (D₂O) δ 3.49, (3H, s, OCH₃); 4.08, (2H, m, CH₂-5'); 4.57, (2H, bs, H2'+H4'); 4.92, (1H, m, H3'); 6.22, (1H, d, *J*=5.9 Hz, H1'); 8.25, (1H, s, H2); 8.59, (1H, s, H8).

³¹P-NMR (D₂O) δ 1.470 (d, *J* = 19.44 Hz, P-3'); 2.016, (pst, P-5').

5

2'-Deoxyadenosine-3',5'-bis(diammonium thiophosphate) (Compound 21)

Starting from 50 mg (0.2 mmol) of 2'-deoxyadenosine and following the general procedure, but using thiophosphoryl chloride (81 μL, 0.8 mmol), 18 mg (0.036 mmol, 18% yield) of the desired compound **21** was obtained.

10

¹H-NMR (D₂O) δ 2.42, (1H, m, CH₂-2'); 2.61, (1H, m, CH₂-2'); 3.83, (2H, pt, CH₂-5'); 4.11, (1H, m, H4'); 4.55, (1H, m, H3'); 6.21, (1H, t, *J*=6.8 Hz, H1'); 7.89, (1H, s, H2); 8.32, (1H, s, H8).

³¹P-NMR (D₂O) δ 37.716 (pd, P-3'); 43.637, (pt, P-5').

15

2'-Deoxy-N¹,N⁶-ethenoadenosine-3',5'-bis(diammonium phosphate) (Compound 22)

Starting from 25 mg (0.091 mmol) of 2'-deoxy-N¹,N⁶-ethenoadenosine and following the general procedure 19.3 mg (0.038 mmol 42% yield) of the desired compound **22** was obtained.

20

¹H-NMR (D₂O) δ 2.88 (2H, m, CH₂-2'); 4.10, (2H, m, CH₂-5'); 4.46, (1H, s, H4'); 5.02, (1H, m, H3'); 6.51, (1H, t *J*=6.8 Hz, H1'); 7.46, (1H, bs, etheno); 7.84, (1H, bs, etheno); 8.43, (1H, s, H2); 8.93, (1H, s, H8).

³¹P-NMR (D₂O) δ 0.113 (d *J* = 16.59 Hz, P-3'); 0.779, (pt, P-5').

Table 4. Synthetic data for nucleotide derivatives, including structural verification using high resolution mass spectroscopy and purity verification using HPLC.

No.	Formula	FAB (M-H ⁺)		HPLC (rt; min) ^a		Yields (%)
		Calcd	Found	System A	System B	
4	C ₁₀ H ₁₅ O ₉ N ₅ P ₂	410.0267	410.0249	5.98	11.51	44
5	C ₁₁ H ₁₇ O ₉ N ₅ P ₂	424.0423	424.0428	3.66	6.42	69
6	C ₁₀ H ₁₄ ClO ₉ N ₅ P ₂	443.9877	443.9872	7.20	12.14	58
7	C ₁₁ H ₁₇ O ₉ N ₅ P ₂ S	456.0144	456.0122	8.14	12.98	48
8	C ₁₀ H ₁₄ BrO ₉ N ₅ P ₂	487.9372	487.9355	6.66	12.06	33
9	C ₁₁ H ₁₇ O ₉ N ₅ P ₂	424.0423	424.0404	6.93	12.32	41
10	C ₁₂ H ₁₉ O ₉ N ₅ P ₂	438.0580	438.0580	7.95	12.83	40
11	C ₁₃ H ₂₁ O ₉ N ₅ P ₂	452.0736	452.0734	8.97	13.55	41
12	C ₁₇ H ₁₉ O ₁₀ N ₅ P ₂	514.0529	514.0538	9.58	13.67	54
13	C ₁₂ H ₁₉ O ₉ N ₅ P ₂	438.0580	438.0582	8.30	12.72	46
14	C ₁₀ H ₁₃ ClO ₉ N ₄ P ₂	428.9768	428.9752	6.87	12.54	65
15	C ₁₀ H ₁₄ O ₁₀ N ₅ P ₂	411.0107	411.0102	4.58	11.64	22
16	C ₁₁ H ₁₆ O ₉ N ₄ P ₂ S	441.0035	441.0026	8.48	13.05	39
17	C ₁₀ H ₁₅ O ₉ N ₅ P ₂	410.0267	410.0262	5.80	11.51	34
18	C ₁₁ H ₁₇ O ₉ N ₅ P ₂	424.0423	424.0437	8.95	12.16	21
19	C ₁₀ H ₁₃ ClO ₅ N ₅ P	348.0265	348.0272	6.89	9.82	22
20	C ₁₁ H ₁₇ O ₁₀ N ₅ P ₂	440.0372	440.3660	6.40	12.20	17
21	C ₁₀ H ₁₅ O ₇ N ₅ P ₂ S ₂	441.9810	441.9794	6.60	10.10	18
22	C ₁₂ H ₁₅ O ₉ N ₅ P ₂	434.0267	434.0257	7.02	12.33	42

^apurity of each derivative was > 95%, as determined using HPLC with two different mobile phases.

System A: gradient of 0.1M TEAA/CH₃CN from 95/5 to 40/60 and system B: gradient of 5nM TBAP/CH₃CN from 80/20 to 40/60.

^bThe percent yields refer to phosphorylation reactions.

Example 12
Biological Activity of Deoxyadenosine -Based Compounds

The deoxyadenosine nucleoside analogues prepared according to Example 11 above were tested for agonist and antagonist activity in the Phospholipase C (PLC) assay at the P2Y₁ receptor in turkey erythrocyte membranes. Concentration-response curves were obtained for each compound alone, and in combination with a given concentration of 2-MeSATP (10 nM). In the case of all the compounds tested, essentially no basal inositol phosphate activity was observed, and a very small stimulation occurred in the presence of 1 μ M GTP γ S (data not shown). Addition of 10nM 2-MeSATP resulted in a marked and concentration-dependent activation of the turkey erythrocyte phospholipase C.

The removal of the unphosphorylated hydroxyl group from adenosine 3',-5'-bisphosphate and adenosine 2',3'-bisphosphate resulted in compounds **4** and **17**, respectively. These compounds are similar to their parent hydroxylated compounds in biological activity, *i.e.* they were both partial agonists with slightly less potency as agonists (5- to 6-fold) at P2Y₁ receptors (**FIG. 7**), but were equipotent as antagonists. The 3',5'-bisphosphate analogue, **4**, was two-fold more potent as an antagonist compared to the 2',5' isomer, **17**. Thus, both 2'- and 3'-deoxy modifications were tolerated in P2Y₁ receptor antagonists, and the potencies of each were approximately equal to the corresponding hydroxy analogue.

Modifications of these two lead structures were then studied. Structural modifications were made at the adenine ring at the 1- (Compound **5**), 2- (Compounds **6** and **7**), 8- (Compound **8**), and 6- (Compounds **9- 16** and **18**) positions, on the ribose moiety (Compounds **19,20**), and on the phosphate groups (Compound **21**). Additional modifications of the purine moiety such as etheno (Compound **22**) and N¹-methyl (Compound **5**) were also made.

Table 4. In vitro pharmacological data for stimulation of PLC at turkey erythrocyte P2Y₁ receptors (agonist effect) and the inhibition of PLC stimulation elicited by 10 nM 2-MeSATP (antagonist effect), for at least three separate determinations.

Compound	Agonist Effect (% of maximal increase) ^a	EC ₅₀ (μM) ^a	Antagonist Effect (% of maximal inhibition) ^b	IC ₅₀ (μM) ^b
adenosine 3',5'- bisphosphate	21 ± 5	1.28 ± .028	77 ± 6	4.19 ± 1.22
adenosine 2',5'- bisphosphate	25 ± 8	1.37 ± 0.31	75 ± 8	8.46 ± 0.46
4	12 ± 3	6.26 ± 2.52	87 ± 4	5.76 ± 0.68
5	NE		100	54.9 ± 20.0
6	19 ± 3	0.651 ± 0.160	80 ± 3	2.01 ± 0.83
7	22 ± 2	0.550 ± 0.117	77 ± 2	2.11 ± 0.95
8	NE		100	36.7 ± 8.6
9	NE		99 ± 1	0.330 ± 0.059
10	NE		100	1.08 ± 0.38
11	NE		Small inhib. at 30-100 μM	
12	NE		NE	
13	NE		70 ± 2	46.7 ± 20.8
14	Small or NE		Small inhib. or NE	
15	NE		Small inhib. at 30-100 μM	
16	NE		78 ± 1	29.1 ± 4.1
17	16 ± 6	8.33 ± 2.22	81 ± 6	11.0 ± 4.8
18	13 ± 2	NC	90 ± 1	0.324 ± 0.054
19	NE		NE	
20	35 ± 7	12.9 ± 3.4	65 ± 7	12.4 ± 2.9
21	9 ± 3	NC	91 ± 3	88.0 ± 30.3
22	NE		NE	
23	NE		NE	
24	≈ 50	≥ 100	≈ 50	≥ 100
25	43	40.9	73 ± 5	12.7 ± 3.2
26	43 ± 3	≥ 100	72 ± 6	73.0 ± 15.0

^aAgonist potencies were calculated using a four-parameter logistic equation and the GraphPad software package (GraphPad, SanDiego,CA). EC₅₀ values (mean ± standard error) represent the concentration at which 50% of the maximal effect is achieved. Relative efficacies (%) were determined by comparison with the effect produced by a maximal effective concentration of 2-MeSATP in the same experiment.

^bAntagonist IC₅₀ values (mean ± standard error) represent the concentration needed to inhibit by 50% the effect elicited by 10 nM 2-MeSATP. The percent of maximal inhibition is equal to 100 minus the residual fraction of stimulation at the highest antagonist concentration.

NE no effect

NC not calculated

The 2-chloro-, (Compound 6), and 2-methylthio-, (Compound 7) modifications of compound 4 were nearly 3-fold more potent as P2Y₁ antagonists. In both cases the residual agonist stimulation of PLC, as observed for compounds 1-4 at the P2Y₁ receptor, was still present. The potency of compounds 6 and 7 in activating the P2Y₁ receptor was also enhanced, by approximately 2-fold. The 8-bromo modified analogue, 8, was 6.4-fold less potent than 4 as an antagonist at the P2Y₁ receptor, and agonist activity was abolished. An N¹-methyl analogue, 5, was a weak antagonist (9.5-fold loss of potency vs. 4) with no agonist activity.

Alkylation of the exocyclic amine had highly consequential effects on biological activity. N⁶-Me-2'-deoxyadenosine 3', 5'-bisphosphate, 9, was considerably more potent (IC₅₀ value 330 nM) as an antagonist than either deoxyadenosine 3', 5'-bisphosphate (17-fold), 4, or adenosine 3', 5'-bisphosphate (13-fold), 1 (FIG. 8A). This analogue displayed only antagonistic properties, since in the absence of 2-MeSATP it did not activate PLC at a concentration as high as 100 μM. A dramatic dependence of the antagonist potency on the size of the N⁶-substituent was observed (FIG. 8). The methyl analogue, 9, was 3-fold more potent as an antagonist than the ethyl analogue, 10. In compound 11 the N⁶-propyl group completely abolished both agonist and antagonist properties. Similarly, the longer chain terminating in an amino group in compound 23 (containing also 3'-hydroxyl and thus an analogue of compound 3) completely abolished interaction with the P2Y₁ receptor. Compound 18, the 3'-deoxy isomer of 9, also acted as an antagonist at the turkey P2Y₁ receptor and was approximately equipotent to 9. Compound 18 showed a small activation of the receptor at high concentration. The N⁶-benzoyl modification, 12, completely abolished the ability of the compound to interact with P2Y₁ receptors as either an agonist or an antagonist.

Double alkylation of the N⁶ amino group in compound 13 resulted in a ≥ 300-fold loss of potency as an antagonist vs 9, and no agonist activity was observed. 6-Chloro and 6-hydroxy analogues, 14 and 15, respectively, were essentially inactive at P2Y₁ receptors. The 6-methylthio-analogue, 16, was only a very weak antagonist at P2Y₁ receptors.

Replacement of the 5'-phosphate ester with chloro, as in compound **19**, abolished antagonist and agonist activity at P2Y₁ receptors. The presence of a 2'-methoxy group on the ribose moiety, **20**, decreased potency by 2.2-fold vs **4**, although a restoration of the agonist effect to a very substantial fraction (33% of maximal) was achieved. Replacement of both phosphate groups with thiophosphate, **21**, diminished potency as a P2Y₁ receptor antagonist by 15-fold. The etheno derivative **22** was inactive at the P2Y₁ receptor.

Example 13

Competitive Antagonist Activity of Compound 9

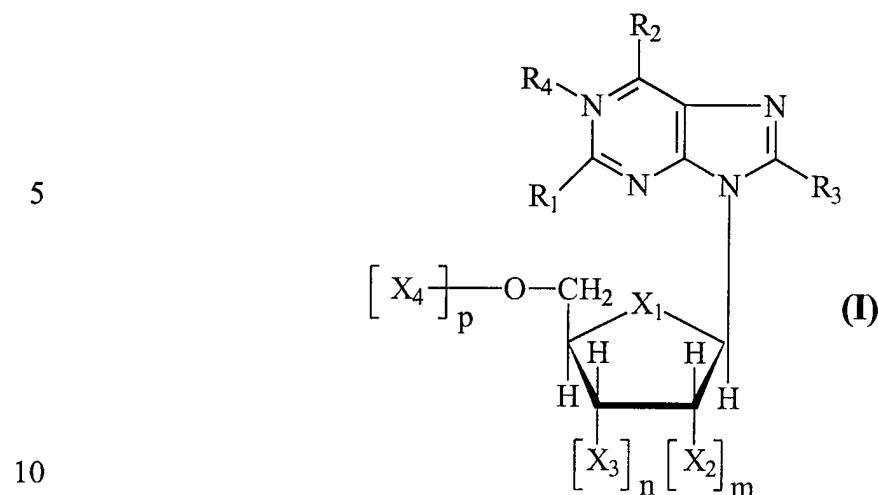
The compound 2'-Deoxy-N⁶-methyladenosine-3',5'-bisphosphate (compound **9**) was determined to be a potent and competitive antagonist of the P2Y₁ receptor. Referring now to **FIG. 9**, log dose response curves of the P2Y₁ agonist 2MeSATP were determined in the presence of a control (circles), or 0.1 μM (diamonds), 0.3 μM (triangles), 1 μM (squares), 3 μM (asterisks), 10 μM (crosses) and 30 μM (X-es) of Compound **9**. The parallel shifting of the agonist log dose response curve to the right illustrates the competitive antagonist activity of Compound **9**. Data in **FIG. 9** is shown in terms of [³H] Inositol Phosphates (percentage of maximum) as a function of the log Molar concentration of 2MeSATP. **FIG. 10** is the Schild regression of the data presented in **FIG. 9**. The calculation of the ratio of the agonist (2MeSATP) concentration that elicits equal responses in the absence and presence of antagonist (Compound **9**) at increasing concentrations (termed the dose ratio) was plotted according to the relationship log(dose ratio-1) vs. log concentration (in Molar) of Compound **9**. In that the slope of the line produced by the Schild regression is approximately equal to 1 (slope = 0.912), the compound **9** is indicated to be a competitive antagonist of the P2Y₁ receptor. **FIG. 11** illustrates the selective binding and specificity of Compound **9** to the P2Y₁ receptor. Inositol phosphate accumulation in 1321N1 human astrocytoma cells expressing the cloned, human P2Y₁ (far left), P2Y₂ (second from left), P2Y₄ (second from right) or P2Y₆ (far right) receptor was measured in the absence (open bars) or presence (filled bars) of Compound **9**. While the presence of Compound **9** had no effect on the accumulation of inositol phosphates in cells expressing P2Y₂ and P2Y₆, and had the slight effect of increasing the amount of inositol

phosphates detected in cells expressing the P2Y₄ receptor, the presence of Compound 9 completely inhibited the generation of inositol phosphates in cells expressing P2Y₁. This result indicates that Compound 9 binds selectively to the P2Y₁ receptor, and further that the compound is a potent antagonist of the P2Y₁ receptor.

- 5 The foregoing Examples are illustrative of the present invention, and are not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

That Which is Claimed:

1. A compound according to Formula I:



wherein:

- m is 1, 2, or 3;
- n is 1, 2, or 3;
- p is 0 or 1;
- 15 q is 0, 1, 2, or 3, with the proviso that when p is zero, q is not zero;
- R₁ is selected from the group consisting of H, lower alkyl, halo, alkoxy, and alkylthio;
- R₂ is selected from the group consisting of hydroxy, halo, alkylthio, lower alkyl, substituted lower alkyl, and -NR'R'', wherein R' and R'' are each independently selected from the group consisting of H, lower
- 20 alkyl, aroyl, alkoxy, alkoxy, and alkoxyalkyl;
- R₃ is selected from the group consisting of H, halo, and lower alkyl;
- R₄ is selected from the group consisting of H and lower alkyl,
- X₁ is selected from the group consisting of O, S, N, and CH₂; and
- 25 X₂, X₃ and X₄ are each independently selected from the group consisting H, hydroxy, amino, lower alkyl, halo, alkoxy, phosphate, thiophosphate, carboxylate, and nitro, or X₂ and X₃ together are cyclophosphate;
- and the pharmaceutically acceptable salts thereof, with the proviso that:
- when R₁, R₃, and R₄ are each H, and m, n, and p are each 1, and q is 1, 2, or 3,
- 30 and X₁ is O, and X₄ is phosphate, and X₂ and X₃ are each either hydroxy, H or phosphate or together are cyclophosphate, R₂ is not -NH₂.

2. A compound according to Claim 1, wherein p and q are each 1.
3. A compound according to Claim 1, wherein n is 1 and X_3 is H.
- 5 4. A compound according to Claim 1, wherein m is 1 and X_2 is H.
5. A compound according to Claim 1, wherein R_2 is $-NR'R''$, and wherein R' and R'' are each lower alkyl.
- 10 6. A compound according to Claim 1, wherein R_2 is $-NR'R''$, and wherein R' is H and R'' is lower alkyl.
7. A compound according to Claim 6, wherein R'' is methyl.
- 15 8. A compound according to Claim 1, wherein R_3 is halo.
9. A compound according to Claim 1, wherein p is 0, q is 1, and X_4 is Cl.
10. A compound according to Claim 1, wherein R_2 is selected from the
20 group consisting of aroyl, halo, hydroxy and alkylthio.
11. A compound according to Claim 1, wherein R_1 is selected from the
group consisting of halo and alkylthio.
- 25 12. A compound according to Claim 1, wherein X_2 is alkoxy.
13. A compound according to Claim 1, wherein X_3 and X_4 are each selected
from the group consisting of thiophosphate and phosphate .
- 30 14. A compound according to Claim 1 selected from the group consisting of
2'-Deoxy-(N¹-methyl)adenosine-3',5'-bisphosphate; 2-Chloro-2'-deoxyadenosine-3',5'-
bisphosphate; 2'-Deoxy-2-methylthioadenosine-3',5'-bisphosphate; 8-Bromo-2'-

deoxyadenosine-3',5'-bisphosphate; 2'-Deoxy-N⁶-methyladenosine-3',5'-bisphosphate;
 2'-Deoxy-N⁶-ethyladenosine-3',5'-bisphosphate; 2'-Deoxy-N⁶-propyladenosine-3',5'-
 bisphosphate; N⁶-Benzoyl-2'-deoxyadenosine-3',5'-bisphosphate; 2'-Deoxy-N⁶-
 dimethyladenosine-3',5'-bisphosphate; 6-Chloro-2'-deoxypurineriboside-3',5'-
 5 bisphosphate; 2'-Deoxyadenosine-3',5'-bisphosphate; 2'-Deoxy-6-
 methylthiopurineriboside-3',5'-bisphosphate; 5'-chloro-3'-deoxyadenosine-2'-phosphate;
 3'-Deoxy-N⁶-methyladenosine-2',5'-bisphosphate; 2'-Deoxy-2'-O-methyladenosine-3',5'-
 bisphosphate; 2'-Deoxyadenosine-3',5'-bis(thiophosphate),
 2'-Deoxy-N¹,N⁶-ethenoadenosine-3',5'-bisphosphate, and the pharmaceutically
 10 acceptable salts thereof.

15. A compound according to Claim 1 selected from the group consisting of
 2'-Deoxy-(N¹-methyl)adenosine-3',5'-bis(ammonium phosphate); 2-Chloro-2'-
 deoxyadenosine-3',5'-bis(diammonium phosphate); 2'-Deoxy-2-methylthioadenosine-
 15 3',5'-bis(diammonium phosphate); 8-Bromo-2'-deoxyadenosine-3',5'-bis(diammonium
 phosphate); 2'-Deoxy-N⁶-methyladenosine-3',5'-bis(diammonium phosphate); 2'-Deoxy-
 N⁶-ethyladenosine-3',5'-bis(diammonium phosphate); 2'-Deoxy-N⁶-propyladenosine-
 3',5'-bis(diammonium phosphate); N⁶-Benzoyl-2'-deoxyadenosine-3',5'-bis(diammonium
 phosphate); 2'-Deoxy-N⁶-dimethyladenosine-3',5'-bis(diammonium phosphate); 6-
 20 Chloro-2'-deoxypurineriboside-3',5'-bis(diammonium phosphate); 2'-Deoxyinosine-3',5'-
 bis(diammonium phosphate); 2'-Deoxy-6-methylthiopurineriboside-3',5'-
 bis(diammonium phosphate); 5'-chloro-3'-deoxyadenosine-2'-(diammonium phosphate);
 3'-Deoxy-N⁶-methyladenosine-2',5'-bis(diammonium phosphate); 2'-Deoxy-2'-O-
 methyladenosine-3',5'-bis(diammonium phosphate); 2'-Deoxyadenosine-3',5'-
 25 bis(diammonium thiophosphate) and
 2'-Deoxy-N¹,N⁶-ethenoadenosine-3',5'-bis(diammonium phosphate).

16. A compound according to claim 1, wherein said compound has
 antagonist activity at the P2Y₁ receptor.

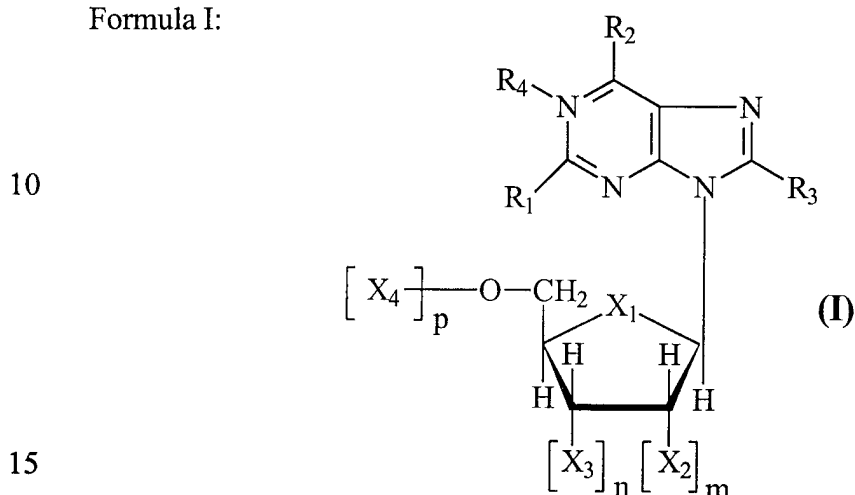
30

17. A compound according to claim 1, wherein said compound has
 competitive antagonist activity at the P2Y₁ receptor.

18. A compound according to claim 1, wherein said compound binds selectively to the P2Y₁ receptor.

19. A method of detecting the P2Y₁ receptor in a biological sample suspected of containing said P2Y₁ receptor, the method comprising:

contacting said biological sample with a compound according to Formula I:



wherein:

m is 1, 2, or 3;

n is 1, 2, or 3;

p is 0 or 1;

20 q is 0, 1, 2, or 3, with the proviso that when p is zero, q is not zero;

R₁ is selected from the group consisting of H, lower alkyl, halo, alkoxy, and alkylthio;

25 R₂ is selected from the group consisting of hydroxy, halo, alkylthio, lower alkyl, substituted lower alkyl, and -NR'R'', wherein R' and R'' are each independently selected from the group consisting of H, lower alkyl, aroyl, alkoxy, alkoxy, and alkoxyalkyl;

R₃ is selected from the group consisting of H, halo, and lower alkyl;

R₄ is selected from the group consisting of H and lower alkyl,

30 X₁ is selected from the group consisting of O, S, N, and CH₂; and

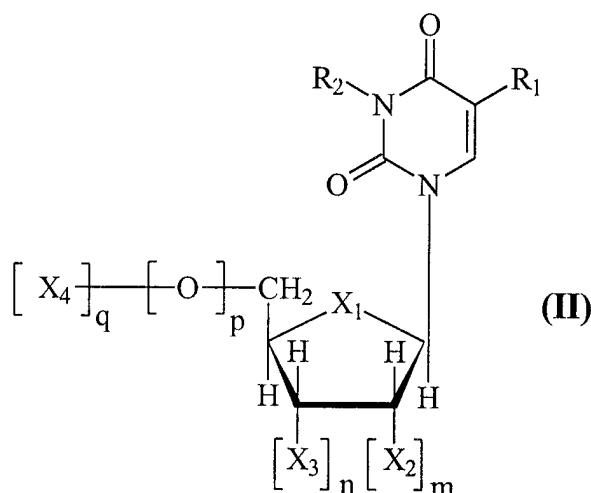
X₂, X₃ and X₄ are each independently selected from the group consisting H, hydroxy, amino, lower alkyl, halo, alkoxy, phosphate, thiophosphate, carboxylate, and nitro, or X₂ and X₃ together are cyclophosphate;

and the pharmaceutically acceptable salts thereof, wherein said compound of Formula I selectively binds the P2Y₁ receptor; and then detecting the presence or absence of binding of said P2Y₁ receptor antagonist to a receptor in said biological sample, the presence of binding indicating the presence of the P2Y₁ receptor.

20. A method according to Claim 19, wherein said compound of Formula I is selected from the group consisting of adenosine 3',5'-bisphosphate and adenosine 2',5'-bisphosphate.

21. A method according to Claim 19, wherein said compound is 2'-Deoxy-N⁶-methyladenosine-3',5'-bisphosphate.

22. A compound according to Formula II:



wherein:

m is 1, 2, or 3;

n is 1, 2, or 3;

p is 0 or 1;

q is 0, 1, 2, or 3, with the proviso that when p is zero, q is not zero;

R₁ is selected from the group consisting of H, lower alkyl, and halo; R₂ is

selected from the group consisting of H, and lower alkyl;

X₁ is selected from the group consisting of O, S, N and CH₂; and

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X_2 , X_3 and X_4 are each independently selected from the group consisting H, hydroxy, lower alkyl, amino, halo, alkoxy, phosphate, thiophosphate, carboxylate, and nitro, or X_2 and X_3 together are cyclophosphate; and the pharmaceutically acceptable salts thereof, with the proviso that:

5 when R_1 and R_2 are H, and p, m, and n are each 1, and q is 1, 2 or 3, and X_1 is O, neither X_2 nor X_3 are H, neither X_2 and X_3 are $-OH$, and X_2 and X_3 together are not cyclophosphate.

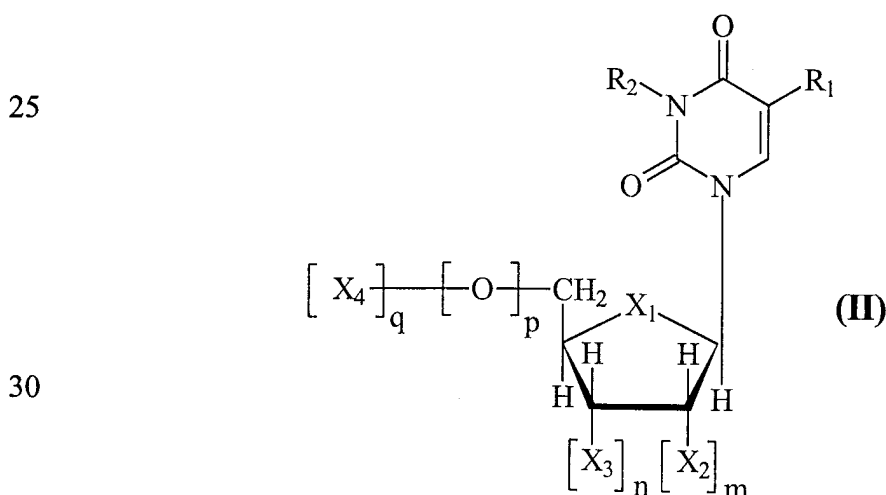
10 23. A compound according to claim 22, wherein p is 0, q is 1, and X_4 is Cl.

24. A compound according to claim 22, wherein said compound has antagonist activity at a P2Y receptor.

15 25. A compound according to claim 22, wherein said compound has competitive antagonist activity at a P2Y receptor.

26. A compound according to claim 22, wherein said compound binds selectively to a P2Y receptor.

20 27. A method of detecting a P2Y receptor in a biological sample suspected of containing a P2Y receptor, comprising;
contacting said biological sample with a compound according to Formula II:



wherein:

m is 1, 2, or 3;

n is 1, 2, or 3;

p is 0 or 1;

5 q is 0, 1, 2, or 3, with the proviso that when p is zero, q is not zero;

R₁ is selected from the group consisting of H, lower alkyl, and halo; R₂ is selected from the group consisting of H, and lower alkyl;

X₁ is selected from the group consisting of O, S, N and CH₂; and

10 X₂, X₃ and X₄ are each independently selected from the group consisting H, hydroxy, lower alkyl, amino, halo, alkoxy, phosphate, thiophosphate, carboxylate, and nitro, or X₂ and X₃ together are cyclophosphate, and the pharmaceutically acceptable salts thereof, wherein said compound of Formula II selectively binds a P2Y receptor; and then

15 detecting the presence or absence of binding of said P2Y receptor antagonist to a receptor in said biological sample, the presence of binding indicating the presence of the P2Y receptor.

28. A method according to Claim 27, wherein said P2Y receptor is the P2Y₂ receptor.

20 29. A method according to Claim 27, wherein said P2Y receptor is the P2Y₄ receptor.

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FIG. 1

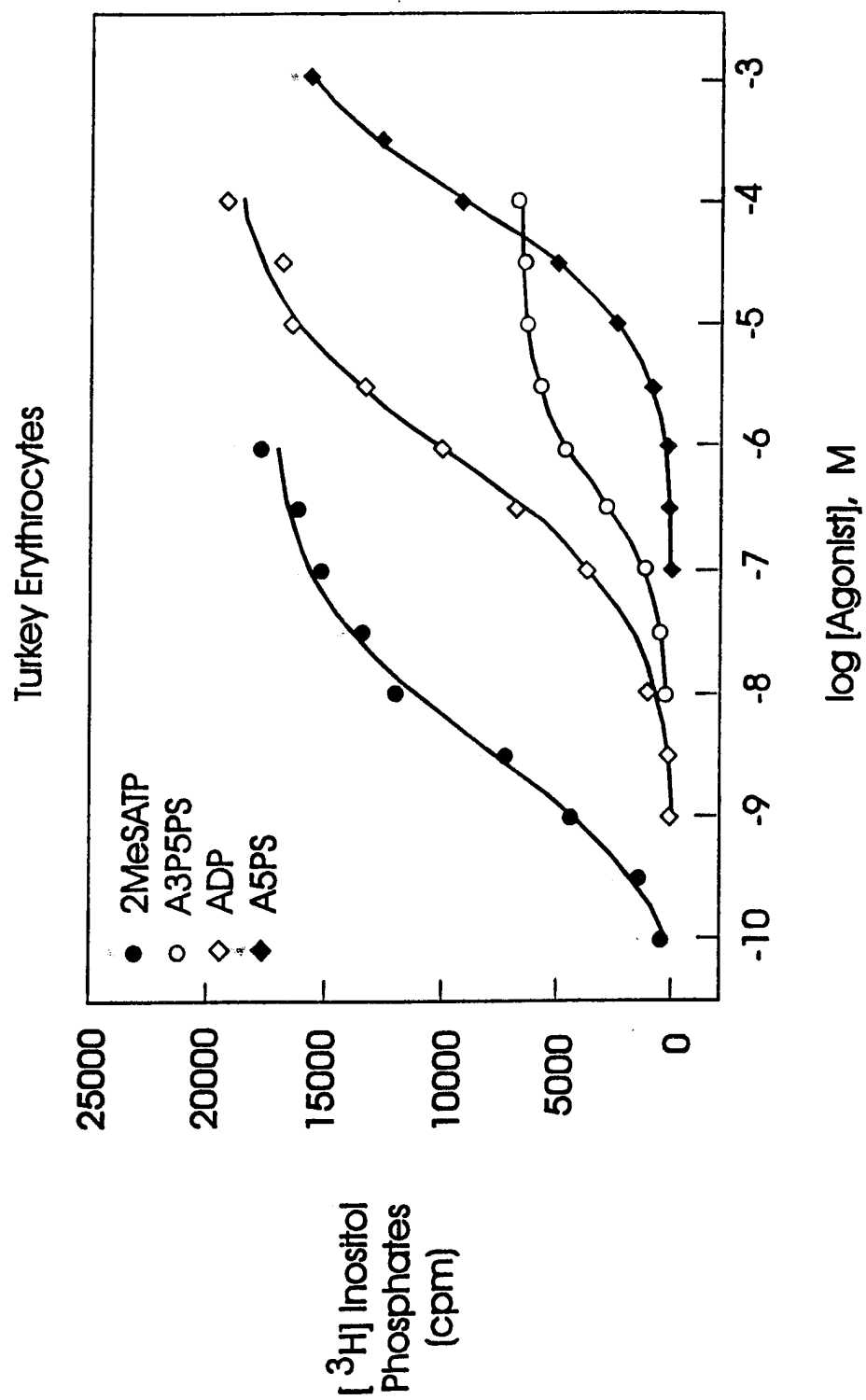


FIG. 2

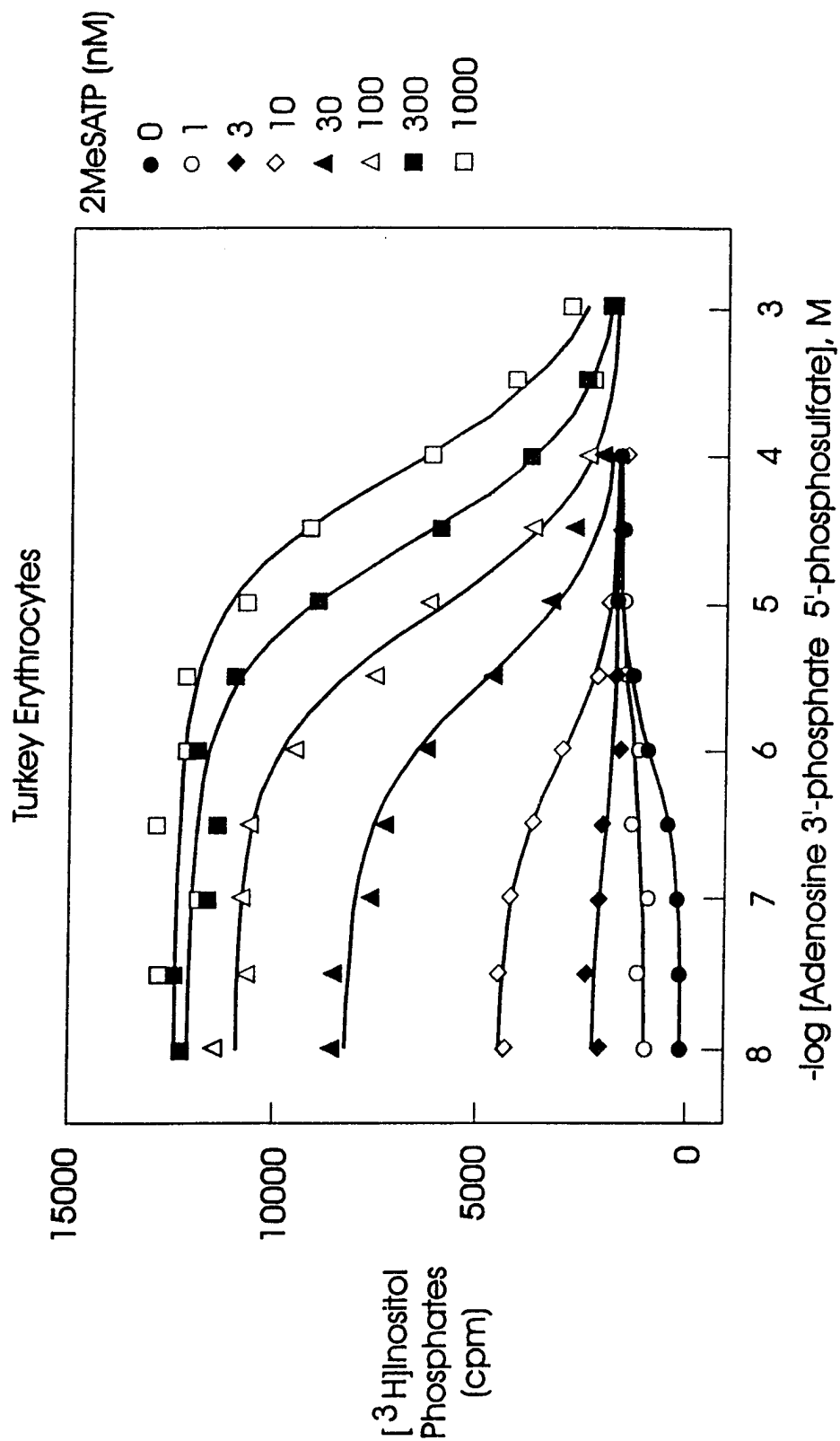


FIG. 3A

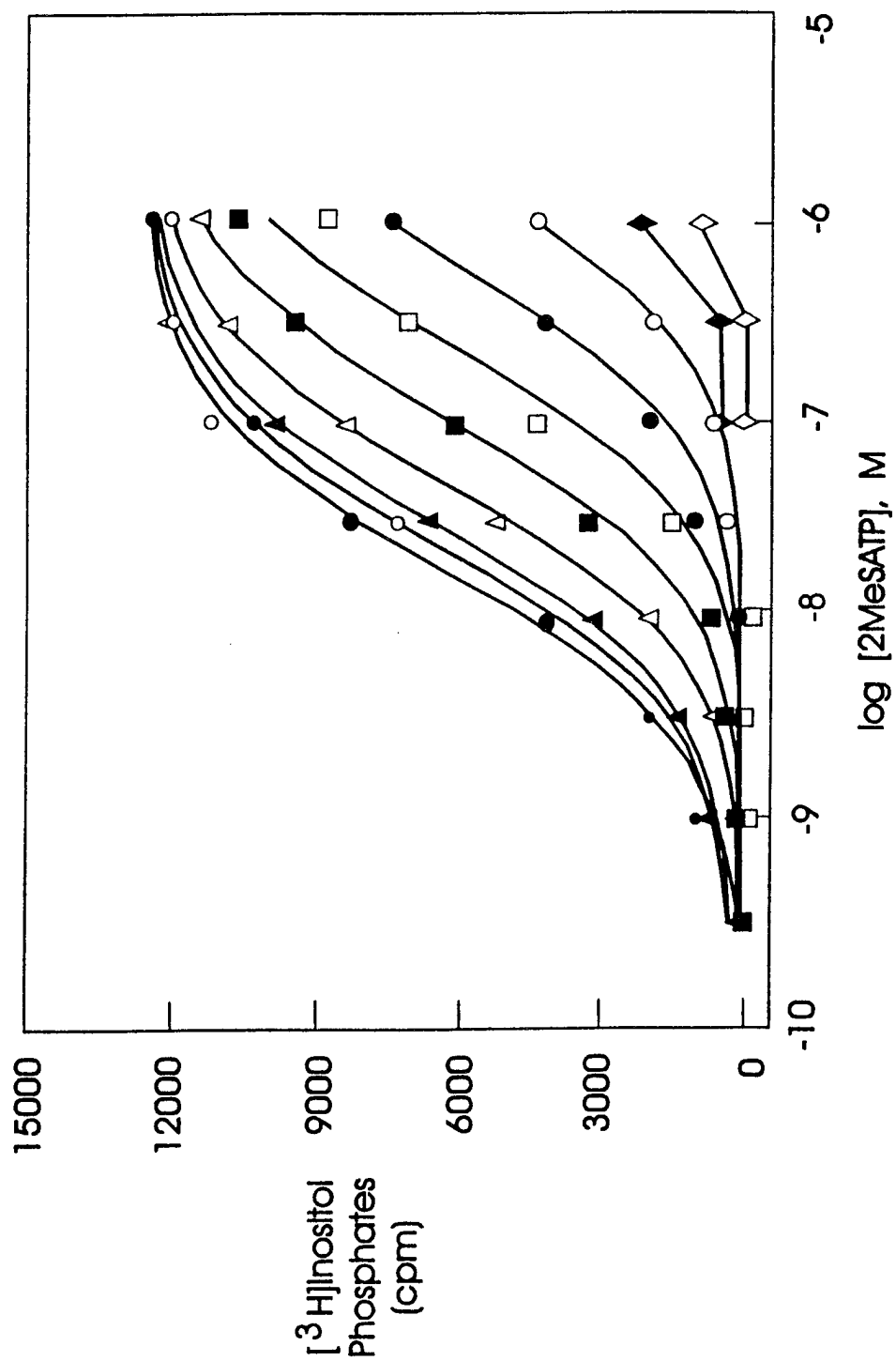


FIG. 3B

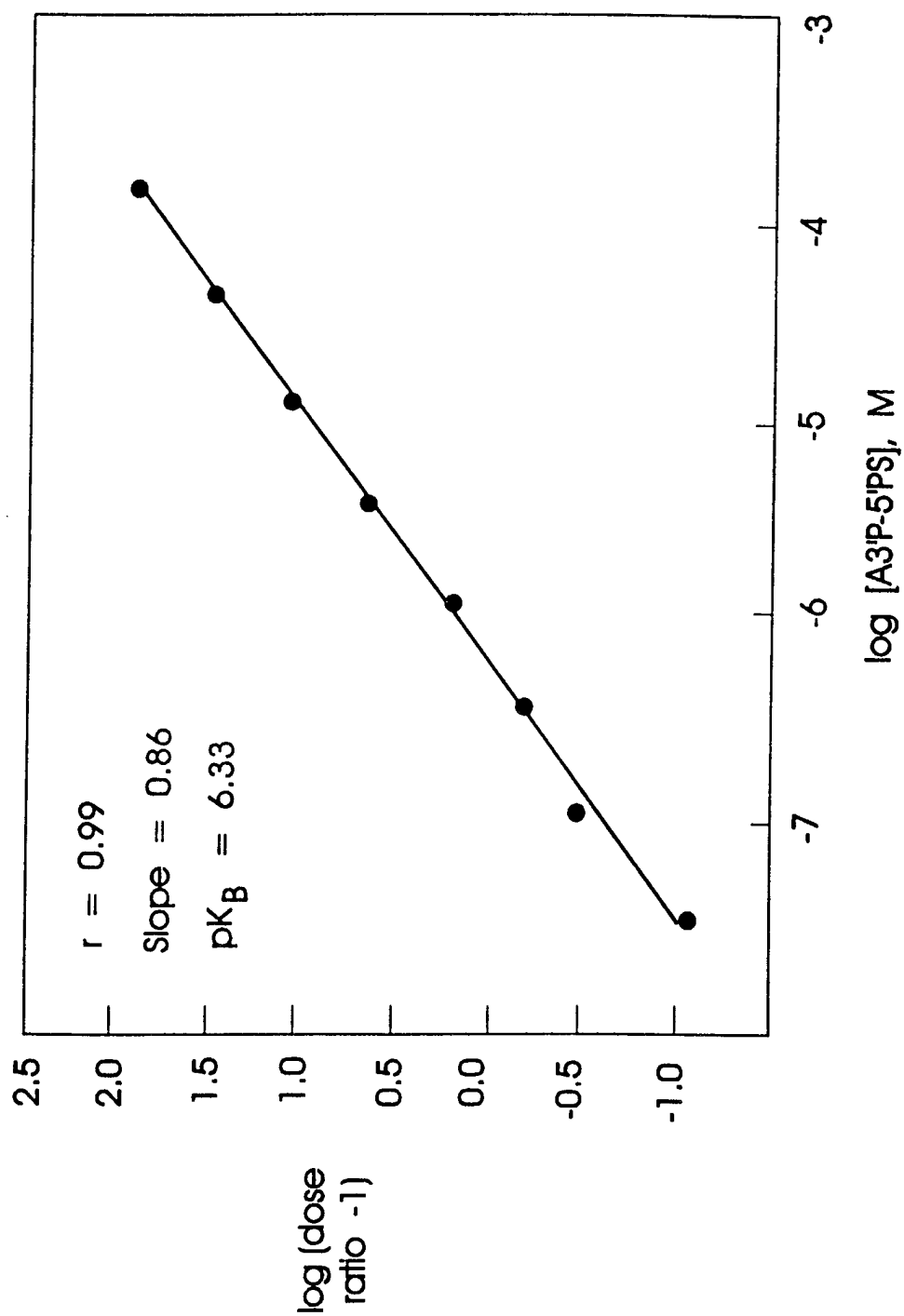


FIG. 3C

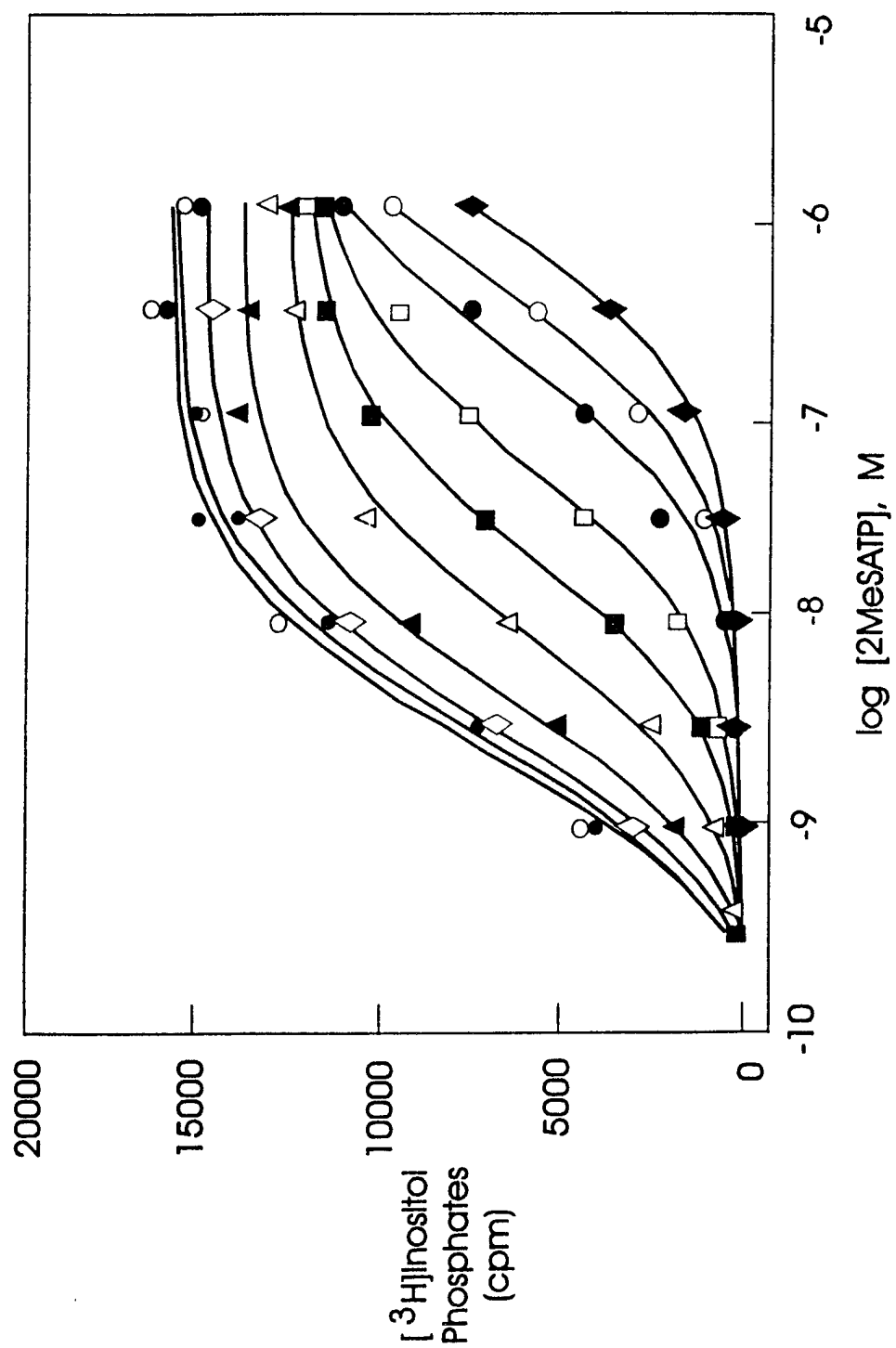
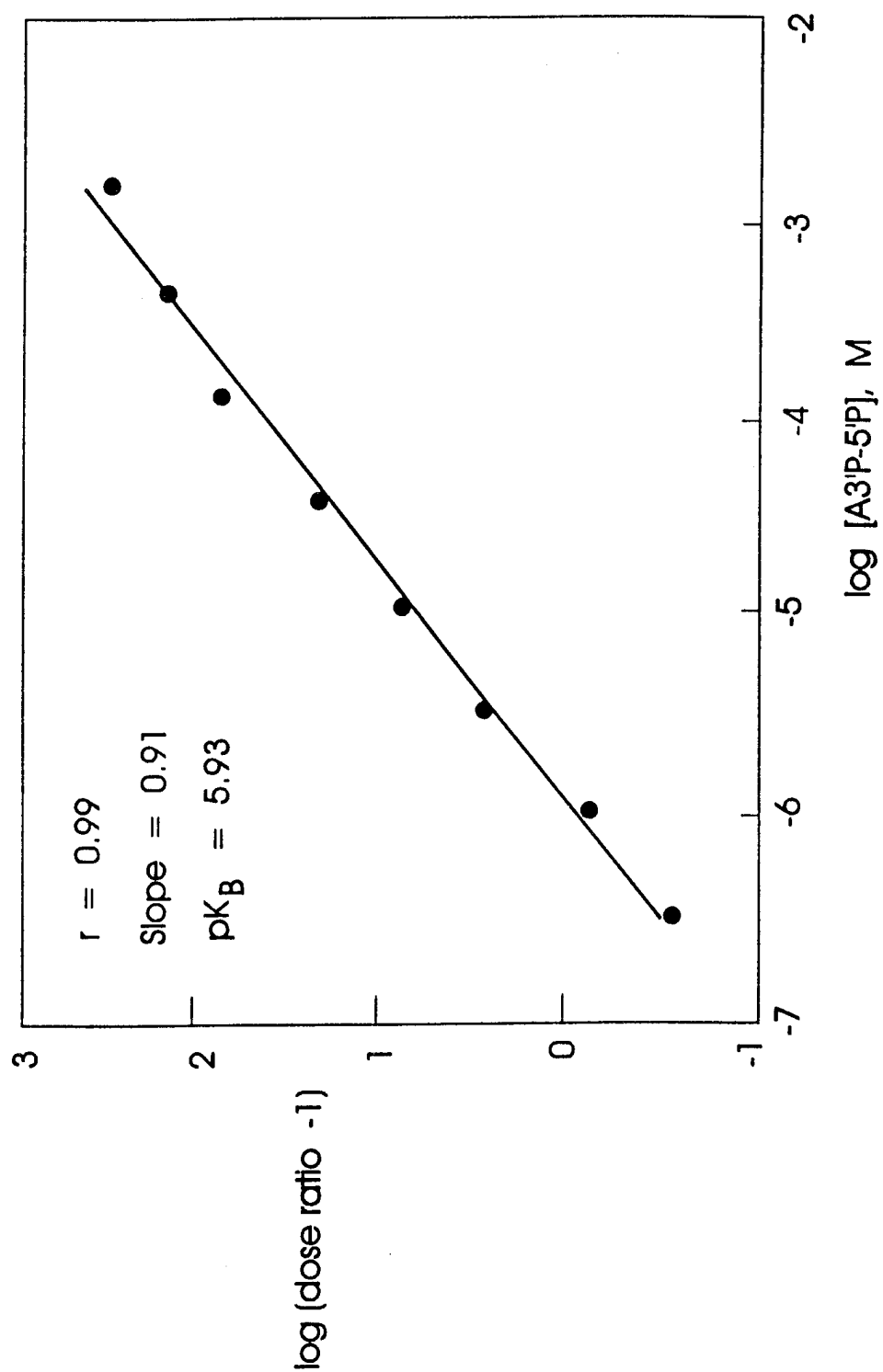
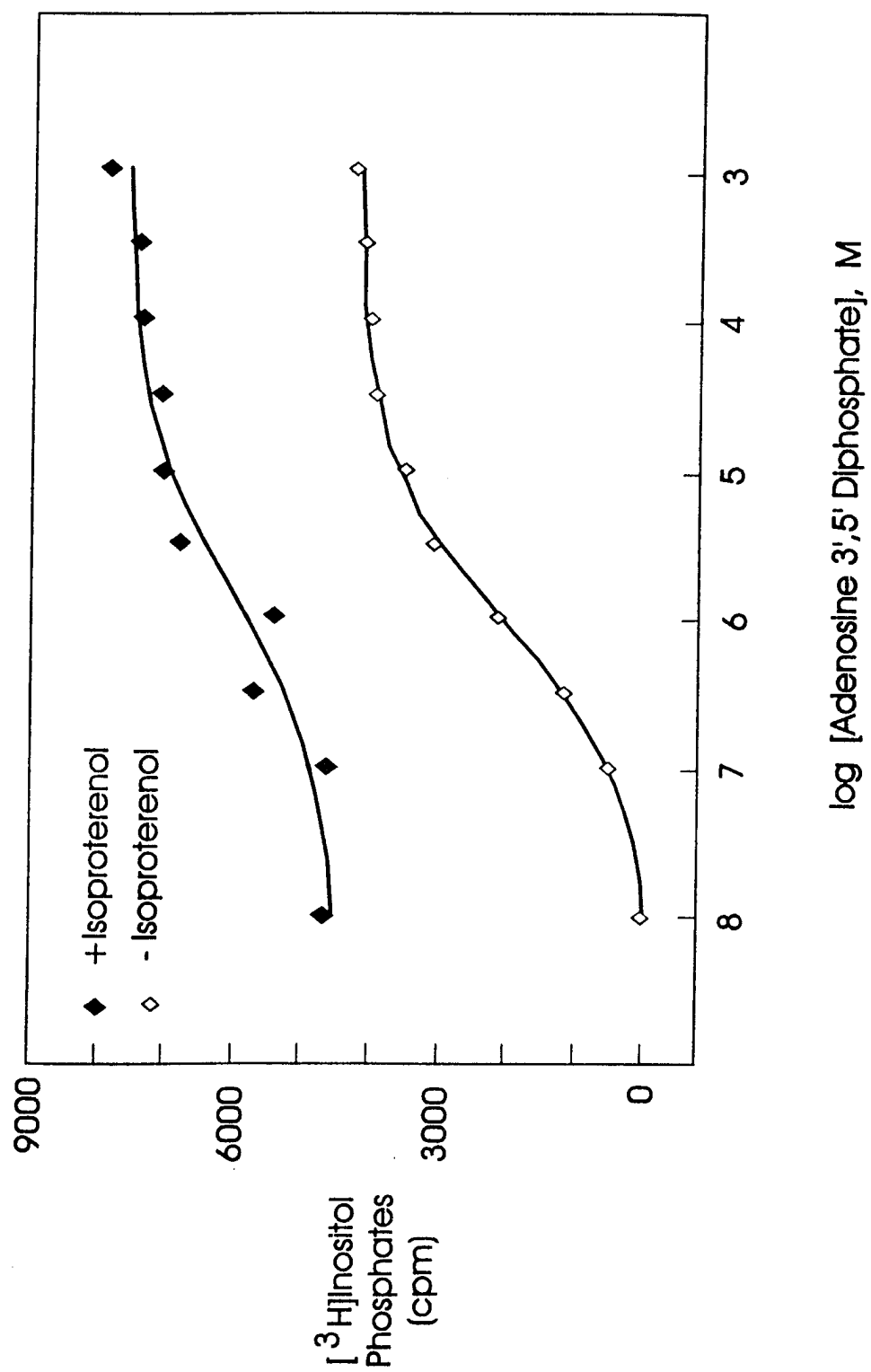


FIG. 3D



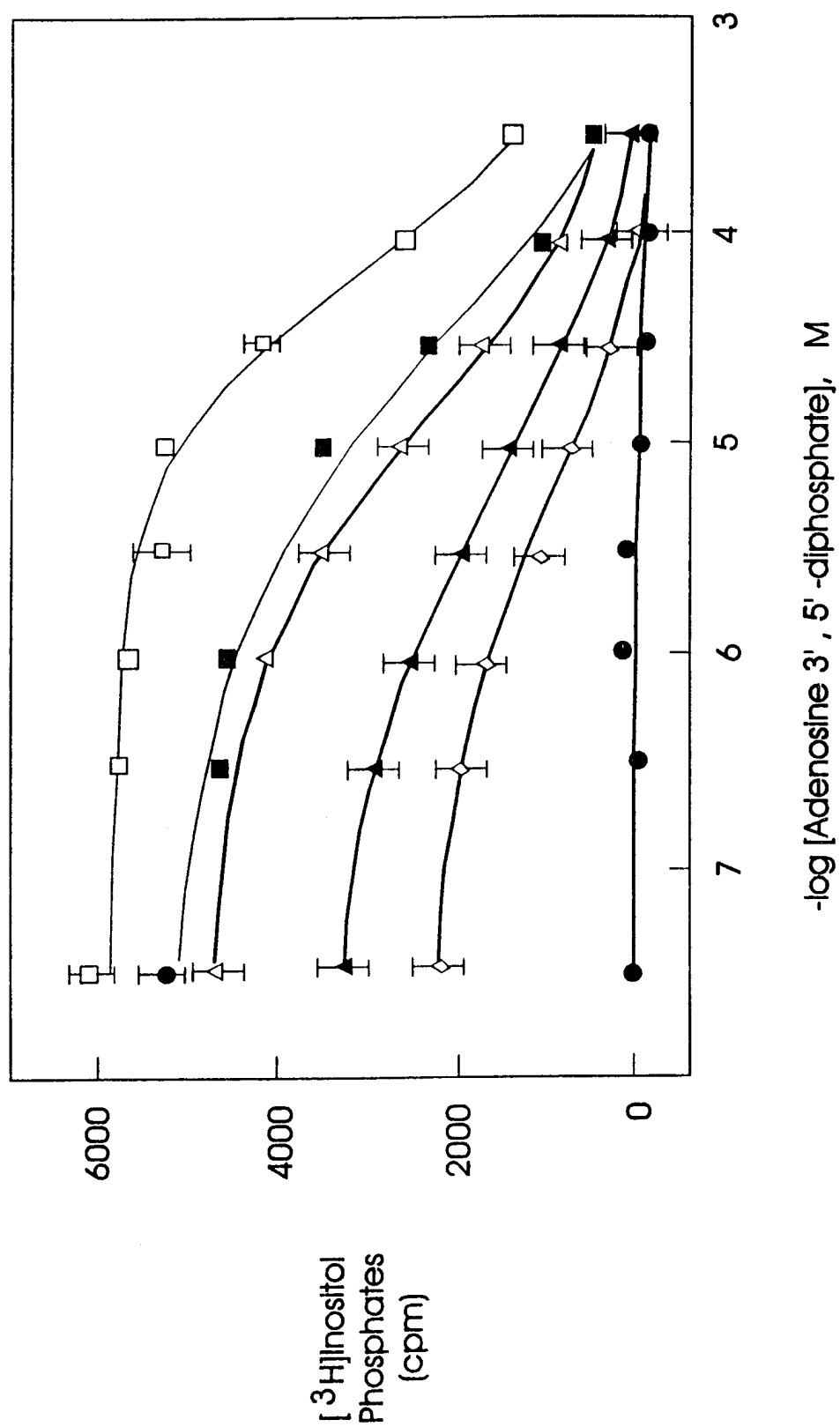
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FIG. 4



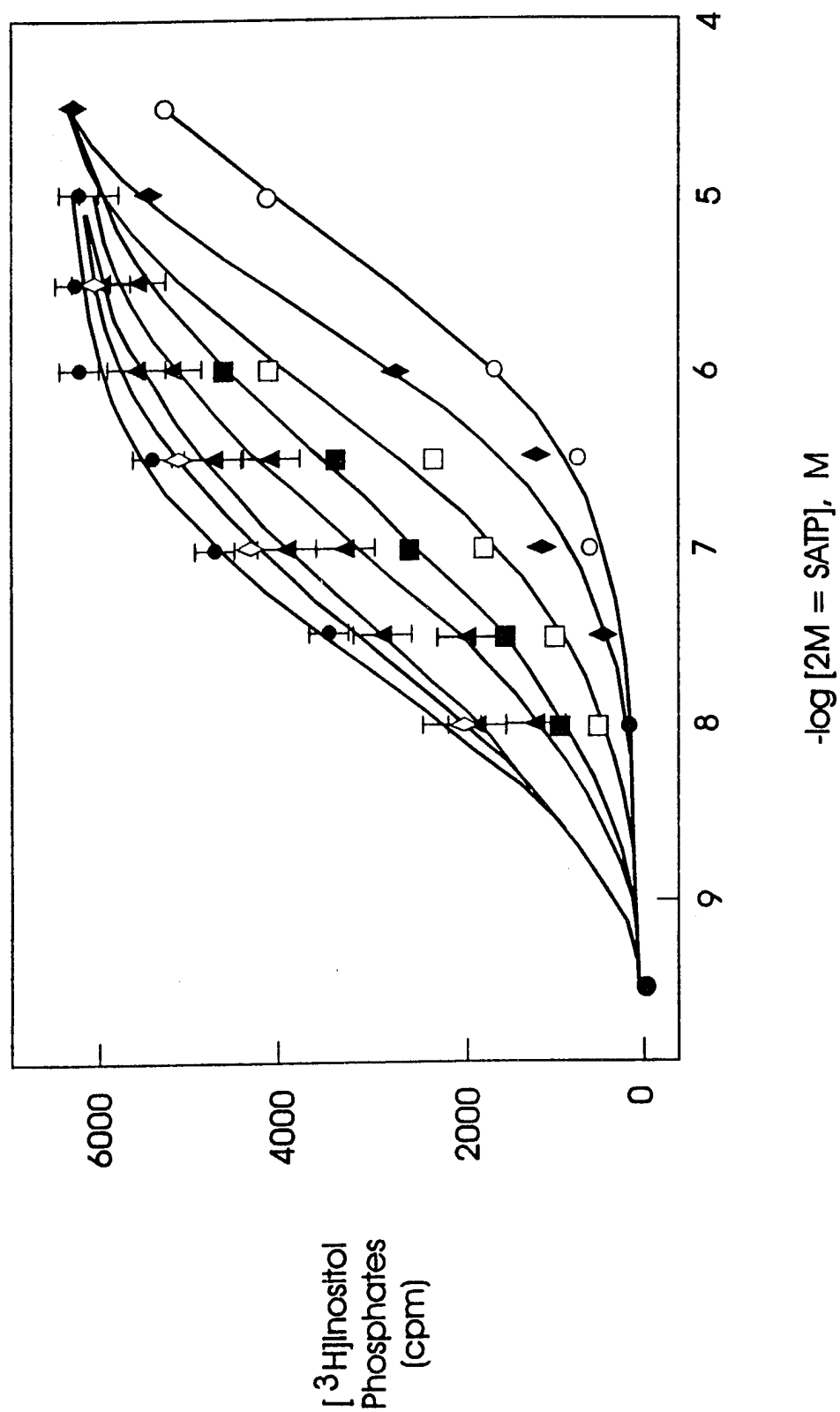
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FIG. 5A



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FIG. 5B



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FIG. 5C

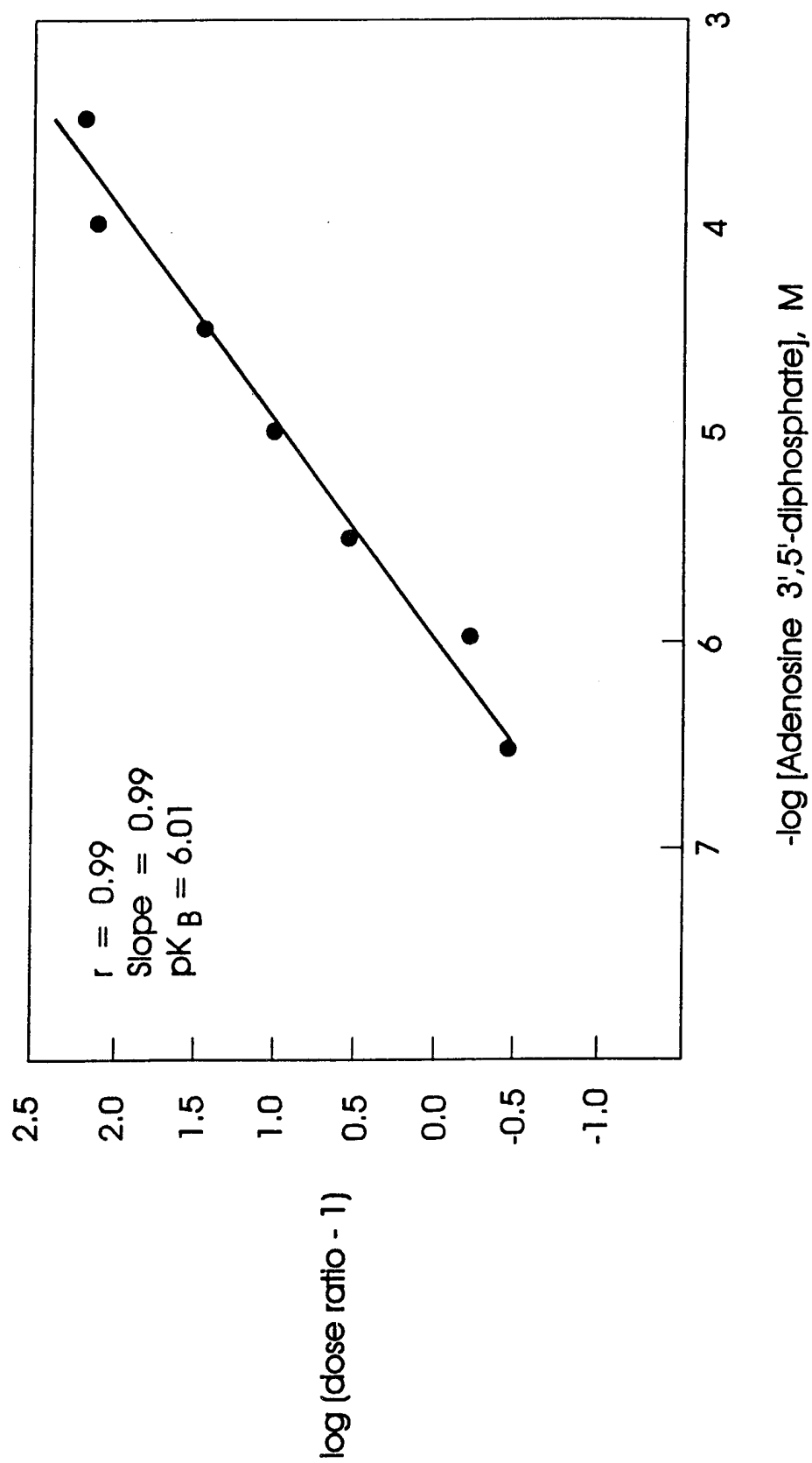


FIG. 6A

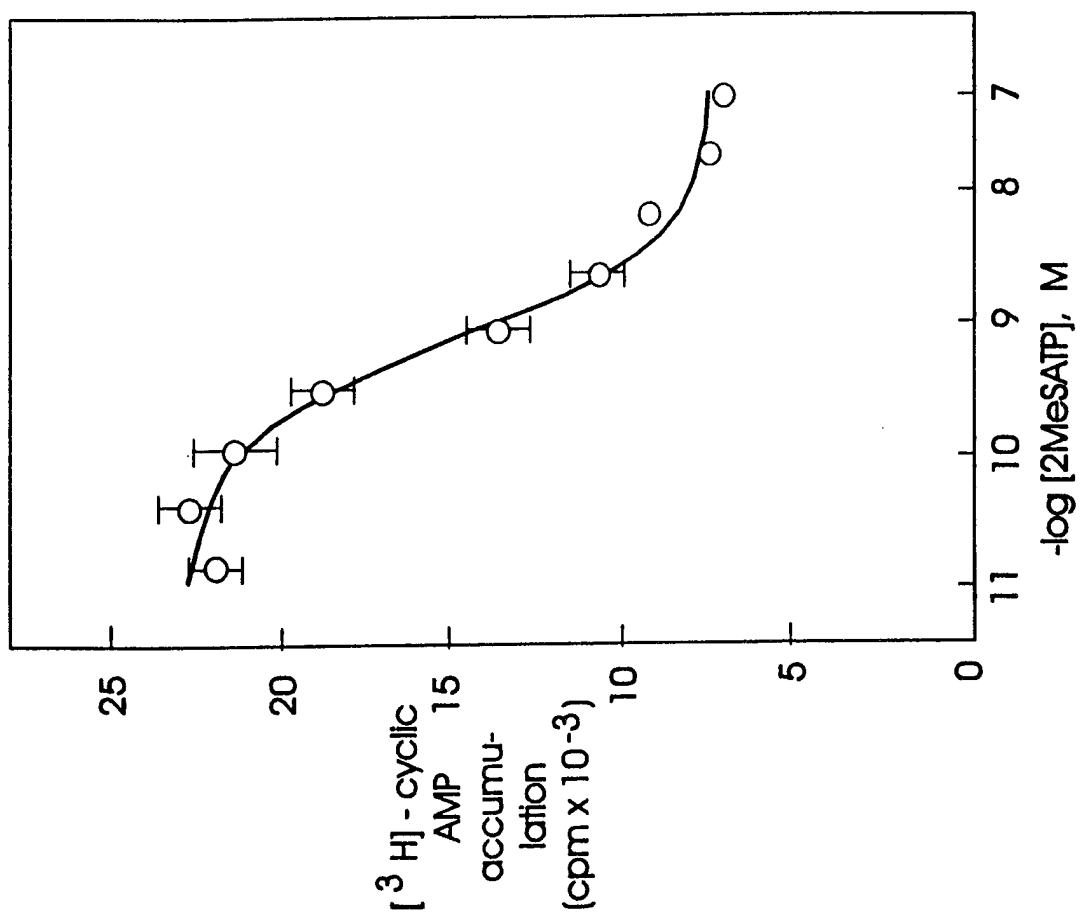
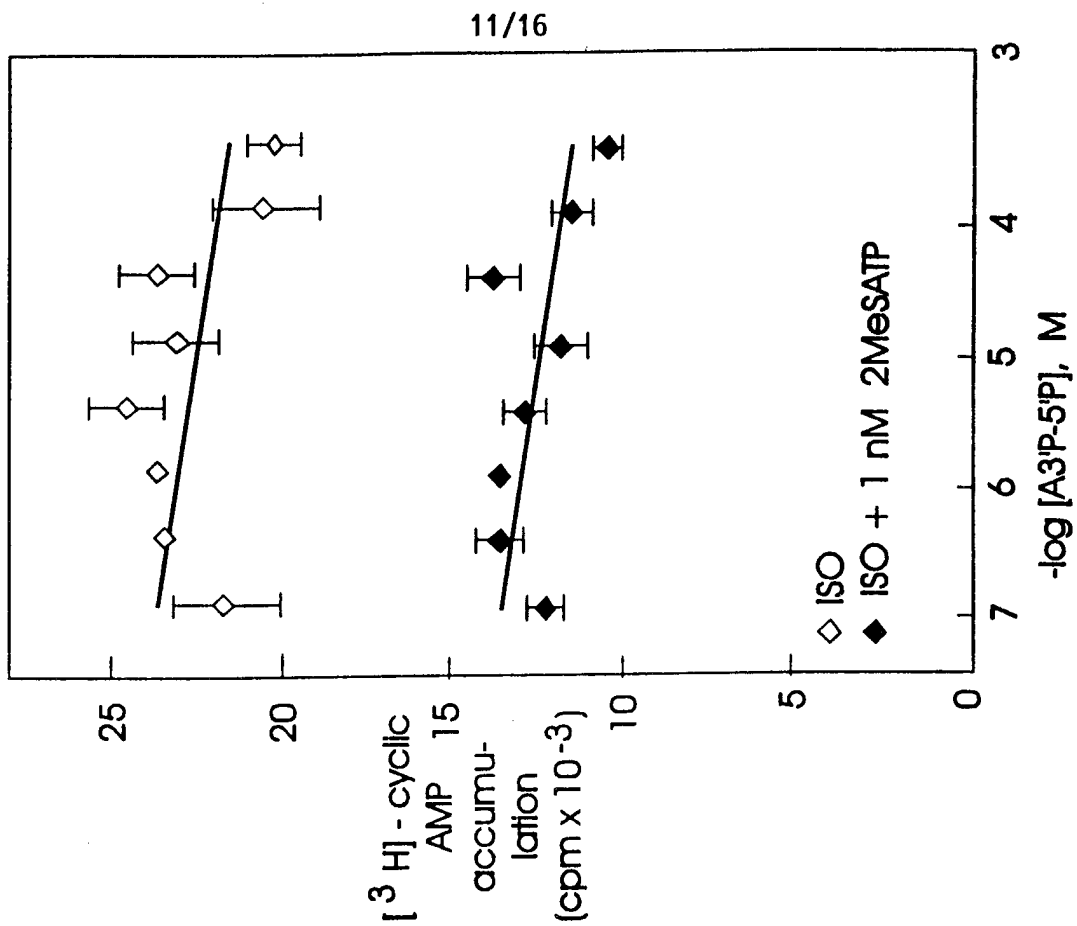


FIG. 6B



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FIG. 7

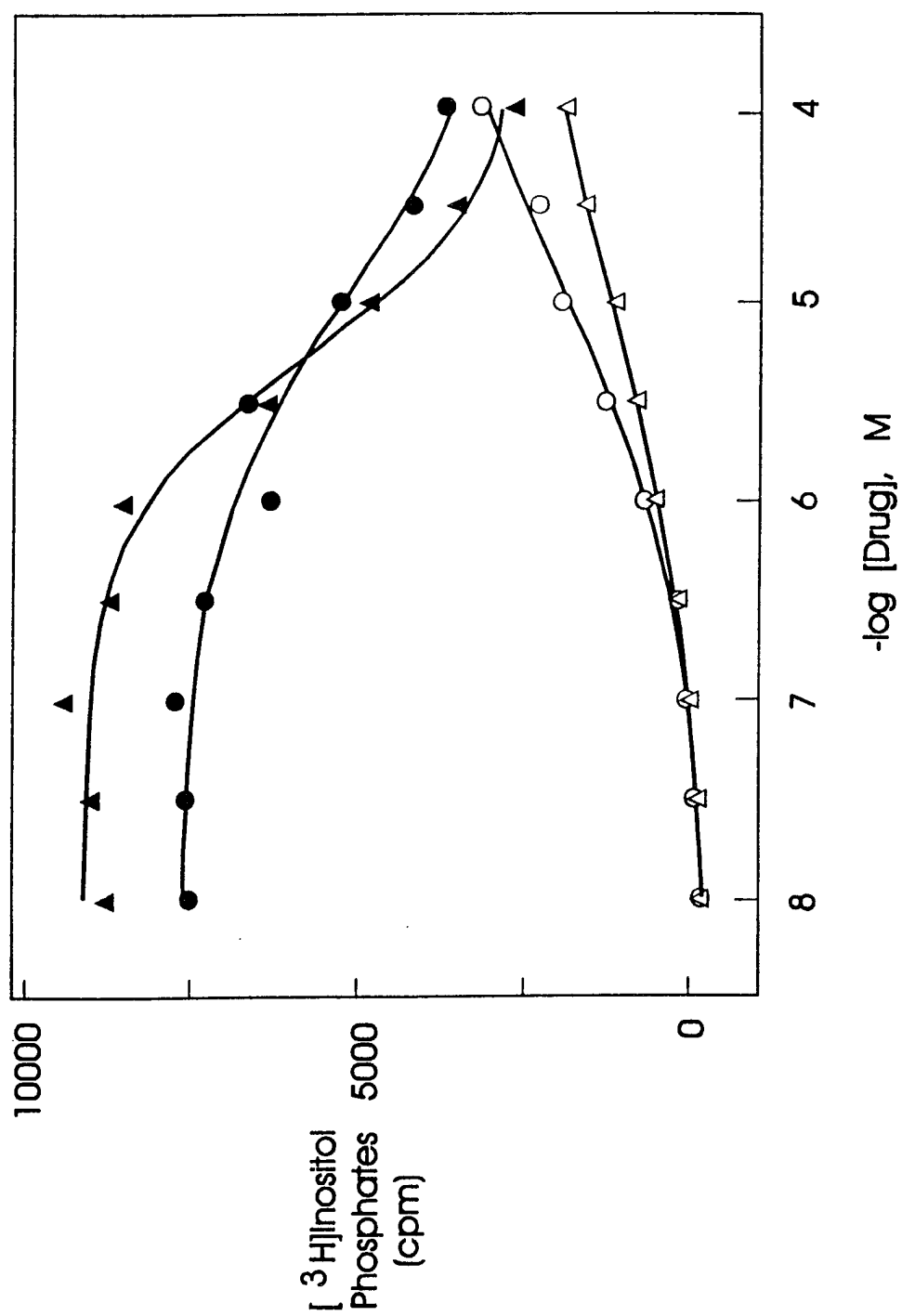


FIG. 8

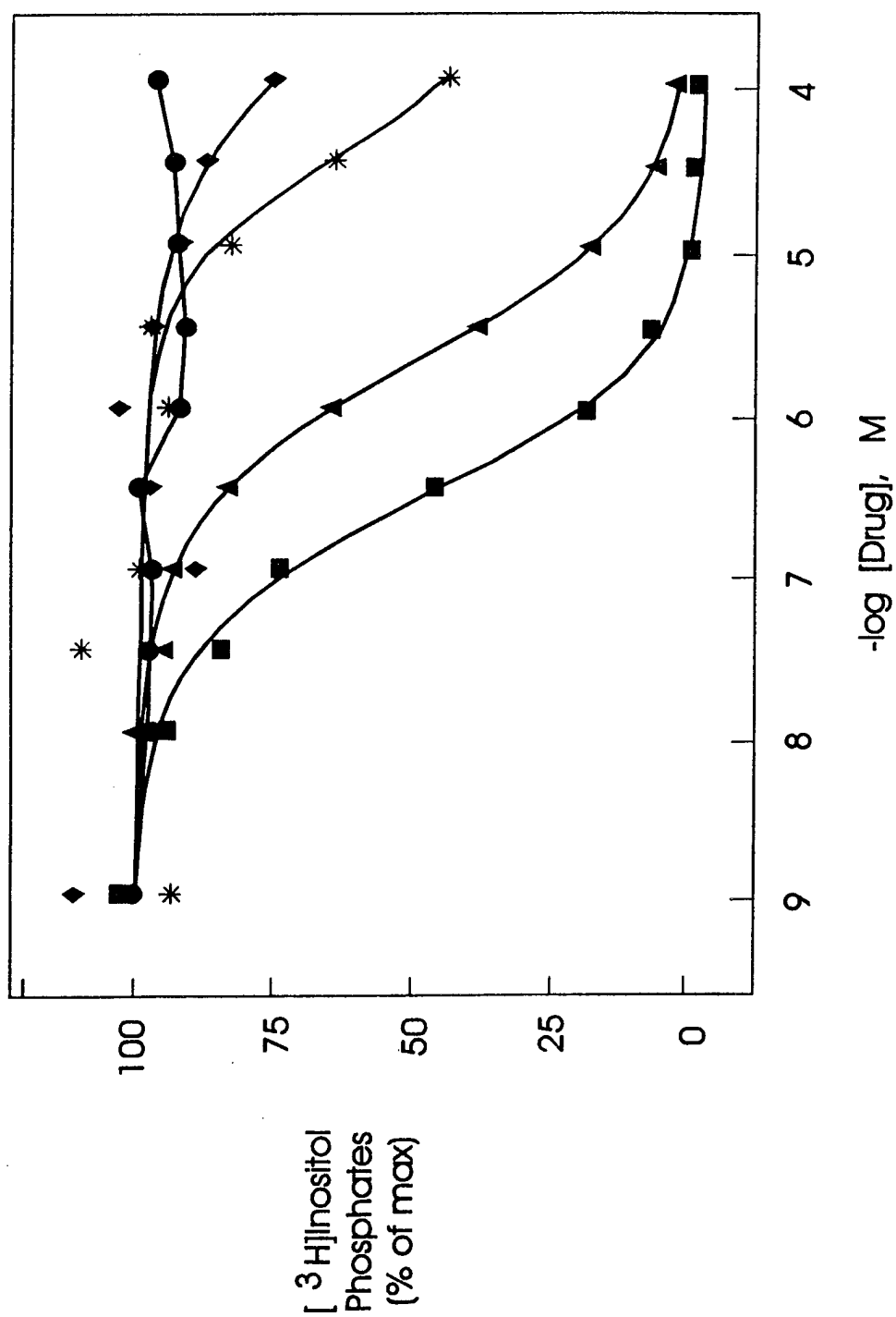


FIG. 9

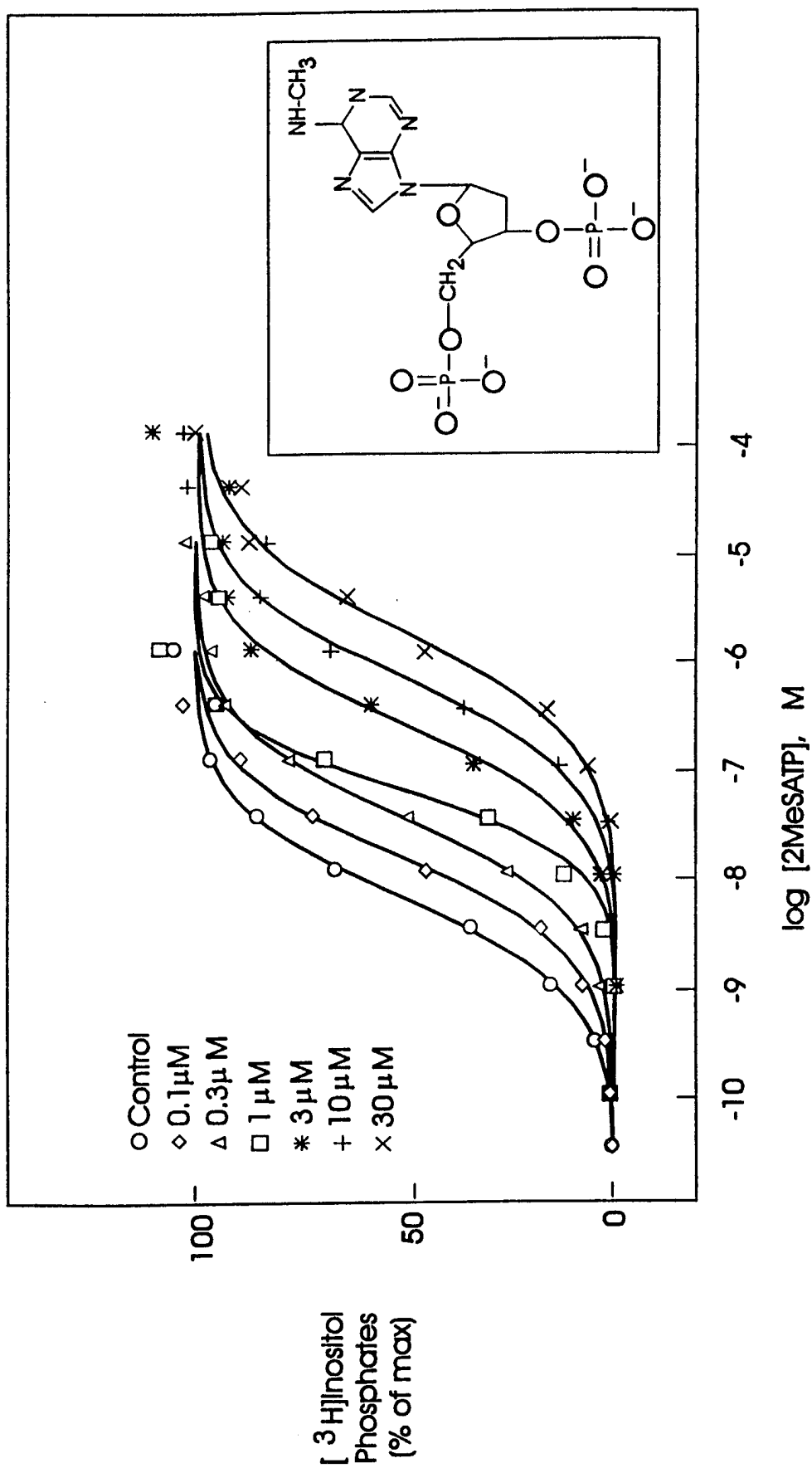


FIG. 10

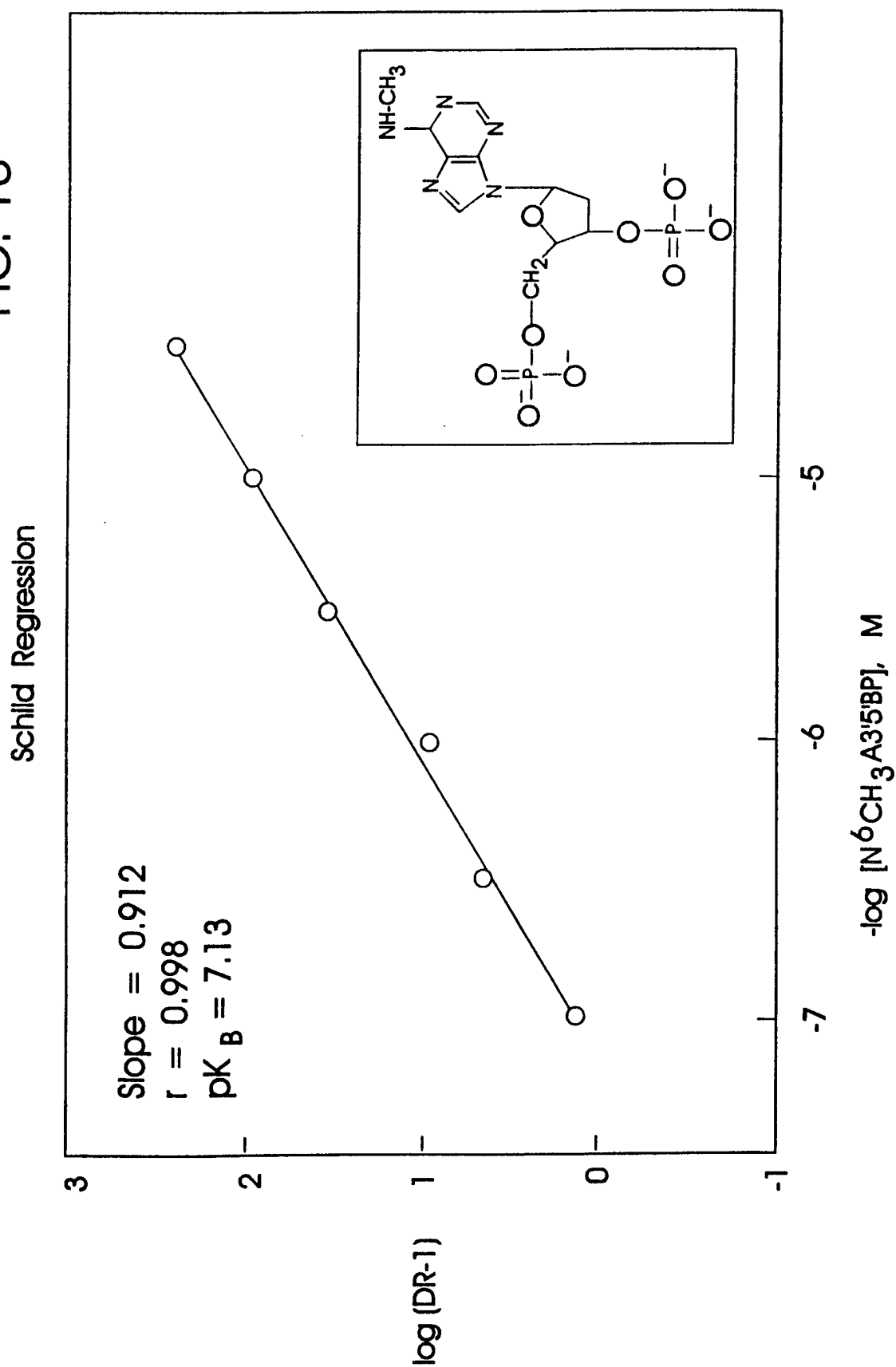


FIG. 11

