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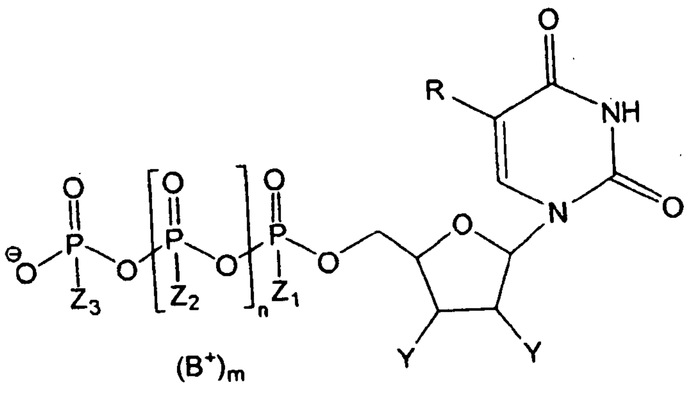
(54) **Title**: URIDINE DI- OR TRI-PHOSPHATE DERIVATIVES AND USES THEREOF



(57) **Abstract**: The invention provides particular uridine di- and tri-phosphate derivatives, and pharmaceutical compositions thereof. These compounds are useful for treatment of diseases, disorders and conditions modulated by P2Y6 receptors, and particularly for lowering intraocular pressure and thereby treating ocular hypertension and/or glaucoma.

URIDINE DI- OR TRI-PHOSPHATE DERIVATIVES AND USES THEREOF

TECHNICAL FIELD

[0001] The present invention relates to uridine di- and tri-phosphate derivatives and to pharmaceutical compositions thereof. The compounds are useful for treatment of diseases, disorders and conditions modulated by $P2Y_6$ receptors, and particularly for lowering intraocular pressure and thereby treating ocular hypertension and/or glaucoma.

BACKGROUND ART

[0002] Extracellular nucleotides that activate G protein-coupled P2Y receptors (P2YRs) are attractive pharmaceutical targets due to their ability to modulate various functions in many tissues and organs under normal and pathophysiological conditions (Hillmann *et al.*, 2009; Burnstock and Verkhratsky, 2009). Extracellular nucleotides and dinucleotides have been shown to play a role in ocular physiology and physiopathology (Crooke *et al.*, 2008), and have been suggested as therapeutic agents for dry eye, retinal detachment and glaucoma (Guzman-Aranguez *et al.*, 2007).

[0003] Ocular hypertension, the most common cause of glaucoma, is a target for agents that reduce intraocular pressure (IOP) (Pintor, 2005). When topically applied to New Zealand white rabbits, some nucleotides, e.g., diadenosine triphosphate and diadenosine pentaphosphate, produce an increase in IOP while others such as ATP, adenosine tetraphosphate and diadenosine tetraphosphate decrease IOP (Peral *et al.*, 2009; Pintor *et al.*, 2003, 2004).

[0004] Receptors for extracellular nucleotides including $P2Y_1$, $P2Y_2$ and $P2Y_4$ have been identified in trabecular meshwork (TM) cells, an area of tissue in the eye that is responsible for draining the aqueous humor (Soto *et al.*, 2005). Among these P2YR subtypes, activation of the $P2Y_1R$ by the selective agonist 2-MeS-ADP reduces aqueous humor outflow in bovine ocular. Other studies have reported the presence of $P2Y_1$ and $P2Y_2$ receptors in bovine TM cells, and of $P2Y_1$, $P2Y_4$ and $P2Y_{11}$ receptors in a human TM cell line (Crosson *et al.*, 2004).

[0005] Lately, the structure-activity relationship of $P2Y_6$-R agonists and antagonists, and the molecular modeling of the $P2Y_6$-R involved in the reduction of IOP have been extensively investigated (El-Tayeb *et al.*, 2006; Jacobson *et al.*, 2009; Costanzi *et al.*, 2005; Maruoka *et al.*, 2010; Besada *et al.*, 2006); however, no potent and selective $P2Y_6$-R agonist or antagonist has yet been identified. The development of agonists for the $P2Y_6$-R included modification of the UDP phosphate chain, ribose ring, and base. Different uracil modifications have been performed over the last years in an attempt to identify agonists which will be more potent than the endogenous ligand UDP (Maruoka *et al.*, 2010; Ginsburg-Shmuel *et al.*, 2010; Ko *et al.*, 2008).

[0006] The therapeutic potential of nucleotides in general, and for the treatment of glaucoma in particular, is limited, since they are degraded by extracellular enzymes, which reduce their potency, efficacy and duration of action. In addition, although nucleotides are chemically stable in a pH range of 4-11 (El-Tayeb *et al.*, 2006), they are rapidly degraded at a more acidic or basic pH. Nucleotides are hydrolyzed enzymatically by the ecto-nucleoside triphosphate diphosphohydrolase family of ectonucleotidases, i.e., e-NTPDase and alkaline phosphatases (Nahum *et al.*, 2002), and ecto-nucleotide pyrophosphatases/phosphodiesterases, i.e., e-NPPs (Grobben *et al.*, 2000; Zimmermann, 2001). Therefore, there is a need for identification of both enzymatically and chemically stable nucleotide scaffolds that can be used to develop selective and potent P2YR agonists.

[0007] A few attempts to improve the stability of nucleotides have been reported (Cusack *et al.*, 1987; Misiura *et al.*, 2005; Kowalska *et al.*, 2007), including the use of phosphate bioisosteres of nucleotides such as phosphonate (Eliahu *et al.*, 2009; Joseph *et al.*, 2004), phosphoramide (Zhou *et al.*, 2005), and boranophosphate analogues (Nahum *et al.*, 2002; Boyle *et al.*, 2005; Barral *et al.*, 2006; Eliahu *et al.*, 2009).

[0008] A second strategy for enhancing stability of potential P2Y-R agonists is the use of dinucleotides, e.g., diuridine triphosphates, which show greater stability than analogues of mononucleotides (Shaver *et al.*, 2005; Yerxa *et al.*, 2002). Indeed, dinucleotides have been successfully developed before as $P2Y_2$-R agonists. Thus, Up_4U (INS365, Diquafosol) and Up_4dC (INS37217, Denufosol) have been clinically tested for the treatment of dry eye disease and cystic fibrosis, respectively, however, both compounds did not show satisfying results at phase 3 clinical trial (http://www.businesswire.com/news/home/20110103005364/en; Jacobson and Boeynaems, 2010).

[0009] Ginsburg-Shmuel *et al.* (2010) discloses 5-OMe-UDP as a $P2Y_6$-receptor agonist. As particularly shown, 5-OMe-UDP adopts the anti-conformation that is favored by the receptors, and the S sugar puckering that is the conformation preferred by the $P2Y_6$-receptors but not the $P2Y_2$- or $P2Y_4$-receptors, and thus fulfills the conformational and H-bonding requirements of $P2Y_6$-receptors and making a potent $P2Y_6$-receptor agonist (EC_{50}=0.08 μM *vs.* 0.14 μM for UDP).

[0010] US 7,084,128 discloses a method of reducing IOP by administration of certain mono- or di- nucleotides, preferably mono- or diadenosine, mono-, di-, tri-, tetra-, penta- or hexaphosphate derivatives, or a pharmaceutically-acceptable salt thereof. The particular compounds exemplified are 2'-(O)-,3'-(O)-(benzyl) methylenedioxy-adenosine-5'-triphosphate and 2'-(O)-,3'-(O)-(benzyl)methylene dioxy-2''-(O)-,3''-(O)-benzyl methylene dioxy-P^1,P^4-di(adenosine 5'-)tetra phosphate, and as shown, at a concentration of 0.25 mM, these compounds produced a time dependent reduction in IOP, which was maximal at 1-2 hours with a reduction of 21-22%.

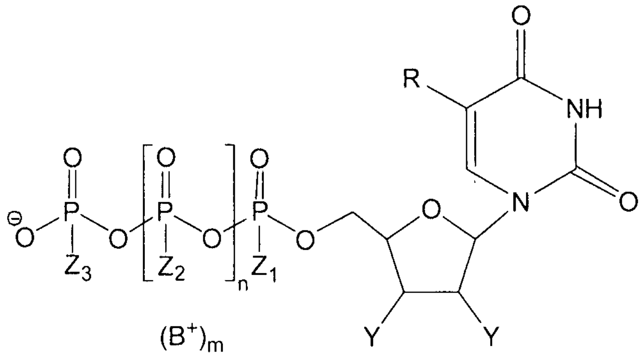
[0011] Eliahu *et al.* (2010) discloses certain non-hydrolyzable adenosine di- or triphosphate analogues such as 2MeS-adenosine-β,γ-CH_2-5'-triphosphate and 2MeS-adenosine-β,γ-CCl_2-5'-triphosphate as potent agents for reducing IOP. As stated in this publication, 2MeS-adenosine-β,γ-CCl_2-5'-triphosphate reduced IOP in normotense rabbits by 32% (EC_{50}=95.5 nM), wherein the duration of effect was about 5 hours, i.e., was found to be more effective at reducing IOP than several common glaucoma drugs and thus represents a promising alternative to timolol maleate, which cannot be used for the treatment of patients suffering from asthma or cardiac problems.

SUMMARY OF INVENTION

[0012] It has now been found, in accordance with the present invention, that certain 5-methoxyuridine nucleotides are capable, upon administration to the cornea, to significantly reduce intraocular pressure (IOP) in male New Zealand white rabbits, and are thus considered promising candidates for treatment of ocular hypertension and/or glaucoma through selective activation of $P2Y_6$ receptors. The particular compound exhibiting the strongest hypotensive effect was one of the two diastereoisomers of 5-methoxyuridine-5'-*O*-(α-boranodiphosphate), which reduced IOP in normotense rabbits by 45%, more than any drug currently available, wherein the duration of effect was about 4 hours.

[0013] In one aspect, the present invention provides a compound of the general formula I:

I



or a diastereoisomer or mixture of diastereoisomers thereof,

wherein

R is -O-(C_1-C_8)alkyl, or -S-(C_1-C_8)alkyl;

Y each independently is H, or -OH;

Z_1, Z_2 and Z_3 each independently is O^-, or BH_3^-;

n is 0 or 1;

m is 3 or 4; and

B^+ represents a pharmaceutically acceptable cation,

but excluding the compounds wherein Z_1, Z_2, if present, and Z_3 are each O^-.

[0014] In another aspect, the present invention provides a pharmaceutical composition comprising a compound of the general formula I but excluding the compounds wherein Z_1, Z_2, if present, and Z_3 are each O^-, or a diastereoisomer or mixture of diastereoisomers thereof, and a pharmaceutically acceptable carrier or diluent.

[0015] In a further aspect, the present invention provides a pharmaceutical composition for reducing intraocular pressure, more particularly, for prevention or treatment of intraocular hypertension and/or glaucoma, comprising a compound of the general formula I, including the compounds wherein Z_1, Z_2, if present, and Z_3 are each O^-, or a diastereoisomer or mixture of diastereoisomers thereof, and a pharmaceutically acceptable carrier or diluent.

[0016] In still another aspect, the present invention provides a compound of the general formula I, including the compounds wherein Z_1, Z_2, if present, and Z_3 are each O^-, or a diastereoisomer or mixture of diastereoisomers thereof, for use in reducing intraocular pressure.

[0017] In yet another aspect, the present invention relates to use of a compound of the general formula I, including the compounds wherein Z_1, Z_2, if present, and Z_3 are each O^-, or a diastereoisomer or mixture of diastereoisomers thereof, for the preparation of a pharmaceutical composition for reducing intraocular pressure.

[0018] In still a further aspect, the present invention relates to a method for reducing intraocular pressure in an individual in need thereof comprising administering to said individual a therapeutically effective amount of a compound of the general formula I, including the compounds wherein Z_1, Z_2, if present, and Z_3 are each O^-, or a diastereoisomer or mixture of diastereoisomers thereof.

BRIEF DESCRIPTION OF DRAWINGS

[0019] **Fig. 1** shows the rate of hydrolysis of **13A** at gastric-juice simulating conditions monitored by ^{31}P NMR at 240 MHz. The different curves represent the species that exist in the solution at different times due to the hydrolysis.

[0020] **Figs. 2A-2B** show time-dependent hydrolysis curves of UDP (**2A**) and of **11**, **12** and **13A** (**2B**) in human blood serum (180 µl) and RPMI-1640 medium (540 µl) over 24 h at 37°C, as monitored by HPLC.

[0021] **Figs. 3A-3D** show concentration-response curves for nucleotides **13A** (**3A**), **15** (**3C**), **16** (**3B**) and **17** (**3D**), and the endogenous agonist UDP at the $P2Y_6$-R. Data were obtained from 1321N1 cells stably expressing the $P2Y_6$GFP receptor determining the ligand induced change in $[Ca^{2+}]_i$. Cells were pre-incubated with 2 µM fura-2 AM for 30 min and change in fluorescence (Δ F340 nm/F380 nm) was detected. Concentration-response curves are from one data set but, for clarity, are represented in separate diagrams with the UDP response curve for common reference.

[0022] **Figs. 4A-4C** show the reduction of intraocular pressure (IOP) in normotense rabbits by the various 5-methoxyuridine nucleotide and dinucleotide derivatives as compared to control, UDP, i.e., the endogenous $P2Y_6$-receptor ligand, and marketed drugs. **4A** shows time-course for the effect of compounds **12**, **13A** and **16A** on rabbit IOP measured over 6 h; **4B** shows the reduction of IOP in normotense rabbits by **12**, **13A** and **16A** *vs*. UDP and control; and **4C** shows the reduction of IOP in normotense rabbits by **12**, **13A** and **16A** *vs*. Xalatan®, Trusopt®, Pilocarpine and control.

DETAILED DESCRIPTION OF THE INVENTION

[0023] The present invention provides, in one aspect, certain uridine nucleotides of the general formula I as defined above, in which the carbon atom at position 5 of the uracil ring is substituted by -O-alkyl or -S-alkyl, and at least one of the non-bridging oxygen atoms of the di- or tri-phosphate is replaced by a borano group.

[0024] The term "$(C_1$-$C_8)$alkyl" as used herein typically means a straight or branched hydrocarbon radical having 1-8 carbon atoms and includes, e.g., methyl, ethyl, n-propyl, isopropyl, n-butyl, sec-butyl, isobutyl, tert-butyl, n-pentyl, 2,2-dimethylpropyl, n-hexyl, n-heptyl, n-octyl, and the like. Preferred are $(C_1$-$C_6)$alkyl groups, more preferably $(C_1$-$C_4)$alkyl groups, most preferably methyl and ethyl.

[0025] In certain embodiments, the compound of the present invention is a compound of the general formula I, wherein R is -O-(C_1-C_8)alkyl, preferably -O-(C_1-C_6)alkyl, more preferably -O-(C_1-C_4)alkyl, most preferably -OCH_3 or -OC_2H_5.

[0026] In certain embodiments, the compound of the present invention is a compound of the general formula I, wherein R is -S-(C_1-C_8)alkyl, -S-(C_1-C_6)alkyl, more preferably -S-(C_1-C_4)alkyl, most preferably -SCH_3 or -SC_2H_5.

[0027] In certain embodiments, the compound of the present invention is a compound of the general formula I, wherein n is 0. Such compounds may be uridine diphosphate derivatives, i.e., compounds wherein Y each is OH, as well as deoxy- or dideoxy-uridine diphosphate derivatives, i.e., compounds wherein one or both of the Ys, respectively, is H. Particular such compounds are those comprising (i) a sole borano group at position α, i.e., wherein Z_1 is BH_3^-, and Z_3 is O^-; (ii) a sole borano group at position β, i.e., wherein Z_3 is BH_3^-, and Z_1 is O^-; or (iii) two borano groups at positions α and β, i.e., wherein Z_1 and Z_3 are BH_3^-.

[0028] In certain embodiments, the compound of the present invention is a compound of the general formula I, wherein n is 1. Such compounds may be uridine triphosphate derivatives, i.e., compounds wherein Y each is OH, as well as deoxy- or dideoxy-uridine triphosphate derivatives, i.e., compounds wherein one or both of the Ys, respectively, is H. Particular such compounds are those comprising (i) a sole borano group at position α, i.e., wherein Z_1 is BH_3^-, and Z_2 and Z_3 are O^-; at position β, i.e., wherein Z_2 is BH_3^-, and Z_1 and Z_3 are O^-; or at position γ, i.e., wherein Z_3 is BH_3^-, and Z_1 and Z_2 are O^-; (ii) two borano groups at positions α and β, i.e., wherein Z_1 and Z_2 are BH_3^-, and Z_3 is O^-; at positions α and γ, i.e., wherein Z_1 and Z_3 are BH_3^-, and Z_2 is O^-; or at positions β and γ, i.e., wherein Z_2 and Z_3 are BH_3^-, and Z_1 is O^-; or (iii) three borano groups at positions α, β and γ, i.e., wherein Z_1 to Z_3 are BH_3^-.

[0029] In particular embodiments, the compound of the present invention is a compound of the general formula I, wherein R is -O-(C_1-C_4)alkyl, preferably -OCH_3

or -OC_2H_5, n is 0, and (i) Z_1 is BH_3^-, and Z_3 is O^-; (ii) Z_1 is O^-, and Z_3 is BH_3^-; or (iii) Z_1 and Z_3 are BH_3^-.

[0030] In other particular embodiments, the compound of the present invention is a compound of the general formula I, wherein R is -O-(C_1-C_4)alkyl, preferably -OCH_3 or -OC_2H_5, n is 1, and (i) Z_1 is BH_3^-, and Z_2 and Z_3 is O^-; (ii) Z_2 is BH_3^-, and Z_1 and Z_3 are O^-; (iii) Z_3 is BH_3^-, and Z_1 and Z_2 are O^-; (iv) Z_1 and Z_2 are BH_3^-, and Z_3 is O^-; (v) Z_1 and Z_3 are BH_3^-, and Z_2 is O^-; (vi) Z_2 and Z_3 are BH_3^-, and Z_1 is O^-; or (vii) Z_1 to Z_3 are BH_3^-.

[0031] The specific uridine nucleotide derivatives of the general formula I described in the specification are herein identified compounds/analogues **12**, **13** and **14** in bold, and the specific uridine dinucleotide derivatives described in the specification, not encompassed by the general formula I, are herein identified compounds/analogues **15**, **16** and **17** in bold. Compound **12** is also identified by the name 5-methoxyuridine diphosphate (5-OMe-UDP); compound **13** is also identified by the name 5-methoxyuridine-5'-*O*-(α-boranodiphosphate); compound **14** is also identified by the name 5-methoxyuridine-5'-*O*-(α-boranotriphosphate); compound **15** is also identified by the name di-(5-OMe)-uridine 5',5''-P^1,P^3, triphosphate; compound **16** is also identified by the name di-(5-OMe)-uridine 5',5''-P^1,P^3,α-boranotriphosphate; and compound **17** is also identified by the name di-(5-OMe)-uridine 5''5''-P^1,P^3,β-boranotriphosphate. In cases a pair of diastereoisomers exist for a certain analogue, such as in the case of analogue **13**, those diastereoisomers are herein identified **A** and **B**, e.g., analogues/diastereoisomers **13A** and **13B**. Particular intermediates described in the specification are herein identified by the Arabic numbers **1-7**. Uridine-5'-*O*-(α-boranodiphophate) is herein identified by the Arabic number **11**. The chemical structures of all these compounds/analogues are depicted in Appendix A and/or in **Scheme 1** hereinafter.

[0032] In one specific embodiment, the compound of the present invention is 5-methoxyuridine-5'-*O*-(α-boranodiphosphate), i.e., a compound of the general formula I, wherein R is -OCH_3, n is 0, Y each is -OH, Z_1 is BH_3^-, and Z_3 is O^- (compound **13**). A preferred compound is that characterized by being the isomer with a retention time (R_t) of 8.97 min when separated from a mixture of diastereoisomers using a semi-preparative reverse-phase Gemini 5μ column (C-18 110A, 250×10 mm, 5 micron), and isocratic elution [100 mM triethylammonium acetate, pH 7: CH_3CN, 94:6] with flow rate of 5 ml/min (compound **13A**).

[0033] In another specific embodiment, the compound of the present invention is 5-methoxyuridine-5'-*O*-(α-boranotriphosphate), i.e., a compound of the general formula I, wherein R is $-OCH_3$, n is 1, Y each is -OH, Z_1 is BH_3^-, and Z_2 and Z_3 are O^- (compound **14**).

[0034] The compounds of the general formula I may be synthesized according to any technology or procedure known in the art, e.g., as described in detail in the Examples section hereinafter. The compounds of the invention may have an asymmetric center, e.g., in the Pα, and may accordingly exist as pairs of diastereoisomers. In cases a pair of diastereoisomers exists, the separation and characterization of the different diastereoisomers may be accomplished using any technology known in the art, e.g., using a semi-preparative reverse-phase column and isocratic solution as described in the Examples section.

[0035] The compounds of the general formula I are in the form of pharmaceutically acceptable salts.

[0036] In certain embodiments, the cation B is an inorganic cation of an alkali metal such as, but not limited to, Na^+, K^+ and Li^+.

[0037] In other embodiments, the cation B is ammonium (NH_4^+) or is an organic cation derived from an amine of the formula R_4N^+, wherein each one of the Rs independently is selected from H, C_1-C_{22}, preferably C_1-C_6 alkyl, such as methyl, ethyl, propyl, isopropyl, butyl, and the like, phenyl, or heteroaryl such as pyridyl, imidazolyl, pyrimidinyl, and the like, or two of the Rs together with the nitrogen atom to which they are attached form a 3-7 membered ring optionally containing a further heteroatom selected from N, S and O, such as pyrrolydine, piperidine and morpholine.

[0038] In further embodiments, the cation B is a cationic lipid or a mixture of cationic lipids. Cationic lipids are often mixed with neutral lipids prior to use as delivery agents. Neutral lipids include, but are not limited to, lecithins; phosphatidyl-ethanolamine; diacyl phosphatidylethanolamines such as dioleoyl phosphatidylethanolamine, dipalmitoyl phosphatidylethanolamine, palmitoyloleoyl phosphatidylethanolamine and distearoyl phosphatidylethanolamine; phosphatidyl-choline; diacyl phosphatidylcholines such as dioleoyl phosphatidylcholine, dipalmitoyl phosphatidylcholine, palmitoyloleoyl phosphatidylcholine and distearoyl phosphatidylcholine; fatty acid esters; glycerol esters; sphingolipids; cardiolipin; cerebrosides; ceramides; and mixtures thereof. Neutral lipids also include cholesterol and other 3β hydroxy-sterols. Other neutral lipids contemplated herein include phosphatidylglycerol; diacyl phosphatidylglycerols such as dioleoyl phosphatidylglycerol, dipalmitoyl phosphatidylglycerol and distearoyl phosphatidylglycerol; phosphatidylserine; diacyl phosphatidylserines such as dioleoyl- or dipalmitoyl phosphatidylserine; and diphosphatidylglycerols.

[0039] Examples of cationic lipid compounds include, without being limited to, Lipofectin® (Life Technologies, Burlington, Ontario) (1:1 (*w/w*) formulation of the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride and dioleoylphosphatidyl-ethanolamine); Lipofectamine™ (Life Technologies, Burlington, Ontario) (3:1 (*w/w*) formulation of polycationic lipid 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanamin-iumtrifluoroacetate and dioleoylphosphatidyl-ethanolamine), Lipofectamine Plus (Life Technologies, Burlington, Ontario) (Lipofectamine and Plus reagent), Lipofectamine 2000 (Life Technologies, Burlington, Ontario) (Cationic lipid), Effectene (Qiagen, Mississauga, Ontario) (Non liposomal lipid formulation), Metafectene (Biontex, Munich, Germany) (Polycationic lipid), Eu-fectins (Promega Biosciences, San Luis Obispo, Calif.) (ethanolic cationic lipids numbers 1 through 12: $C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H$, $C_{88}H_{178}N_8O_4S_2 \cdot 4CF_3CO_2H$, $C_{40}H_{84}NO_3P \cdot CF_3CO_2H$, $C_{50}H_{103}N_7O_3 \cdot 4CF_3CO_2H$, $C_{55}H_{116}N_8O_2 \cdot 6CF_3CO_2H$, $C_{49}H_{102}N_6O_3 \cdot 4CF_3CO_2H$, $C_{44}H_{89}N_5O_3 \cdot 2CF_3CO_2H$, $C_{100}H_{206}N_{12}O_4S_2 \cdot 8CF_3CO_2H$, $C_{162}H_{330}N_{22}O_9 \cdot 13CF_3CO_2H$, $C_{43}H_{88}N_4O_2 \cdot 2CF_3CO_2H$, $C_{43}H_{88}N_4O_3 \cdot 2CF_3CO_2H$, $C_{41}H_{78}NO_8P$); Cytofectene (Bio-Rad, Hercules, Calif.) (mixture of a cationic lipid and a neutral lipid), GenePORTER® (Gene Therapy Systems, San Diego, Calif.) (formulation of a neutral lipid (Dope) and a cationic lipid) and FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, Ind.) (Multi-component lipid based non-liposomal reagent).

[0040] As shown in the Examples section hereinafter, analogue **13A** is a potent and selective agonist at the P2Y$_6$ receptor, when expressed in 1321N1 astrocytoma cells, and is significantly more potent than both the endogenous agonist UDP and analogue **12** (Ginsburg-Shmuel *et al.*, 2010). The introduction of a chiral center in 5-OMe-UDP by BH$_3$-substitution of the non-bridging oxygen at Pα reveals stereo-specificity of the receptor for the A-isomer over the B-isomer. The A-isomer of the mono-nucleotide derivative **13** was the most potent among the tested nucleotides with EC$_{50}$=0.008 μM, and was more than 500-fold more potent than the corresponding B-isomer (EC$_{50}$=4.3 μM) and 24-fold more potent than the endogenous agonist UDP (EC$_{50}$=0.15 μM). Although borano-substitution dramatically enhanced the potency of analogue **12** at P2Y$_6$-R, the corresponding triphosphate mono-nucleotide **14** was hardly active at the P2Y$_6$-R due to the preference of the receptor for three phosphate negative charges. A stereo-specificity similar to that of analogue **13**, although less pronounced, could be observed for the dinucleotide derivative **16**, wherein the potency of the A-isomer (EC$_{50}$=0.06 μM) was in the range of that of UDP but only about 40-fold higher than that of the B-isomer (EC$_{50}$=2.2 μM). The non-chiral dinucleotide derivative **17**, with a BH$_3$-substituent at the middle phosphate, showed a potency (EC$_{50}$=0.2 μM) similar to that of the standard agonist UDP; and the dinucleotide derivative **15** without a BH$_3$-substitution showed the weakest potency among the tested nucleotides. None of the nucleotides tested was active at the P2Y$_2$- or P2Y$_4$-receptor in 1321N1 cells and at 1321N1 wild type cells.

[0041] The therapeutic potential of the new P2Y$_6$-R agonists is related to their chemical stability and their resistance to enzymatic hydrolysis. Therefore, in a further study described herein, the stability of the most potent analogue found, **13A**, *vs.* that of UDP, **11** and **12**, under various conditions, was evaluated, and as found:

(i) P_α borano substitution reduces the stability of **13A** under conditions simulating gastric juice acidity (pH 1.4 and 37°C). In particular, under those conditions, analogues **13A** and **12** displayed a half-life of 16.9 h and 13 days, respectively, and similar results were obtained for **11** and UDP (16.9 h and 12 days, respectively). The reduction of chemical stability is explained by the susceptibility of the P-B bond to acidic hydrolysis, as compared to that of the P-O bond (Nahum and Fischer, 2004), but even so, a half-life of 16.9 h is quite satisfactory for a drug candidate; (ii) P_α borano substitution increases resistance to degradation by NPP1 and NPP3. In particular, **13A** was more stable than its non-borano counterparts UDP and **12** in the presence of both NPP1 and NPP3 (at NPP1 - 15% *vs.* 50% and 51% hydrolysis, and at NPP3 - 28% *vs.* 45% and 36% hydrolysis, respectively), indicating that the introduction of the boranophosphate moiety at P_α plays an important role in protecting UDP analogues against NPP1,3 hydrolysis. In fact, although the enzymatic degradation by NPP occurs between P_α and P_β, it is postulated that the BH_3 group in analogue **13A**, which is larger than O in the parent compound, prevents attack by an essential water molecule on P_α and thus makes these analogues poor NPP substrates. Furthermore, analogue **12** bearing only a modification at the uracil ring was hydrolyzed more slowly by NPP3 but not by NPP1 as compared to UDP; (iii) The BH_3 substitution at P_α position and OMe at the C5 position of uracil nucleotides increase resistance to degradation in human blood serum. As particularly shown, **12** bearing a methoxy group at C5 of the uracil ring was more stable than UDP (half-life of 11.9 h *vs.* 2.4 h), and **13A**, bearing both BH_3 and methoxy groups, displayed even greater stability with a half-life of 17 h. Similar results were obtained for uridine-5'-*O*-(α-boranodiphosphate) ($t_{1/2}$=21 h). It is suggested that the methoxy group presents a steric hindrance which causes analogue **12** to be a poor substrate for the various enzymes present in blood serum. Apparently, the borano group in **13A** and **11** renders the nucleotides even more stable to enzymatic degradation than UDP.

[0042] As further shown in the Examples section, analogue **12** reduced IOP in normotense rabbits by 31%, and analogue **13A** reduced IOP in normotense rabbits by 45%, more than any marketed drug, e.g., Xalatan®, Trusopt®, and Pilocarpine, wherein the duration of action was about 4 h.

[0043] In another aspect, the present invention thus provides a pharmaceutical composition comprising a compound of the general formula I but excluding the compounds wherein Z_1, Z_2, if present, and Z_3 are each O^-, or a diastereoisomer or mixture of diastereoisomers thereof, and a pharmaceutically acceptable carrier or diluent.

[0044] In a further aspect, the present invention provides a pharmaceutical composition for reducing, i.e., lowering, intraocular pressure, more particularly, for prevention or treatment of intraocular hypertension and/or glaucoma, comprising a compound of the general formula I, including the compounds wherein Z_1, Z_2, if present, and Z_3 are each O^-, or a diastereoisomer or mixture of diastereoisomers thereof, and a pharmaceutically acceptable carrier or diluent.

[0045] In particular embodiments, the compound comprised within this composition is 5-methoxyuridine diphosphate, i.e., a compound of the general formula I wherein R is $-OCH_3$, n is 0, Y each is -OH, and Z_1 and Z_3 are O^- (**12**), compound **13**, more particularly **13A**, or compound **14**, preferably compound **13A**.

[0046] The terms "intraocular hypertension", "ocular hypertension", or "intraocular pressure", as used herein interchangeably, refer to an intraocular pressure in an eye of a patient that is above a normal level and is correlated as a risk factor for the development of visual field loss and glaucoma.

[0047] Glaucoma is a heterogeneous group of optic neuropathies that share certain clinical features, wherein the loss of vision is due to the selective death of retinal ganglion cells in the neural retina that is clinically diagnosed by characteristic changes in the visual field, nerve fiber layer defects, and a progressive cupping of the optic nerve head (ONH). One of the main risk factors for the development of glaucoma is the presence of intraocular hypertension (elevated intraocular pressure, IOP). IOP also appears to be involved in the pathogenesis of normal tension glaucoma where patients have what is often considered to be normal IOP. The elevated IOP associated with glaucoma is due to elevated aqueous humor outflow resistance in the trabecular meshwork (TM), a small-specialized tissue located in the iris-corneal angle of the ocular anterior chamber. Glaucomatous changes to the TM include a loss in TM cells and the deposition and accumulation of extracellular debris including proteinaceous plaque-like material. In addition, there are also changes that occur in the glaucomatous ONH. In glaucomatous eyes, there are morphological and mobility changes in ONH glial cells. In response to elevated IOP and/or transient ischemic insults, there is a change in the composition of the ONH extracellular matrix and alterations in the glial cell and retinal ganglion cell axon morphologies.

[0048] The term "glaucoma" as used herein is a disease of the eye characterized by increased pressure inside the eye with resultant optic nerve damage. Glaucoma includes, but is not limited to, primary glaucoma, secondary glaucoma, juvenile glaucoma, congenital glaucoma, pseudoexfoliation glaucoma, acute angle closure glaucoma, absolute glaucoma, chronic glaucoma, narrow angle glaucoma, chronic open angle glaucoma, simplex glaucoma and familial glaucomas, including, without limitation, pigmentary glaucoma, high tension glaucoma, and low tension glaucoma and their related diseases.

[0049] The pharmaceutical compositions of the invention can be formulated for any suitable route of administration, e.g., intravenous, intraarterial, intramuscular, subcutaneous or intraperitoneal administration. Nevertheless, when used for reducing intraocular pressure, more particularly, for prevention or treatment of intraocular hypertension and/or glaucoma, the compositions are formulated as ophthalmic compositions, e.g., ophthalmic drops, emulsion, suspension, gel, ointment, or membranous ocular eye patches.

[0050] The ophthalmic compositions of the present invention can be provided in a variety of formulations and dosages. These compositions may be prepared by conventional techniques, e.g., as described in Remington: The Science and Practice of Pharmacy, 19th Ed., 1995. The compositions can be prepared, e.g., by uniformly and intimately bringing the active agent, i.e., the compound of the general formula

I, into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product into the desired formulation.

[0051] The ophthalmic compositions of the invention, intended for direct application to the eye, may be formulated so as to have both pH and tonicity compatible with the eye. This will normally require a buffer to maintain the pH of the composition at or near physiologic pH, i.e., in the range of 5-9, preferably 6 to 8, more preferably 6.8-7.4; and may further require a tonicity agent to bring the osmolality of the composition to a level at or near 210-320 milliosmoles per kilogram (mOsm/kg). In certain embodiments, the composition of the invention has an osmolality in the range of 50-700 mOsm/kg, preferably 100-600 mOsm/kg, more preferably 150-500 mOsm/kg, still more preferably 200-400 mOsm/kg, most preferably 200-350 mOsm/kg.

[0052] The ophthalmic compositions of the invention may be administered to the eye of the subject by any suitable means. In one embodiment, the composition is in the form of a liquid, emulsion, gel or suspension of the compound of the general formula I, and it is administered as drops, spray, or gel. In another embodiment, the active agent, i.e., the compound of the general formula I, is applied to the eye via liposomes. In a further embodiment, the active agent is contained within a continuous or selective-release device, e.g., membranes such as, but not limited to, those employed in the OcusertTM System (Alza Corp., Palo Alto, Calif.).

[0053] In one embodiment, the active agent can be contained within, carried by, or attached to contact lenses, which are placed on the eye. In other embodiments, the active agent is contained within a swab or sponge, or within a liquid spray, which is applied to the ocular surface. In a further embodiment, the active agent is directly injected into the ocular tissues, e.g., by subconjunctival, subscleral, or intravitreal injection, or onto the eye surface.

[0054] In addition to the active agent, the ophthalmic compositions of the present invention contain a physiologically compatible carrier or vehicle as those skilled in the ophthalmic art can select using conventional criteria. Such vehicles may be selected from known ophthalmic vehicles that include, *inter alia*, saline solution, water, polyethers such as polyethylene glycol, polyvinyls such as polyvinyl alcohol and povidone, cellulose derivatives such as methylcellulose and hydroxypropyl methylcellulose, cyclodextrins, in particular betahydroxypropyl cyclodextrin, petroleum derivatives, e.g., mineral oil and white petrolatum, animal fats such as lanolin, polymers of acrylic acid such as carboxypolymethylene gel, vegetable fats such as peanut oil, polysaccharides such as dextrans, an alginate such as sodium alginate optionally comprising guluronic acid and/or mannuronic acid, glycosaminoglycans such as sodium hyaluronate, and salts such as sodium chloride and potassium chloride.

[0055] The optimal dosage for administration will depend on the state of the patient, and will be determined as deemed appropriate by the practitioner. In particular, compositions for the treatment of glaucoma may be administered daily, twice daily, or 3-4 times daily, and/or upon the occurrence of symptoms associated with the condition; and over a period of time consistent with treatment of the ocular hypertension and glaucoma, e.g., for a period of weeks, months, years, or decades.

[0056] In still another aspect, the present invention provides a compound of the general formula I, including the compounds wherein Z_1, Z_2, if present, and Z_3 are each O^-, or a diastereoisomer or mixture of diastereoisomers thereof, for use in reducing intraocular pressure. In particular embodiments, the compound used according to the invention is compound **12**, **13**, more particularly **13A**, or **14**, preferably compound **13A**.

[0057] In yet another aspect, the present invention relates to use of a compound of the general formula I, including the compounds wherein Z_1, Z_2, if present, and Z_3 are each O^-, or a diastereoisomer or mixture of diastereoisomers thereof, for the preparation of a pharmaceutical composition, in particular, an ophthalmic composition, for reducing intraocular pressure. In particular embodiments, the compound used according to the invention is compound **12**, **13**, more particularly **13A**, or **14**, preferably compound **13A**.

[0058] In still a further aspect, the present invention relates to a method for reducing intraocular pressure in an individual in need thereof comprising administering to said individual a therapeutically effective amount of a compound of the general formula I, including the compounds wherein Z_1, Z_2, if present, and Z_3 are each O^-, or a diastereomer or mixture of diastereoisomers thereof. In particular embodiments, the compound used according to the method of the invention is compound **12**, **13**, more particularly **13A**, or **14**, preferably compound **13A**.

[0059] The invention will now be illustrated by the following non-limiting Examples.

EXAMPLES

Materials and Methods

General

[0060] All air and moisture sensitive reactions were carried out in flame-dried, argon flushed, two-neck flasks sealed with rubber septa. All reactants in moisture sensitive reactions were dried overnight in a vacuum oven, and the reagents were introduced by syringe. Progress of reactions was monitored by TLC on precoated Merck silica gel plates (60F-254). Visualization was accomplished by UV light. Flash chromatography was carried out on silica gel (Davisil Art. 1000101501). All commercial reagents were used without further purification, unless otherwise noted. All phosphorylation reactions were carried out in flame-dried, argon-flushed, two-neck flasks sealed with rubber septa. Nucleosides were dried in-vacuo overnight. Proton Sponge® was kept in a desiccator. Phosphorus oxychloride was distilled and kept under nitrogen.[52] Bpi was prepared according to literature.[52] Tri-*n*-butylammonium pyrophosphate solution were prepared as described previously.[53] The preparation of the tri-*n*-butylammonium-tri-*n*-octylammonium and the bis(trioctylammonium) 5'-monophosphate 5-OMe-uridine salts were achieved by eluting the uridine nucleotide derivative (obtained after LC separation) through an activated Dowex-H^+-form using deionized water to an ice-cooled EtOH solution containing 1 eq. tri-*n*-octylamine and 1 eq. tri-*n*-butylamine. The preparation of the tetra-*n*-butylammonium 5'-diphosphate 5-OMe-uridine salt was achieved by eluting the uridine nucleotide derivative (obtained after LC separation) through a CM

Sephadex previously washed with an excess of tetrabutylammonium aquase solution. 5-Methoxy uracil, 5-methoxyuridine, **1**, and 2',3'-*O*-methoxymethylidene-5-OMe-uridine, **2**, were prepared according to literature (Stout and Robins, 1972; Chesterfield *et al.*, 1960; Niedballa and Vorbruggen, 1976; Griffin *et al.*, 1967). 5-OMe-UMP and 5-OMe-UDP, **12**, were prepared as previously described (Niedballa and Vorbruggen, 1976). pH measurements were performed with a Metrohm pH electrode and a Metrohm 827 pH lab pH meter. Compounds were characterized by NMR using Bruker AC-200, DPX-300, or DMX-600 spectrometers. ^{1}H NMR spectra were recorded at 200, 300, or 600 MHz. Chemical shifts are expressed in ppm downfield from Me_4Si (TMS), used as an internal standard. Nucleotides were characterized also by ^{31}P NMR in D_2O, using 85% H_3PO_4 as an external reference on Bruker AC-200 and DMX-600 spectrometers. High resolution mass spectra were recorded on an AutoSpec Premier (Waters UK) spectrometer by chemical ionization. Nucleotides were analyzed under ESI (electron spray ionization) conditions on a Q-TOF micro-instrument (Waters, UK). Primary purification of the nucleotides was achieved on a LC (Isco UA-6) system using a Sephadex DEAE-A25 column, swollen in 1M $NaHCO_3$ at room temperature for 1 day. The resin was washed with deionized water before use. The LC separation was monitored by UV detection at 280 nm. A buffer gradient of NH_4HCO_3 was applied as detailed below. Final purification of the nucleotides was achieved on an HPLC (Hitachi Elite LaChrome) system, using a semi-preparative reverse-phase column (Gemini 5μ C-18 110A, 250 x 10.00 mm, 5 micron, Phenomenex, Torrance, USA). The purity of the nucleotides was evaluated with an analytical reverse-phase column system (Gemini 5μ C-18 110A, 150 mm x 4.60 mm; 5 μm; Phenomenex, Torrance, CA) using two solvent systems: solvent system I, (A) 100 mM triethylammonium acetate (TEAA), pH 7:(B) CH_3CN; solvent system II, (A) 10 mM PBS buffer, pH 7.4:(B) CH_3CN. The details of the solvent system conditions used for the separation of each product are given below. The purity of the nucleotides was generally ≥95%.

Stability assays

[0061] For the chemical stability assays, ^{31}P NMR spectra were recorded (isotope frequency of 240 MHz). NPP 1 and 3 enzymes were provided by the Center of Research in Rheumatology and Immunology, Laval University (Que´bec, Canada). Human blood serum was obtained from a blood bank (Tel-Hashomer Hospital, Israel). All stability experiments were performed in duplicates.

Evaluation of activity of analogues 13A/B, 15, 16 and 17 at $P2Y_{2/4/6}$ receptors

[0062] ***Cell Culture and Transfection***. Green fluorescent protein (GFP) constructs of human $P2Y_2$-R, $P2Y_4$-R and $P2Y_6$-R were expressed in 1321N1 astrocytoma cells, which lack endogenous expression of P2X- and P2Y-receptors. The respective cDNA of the receptor gene was cloned into a pEGFPN1 vector. After transfection using FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Mannheim, Germany), the cells were selected with 0.5 mg/ml G418 (geneticine; Merck Chemicals, Darmstadt, Germany) and grown in Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C and 5% CO_2. The expression and cell membrane localization of the respective P2Y receptors was confirmed through the analysis of the GFP fluorescence. The functionality of the expressed GFP-labeled receptor in cells was verified by recording a change of $[Ca^{2+}]_i$ after stimulation with the appropriate receptor agonist.

[0063] ***Single cell $[Ca^{2+}]$ measurements***. 1321N1 astrocytoma cells transfected with the respective plasmid for P2YR-GFP expression plated on coverslips (22 mm diameter) and grown to approximately 80% density were incubated with 2 μM fura 2/AM and 0.02% pluronic acid in Na-HBS buffer (Hepes buffered saline: 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM glucose, 20 mM Hepes/Tris pH 7.4) for 30 min at 37°C. The cells were superfused (1 ml/min, 37°C) with different concentrations of nucleotide in Na-HBS buffer, and the nucleotide-induced change of $[Ca^{2+}]_i$ was measured by detecting the respective emission intensity of fura 2/AM at 510 nm with after 340 nm and 380 nm excitations (Ubl *et al.*, 1998). The average maximal amplitude of the responses and the respective standard errors were calculated from the ratio of the fura 2/AM fluorescence intensities with excitations at 340 nm and 380 nm (only GFP-labeled cells were analysed). Microsoft Excel (Microsoft Corp., Redmond, WA, USA) and SigmaPlot (SPSS Inc., Chicago, IL, USA) were used to derive the concentration-response curves and EC_{50} values from the average response amplitudes obtained in at least three independent experiments (Ecke *et al.*, 2006, 2008). Only cells with a clear GFP-signal and with the typical calcium response kinetics upon agonist pulse application were included in the data analysis. The GFP-tagged P2Y receptors are suitable for pharmacological and physiological studies, as previously reported (Tulapurkar *et al.*, 2004, 2006; Zylberg *et al.*, 2007).

Evaluation of activity of analogues 14A/B at $P2Y_{2/4/6}$ receptors

[0064] ***Cell culture.*** 1321N1 cell lines stably expressing the human $P2Y_6$ receptors were grown as previously described in DMEM (5% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1X Glutamax, 10 mM Hepes and 0.5 mg/ml G-418) at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air (Gendron *et al.*, 2003).

[0065] ***Cytosolic $[Ca^{2+}]$ Measurement.*** 1321N1 cells ($10x10^6$ cells grown in 10 cm^2 dishes) were harvested by a brief trypsin/EDTA treatment, suspended in complete culture medium, and washed by centrifugation for 3 min at 100x*g* before being incubated with 1 μM Fluo 4/AM in 4.5 ml HBSS with Ca^{2+} and Mg^{2+} (Wisent, St. Bruno, QC) for 25 min at 37°C. Cells were washed by centrifugation (100x*g*, 3 min*)*, suspended in HBSS containing Ca^{2+} and Mg^{2+}, and incubated for 25 min at 37°C. Cells were washed and suspended in 16 ml of HBSS containing Ca^{2+} and Mg^{2+}. Cell suspension (2 ml) was gently stirred in a quartz cuvette while $[Ca^{2+}]_i$ and monitored on a RF-5301 PC Shimadzu spectrofluorometer with Panormama fluorescence 1.1 software (Man-Tech, Guelph, ON). Excitation wavelengths of 488 nm and emission wavelength of 520 nm were used to measure changes in intracellular Fluo 4 fluorescence intensity (F). At the end of each recording, maximal fluorescence (Fmax) and minimal fluorescence (Fmin) were determined by adding successively 0.1% Triton X-100 and 50 mM EDTA to cell suspensions. The following equation from Grynkiewicz *et al.* (1985) was used to relate the fluorescence intensity to Ca^{2+} levels: $[Ca^{2+}] = Kd \times (F\text{-}F\text{min})/(F\text{max-}F)$, wherein Kd is the Ca^{2+} dissociation constant of the indicator (345 nM).

Animals

[0066] Twenty-four male New Zealand white rabbits (2.5±0.5 kg) were kept in individual cages with free access to food and water on controlled 12 h/12 h light/dark cycles. All the protocols used adhere to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmology and Vision Research and also are in accordance with the European Communities Council Directive (86/609/EEC).

Intraocular pressure measurements

[0067] Intraocular pressure (IOP) was measured by means of a TonoVET rebound tonometer supplied by Tiolat Oy (Helsinki, Finland). The application of this tonometer to animals does not require the use of any anaesthetic. For single dose experiments, different analogues were applied unilaterally to the cornea at a concentration of 100 μM and a fixed volume of 10 μl. The contralateral eye received the same volume of saline solution (0.9% NaCl, vehicle). Two IOP measurements were taken before any analogue was instilled. Experiments were performed following a blinded design where no visible indication was given to the experimenter as to the nature of the applied solution. IOP was followed up to 8 h to study the time course of the effect. Some of the analogues were assayed over a range of doses from 1 nM to 100 μM to generate dose-response curves. For these experiments, IOP was measured as the maximal response obtained with each dose of the analogue. Dose-response curves were calculated by plotting the IOP value for given concentration versus that concentration (from 1 nM to 100 μM). pD2 values were obtained by fitting the values to a dose-response-curve equation according to ORIGIN 8.0 software. With the pD2 value it was possible to calculate the EC_{50} by multiplying by -1 and then take the antilogarithm. The obtained value is the EC_{50} expressed in molar concentration. In all experiments, on any given day, only a single dose was tested on a single animal, which was washed out at least 2 days between doses. The commercial hypotensive agents, Xalatan® (latanoprost;

0.005%), Trusopt® (dorzolamide hydrochloride (2%), and Pilocarpine were assayed by applying a volume of 40 µl.

Statistical analysis

[0068] All data are presented as the means±s.e.m. Significant differences were determined by two-tailed Student's t-tests. The plotting and fitting of dose-response curves was carried out with Microcal Origin v.7.0 software (Microcal Software, U.S.A).

Example 1: Synthesis of 5-OMe-uridine-5'-*O*-(α-boranodiphosphate), 13

Synthesis of 5-methoxyuridine, 1

[0069] 5-methoxyuridine, **1**, was synthesized as previously described (Stout and Robins, 1972; Chesterfield *et al.*, 1960; Niedballa and Vorbruggen, 1976), and was used, e.g., for the preparation of 5-OMe-UDP, **12**, as previously described (Ginsburg-Shmuel *et al.*, 2010).

Synthesis of 2',3'-O-methoxymethylidene-5-OMe-uridine, 2

[0070] The protected nucleoside, **2**, was synthesized as previously described (Griffin *et al.*, 1967). In particular, a suspension of 5-OMe-uridine, **1**, (300 mg, 1.09 mmol) and p-TsOH (catalytic amount) in trimethyl orthoformate (1.09 ml, 9.85 mmol, 9 eq) was prepared in a flamed-dried, nitrogen-flushed two-necked round bottom flask, and stirred at room temperature (RT). After 24 h, the solution became almost clear and TLC ($CHCl_3$:MeOH 8:2) showed two less polar spots and the complete disappearance of the starting material. Dowex (weak base) was added (0.31 gr, 1.09 mmol, 1 eq) and the mixture was stirred at RT for 3 h. The liquid was decanted and the MeOH was used for washes. The solution was evaporated to give an oil-like residue. Co evaporations with ether were done and a white solid was obtained (a mixture of two diastereomers: 331.8 mg, 96.3%).

[0071] ***Characterization of 2.*** ^{1}H-NMR (DMSO-d_6, 300 MHz): δ 11.54 (bs, 1H, NH), 7.46, 7.44 (2s, 1H, H-6), 6.101, 6.01 (2s, 1H, *CH*-OCH_3), 6.00, 5.86 (2d, $J = 2.9$ Hz, and $J = 2.7$ Hz, H-1', 1H), 5.21, 5.17 (2m, 1H, OH-5'), 4.99-5.05 (m, 2H, H-2'), 4.88, 4.82 (2dd, $J = 6.6$, 3.4 Hz and $J = 7.6$, 3.8 Hz, 1H, H-3'), 4.18 and 4.09

(2m, 1H, H-4'), 3.58-3.66 (m, 2H, H-5' and H-5"), 3.606 (s, 3H, C-OCH_3), 3.30, 3.21 (2s, 3H, CH-OCH_3) ppm. HR MALDI (positive) calcd for $C_{12}H_{16}N_2Na_1O_8$ 339.080 found 339.080.

Synthesis of analogue 13

[0072] As depicted in **Scheme 1** hereinafter, a solution of 2',3'-*O*-methoxymethylidene 5-OMe-uridine, **2** (327.1 mg, 1.03 mmol) in dry DMF (2 ml) was prepared in a flame-dried, argon flushed, two-neck flask. Dry pyridine (0.42 ml, 5.17 mmol, 5 eq) and a solution of 2-Cl-1,3,2-benzdioxaphosphorin-4-one (230.4 mg, 1.14 mmol, 1.1 eq) in dry dioxane (2 ml) were added and the solution was stirred at RT for 10 min. Then, a mixture of 1 M $(Bu_3NH^+)_2P_2O_7H_2^{-2}$ in DMF (1.55 ml, 1.55 mml, 1.5 eq) and Bu_3N (0.99 ml, 4.14 mmol, 4 eq) was added and the solution turned turbid and then clear again. Then, a 2 M solution of BH_3-SMe_2 complex in THF (5.17 ml, 10.34 mmol, 10 eq) was added. After 15 min, ethylene diamine (0.35 ml, 5.17 mmol, 5 eq) was added and a white precipitate was formed. After an hour at RT, the reaction was quenched with distilled water (1.4 ml) and the clear solution was evaporated and freeze-dried. TLC (isopropanol: 25% NH_4OH: H_2O 11:2:7) of the crude material showed a main polar product (R_f=0.35). The methoxymethylidene protecting group was removed by acidic hydrolysis (10% HCL solution was added until pH 2.3 was obtained). After 3 h at RT, the pH was rapidly raised to 9 by addition of 24% NH_4OH solution (pH 11), and the solution was stirred at RT for 45 min and then freeze-dried. The semisolid obtained after freeze-drying was chromatographed on an activated Sephadex DEAE-A25 column. The resin was washed with deionized water and loaded with the crude reaction residue dissolved in a minimal volume of water. The separation was monitored by UV detection (ISCO, UA-6) at 280 nm. A buffer gradient of 0-0.2 M NH_4HCO_3 (200 ml of each solution) followed by a second buffer gradient of 0.2-0.4 M NH_4HCO_3 (200 ml of each solution) were applied. The different fractions were pooled and freeze-dried three times to yield a white solid. Final separation of the diastereomers and purification of the relevant fractions was carried out on an HPLC system, using a semi-preparative reverse-phase column, under the conditions described bellow. The purity of the nucleotides was evaluated on an analytical reverse-phase column system, in two solvent systems as described below. Finally, aqueous solutions of the products were passed through a Dowex 50WX8-200 ion-exchange resin Na^+-form column and the products were eluted with deionized water to obtain the corresponding sodium salts after freeze-drying.

Separation of diastereoisomers 13A and 13B

[0073] The separation of analogue **13** diastereoisomers, **13A** and **13B**, was accomplished using a semipreparative reverse-phase Gemini 5µ column and isocratic elution with 94:6 (A) 100 mM TEAA, pH 7:(B) CH_3CN at a flow rate of 5 ml/min. Fractions containing purified isomers [R_t=8.97 min (**13A**); 13.45 min (**13B** isomer)] were collected and freeze-dried. Excess buffer was removed by repeated freeze-drying cycles, with the solid residue dissolved each time in deionized water. Diastereoisomers **13A** and **13B** were obtained at 50.9% overall yield (253.5 mg) after LC separation.

Characterization of 13A

[0074] ^{1}H NMR (D_2O; 600 MHz): δ 7.41 (s, 1H, H-6), 6.00 (d, J = 5.6, 1H, H-1'), 4.47 (t, J =5.5, 1H, H-3'), 4.42 (t, J =4.7, 1H, H-3'), 4.32 (m, 1H, H-4'), 4.28 (m, 1H, H-5'), 4.10 (m, 1H, H-5"), 3.83 (s, 3H, CH_3), 0.39 (m, 3H, BH_3) ppm. ^{31}P NMR (240 MHz, D_2O) δ: 80.43 (m, 1P, P_α-BH_3), -7.16 (d, J = 29.48 Hz, 1P, P_β) ppm. HR MALDI (negative) calcd for $C_{10}H_{18}B_1N_2O_{12}P_2$ 431.042 found 431.043. Purity data obtained on an analytical column: retention time: 3.88 min (94.48% purity) using solvent system I isocratic elution of 95:5 A:B over 10 min followed by a gradient from 95:5 to 85:15 over 2 min at a flow rate of 1 ml/min. Retention time: 2.71 min (95.31% purity) isocratic elution of 97.5:2.5 A:B over 8 min followed by a gradient from 97.5:2.5 to 85:15 over 2 min at a flow rate of 1 ml/min.

Characterization of 13B

[0075] ^{1}H NMR (D_2O; 600 MHz): δ 7.41 (s, 1H, H-6), 6.02 (d, J = 5.8, 1H, H-1'), 4.44 (m, 2H, H-3', H-2'), 4.27 (m, 2H, H-4', H-5'), 4.11 (m, 1H, H-5'), 3.83 (s, 3H, CH_3), 0.39 (m, 3H, BH_3) ppm. ^{31}P NMR (240 MHz, D_2O) δ: 80.83 (m, 1P, P_α-BH_3),

-7.51 (d, J = 32.4 Hz, 1P, P_β) ppm. HR MALDI (negative) calcd for $C_{10}H_{18}B_1N_2O_{12}P_2$ 431.042 found 431.043. Purity data obtained on an analytical column: retention time: 6.06 min (95.40% purity) using solvent system I isocratic elution of 95:5 A:B over 10 min followed by a gradient from 95:5 to 85:15 over 2 min at a flow rate of 1 ml/min. Retention time: 4.21 min (95.08% purity) isocratic elution of 99.5:0.5 A:B over 8 min followed by a gradient from 99.5:0.5 to 85:15 over 2 min at a flow rate of 1 ml/min.

Example 2: Synthesis of 5-OMe-uridine-5'-*O*-(α-boranotriphosphate), 14

[0076] 5-OMe-uridine-5'-*O*-(α-boranotriphosphate), **14**, was obtained as a by-product from the synthesis of **13** depicted in **Scheme 1**. After LC separation, the relevant fractions were pooled and freeze-dried three times to yield a white solid. Final separation of the diastereomers and purification of the relevant fractions was carried out on an HPLC system, using a semi-preparative reverse-phase column, under the conditions described below. The purity of the nucleotides was evaluated on an analytical reverse-phase column system, in two solvent systems as described below. Finally, aqueous solutions of the products were passed through a Dowex 50WX8-200 ion-exchange resin Na^+-form column and the products were eluted with deionized water to obtain the corresponding sodium salts after freeze-drying.

Separation of diastereoisomers 14A and 14B

[0077] The separation of analogue **14** diastereoisomers, **14A** and **14B**, was accomplished using a semipreparative reverse-phase Gemini 5µ column and isocratic elution with 93:7 (A) 100 mM TEAA, pH 7:(B) CH_3CN at a flow rate of 5 mL/min. Fractions containing purified isomers [Rt 6.15 min (**14A**); 9.22 min (**14B**) isomer)] were collected and freeze-dried. Excess buffer was removed by repeated freeze-drying cycles, with the solid residue dissolved each time in deionized water. Diastereoisomers **14A** and **14B** were obtained in 8.66% overall yield (51.7 mg) after LC separation.

Characterization of 14A

[0078] ^1H NMR (D_2O; 200 MHz): δ 7.32 (s, 1H, H-6), 6.00 (d, *J* = 5.5, 1H, H-1'), 4.38 (m, 2H, H-2', H-3'), 4.26 (m, 2H, H-4', H-5'), 4.08 (m, 1H, H-5"), 3.78 (s, 3H, CH_3), 0.39 (m, 3H, BH_3) ppm. ^{31}P NMR (81 MHz, D_2O) δ: 84.51 (m, 1P, P_α-BH_3), -10.33 (d, *J* = 19.8 Hz, 1P, P_γ), -22.48 (dd, *J* = 29.4, 19.8 Hz, 1P, P_β) ppm. HR MALDI (negative) calcd for $C_{10}H_{19}BN_2O_{15}P_3$ 511.009 found 511.008. Purity data obtained on an analytical column: retention time: 4.43 min (94.32% purity) using solvent system I isocratic elution of 93:7 A:B over 10 min followed by a gradient from 93:7 to 85:15 over 2 min at a flow rate of 1 mL/min. Retention time: 3.27 min (94.11% purity) isocratic elution of 97.5:2.5 A:B over 8 min followed by a gradient from 97.5:2.5 to 85:15 over 2 min at a flow rate of 1 mL/min.

Characterization of 14B

[0079] ^1H NMR (D_2O; 200 MHz): δ 7.32 (s, 1H, H-6), 6.00 (d, *J* = 6.1, 1H, H-1'), 4.38 (m, 2H, H-2', H-3'), 4.24 (m, 2H, H-4', H-5'), 4.10 (m, 1H, H-5"), 3.78 (s, 3H, CH_3), 0.37 (m, 3H, BH_3) ppm. ^{31}P NMR (81 MHz, D_2O) δ: 84.58 (m, 1P, P_α-BH_3), -10.20 (d, *J* = 19.5 Hz, 1P, P_γ), -22.46 (dd, *J* = 33.3, 19.5 Hz, 1P, P_β) ppm. Purity data obtained on an analytical column: retention time: 6.75 min (96.85% purity) using solvent system I isocratic elution of 93:7 A:B over 10 min followed by a gradient from 93:7 to 85:15 over 2 min at a flow rate of 1 mL/min. Retention time: 4.82 min (95.33% purity) isocratic elution of 97.5:2.5 A:B over 8 min followed by a gradient from 97.5:2.5 to 85:15 over 2 min at a flow rate of 1 mL/min.

Example 3: Synthesis of di-(5-OMe)-uridine 5',5"-P^1,P^3, triphosphate, 15

[0080] The tri-*n*-butylammonium-tri-*n*-octylammonium 5-OMe-uridine mono phosphate salt (160.8 mg, 0.18 mmol) was dissolved in dry DMF (0.7 ml), and added to a flamed-dried, nitrogen-flushed two-necked round bottom flask containing CDI (145.8 mg, 0.9 mmol, 5 eq). The reaction was stirred at RT. After 2 h TLC (isopropanol: 25% NH_4OH: H_2O 11:2:7) showed the presence of a less polar product (R_f=0.62) and the complete disappearance of the starting material (R_f=0.35). MeOH (0.06 ml, 1.62 mmol, 9 eq) was added to destroy CDI leftovers, and after 10 min, a solution of the tetra-*n*-butylammonium 5-OMe-uridine diphosphate, **12**, (0.18 mmol, 1 eq) in dry DMF (0.5 ml) and $MgCl_2$ (68.4 mg, 0.72 mmol, 4 eq) were added. The solution was stirred at RT and TLC monitoring after 24 hours showed the presence of a more polar product (R_f=0.39) and the complete disappearance of the intermediate. The solution was freeze-dried after the addition of water. The semisolid obtained after freeze-drying was chromatographed on an activated Sephadex DEAE-A25 column. The resin was washed with deionized water and loaded with the crude reaction residue dissolved in a minimal volume of water. The separation was monitored by UV detection (ISCO, UA-6) at 280 nm. A buffer gradient of 0-0.2 M NH_4HCO_3 (250 ml of each solution) followed by a second buffer gradient of 0.2-0.4 M NH_4HCO_3 (300 ml of each solution) were applied. The different fractions were pooled and freeze-dried three times to yield a white solid. Final purification of the relevant fractions was carried out on an HPLC system, using a semi-preparative reverse-phase column, under the conditions described below. The purity of the nucleotides was evaluated on an analytical reverse-phase column system, in two solvent systems as described below. Finally, aqueous solutions of the products were passed through a Dowex 50WX8-200 ion-exchange resin Na^+-form column and the products were eluted with deionized water to obtain the corresponding sodium salts after freeze-drying.

Purification of 15

[0081] The purification of analogue **15** was accomplished using a semipreparative reverse-phase Gemini 5μ column and isocratic elution with 96:4 (A) 100 mM TEAA, pH 7:(B) CH_3CN at a flow rate of 5 ml/min. The fraction containing the purified analogues (R_t=11.3 min) was collected and freeze-dried. Excess buffer was removed by repeated freeze-drying cycles, with the solid residue dissolved each time in deionized water. Analogue **15** was obtained at 51.8% overall yield (76.6 mg) after LC separation.

Characterization of 15

[0082] ^1H NMR (D_2O; 600 MHz): δ 7.31 (s, 2H, H-6), 5.90 (d, J = 5.2, 2H, H-1'), 4.38-4.40 (m, 4H, H-2, H-3), 4.21-4.25 (m, 6H, H-4'H-5', H-5"), 3.79 (s, 6H, CH_3)

ppm. ^{31}P NMR (240 MHz, D_2O) δ: -0.89 (d, J = 18.0 Hz, 1P, P_α), -22.26 (dd, J = 18.0, J = 18.3 Hz, 1P, P_β) ppm. HR MALDI (negative) calcd for $C_{20}H_{28}N_4O_{22}P_3$ 769.041 found 769.045. Purity data obtained on an analytical column: retention time: 3.72 min (97.21% purity) using solvent system I isocratic elution of 96:4 A:B over 10 min followed by a gradient from 96:4 to 85:15 over 2 min at a flow rate of 1 ml/min. Retention time: 2.47 min (97.35% purity) isocratic elution of 99.5:0.5 A:B over 8 min followed by a gradient from 99.5:0.5 to 85:15 over 2 min at a flow rate of 1 ml/min.

Example 4: Synthesis of di-(5-OMe)-uridine 5',5''-P^1,P^3,α-boranotriphosphate, 16

[0083] The tri-*n*-butylammonium-tri-*n*-octylammonium 5-OMe-uridine mono phosphate salt (0.201 mmol) was dissolved in dry DMF (0.4 ml), and added to a flamed-dried, nitrogen-flushed two-necked round bottom flask containing CDI (162.8 mg, 1.005 mmol, 5 eq). The reaction was stirred at RT. After 2 h TLC (NH_4OH:H_2O:2-propanol 2:7:11) showed the presence of a less polar product (R_f=0.62) and the complete disappearance of the starting material (R_f=0.35). MeOH (0.07 ml, 1.809 mmol, 9 eq) was added in order to destroy CDI leftovers, and after 10 min, a solution of **13** (0.201 mmol,1 eq) in dry DMF (1 ml) and $MgCl_2$ (76 mg, 0.804 mmol, 4 eq) were added. The solution was stirred at RT and TLC monitoring after 24 hours showed the presence of a more polar product (R_f=0.41) and the complete disappearance of the intermediate. The solution was freeze-dried after the addition of water. The semisolid obtained after freeze-drying was chromatographed on an activated Sephadex DEAE-A25 column. The resin was washed with deionized water and loaded with the crude reaction residue dissolved in a minimal volume of water. The separation was monitored by UV detection (ISCO, UA-6) at 280 nm. A buffer gradient of 0-0.2 M NH_4HCO_3 (200 ml of each solution) followed by a second buffer gradient of 0.2-0.4 M NH_4HCO_3 (250 ml of each solution) were applied. The different fractions were pooled and freeze-dried three times to yield a white solid. Final separation of the diastereomers and purification of the relevant fractions was carried out on an HPLC system, using a semi-preparative reverse-phase column, under the conditions described bellow. The purity of the nucleotides was evaluated on an analytical reverse-phase column system, in two solvent systems as described below. Finally, aqueous solutions of the products were passed through a Dowex 50WX8-200 ion-exchange resin Na^+-form column and the products were eluted with deionized water to obtain the corresponding sodium salts after freeze-drying.

Separation of diastereoisomers 16A and 16B

[0084] The separation of analogue **16** diastereoisomers, **16A** and **16B**, was accomplished using a semipreparative reverse-phase Gemini 5μ column and isocratic elution with 93:7 (A) 100 mM TEAA, pH 7:(B) CH_3CN at a flow rate of 5 mL/min. Fractions containing purified isomers [R_t=6.60 min (**16A**); 10.87 min (**16B** isomer)] were collected and freeze-dried. Excess buffer was removed by repeated freeze-drying cycles, with the solid residue dissolved each time in deionized water. Diastereoisomers **16A** and **16B** were obtained at 45.2% overall yield (74.4 mg) after LC separation.

Characterization of 16A

[0085] ^1H NMR (D_2O; 600 MHz): δ 7.33 (s, 1H, H-6_A), 7.32 (s, 1H, H-6_B), 6.02 (d, J = 6.3, 1H, H-$1'_A$), 6.02 (d, J = 5.6, 1H, H-$1'_B$), 4.37-4.44 (m, 4H, H-$2'_A$, H-$2'_B$, H-$3'_A$, H-$3'_B$), 4.22-4.29 (m, 4H, H-$4'_A$, H-$4'_B$, H-$5'_A$, H-$5'_B$), 4.26 (m, 1H, H-$5''_A$), 4.13 (m, 1H, H-$5''_B$), 3.83 (s, 6H, CH_{3A}, CH_{3B}), 0.49 (m, 3H, BH_3) ppm. ^{31}P NMR (240 MHz, D_2O) δ: 84.29 (m, 1P, P_α-BH_3), -11.03 (d, J = 18.3 Hz, 1P, P_γ), -22.52 (dd, J = 27.8, J = 18.3 Hz, 1P, P_β) ppm. HR MALDI (negative) calcd for $C_{20}H_{31}B_1N_4O_{21}P_3$ 767.078 found 767.079. Purity data obtained on an analytical column: retention time: 3.76 min (96.84% purity) using solvent system I isocratic elution of 94:6 A:B over 10 min followed by a gradient from 94:6 to 85:15 over 2 min at a flow rate of 1 ml/min. Retention time: 2.43 min (95.14% purity) isocratic elution of 97.5:2.5 A:B over 8 min followed by a gradient from 97.5:2.5 to 85:15 over 2 min at a flow rate of 1 ml/min.

Characterization of 16B

[0086] ^{1}H NMR (D_2O; 600 MHz): δ 7.33 (s, 1H, H-6_A), 7.31 (s, 1H, H-6_B), 6.02 (m, 1H, H-$1'_A$, H-$1'_B$), 4.35-4.41 (m, 4H, H-$2'_A$, H-$2'_B$, H-$3'_A$, H-$3'_B$), 4.24-4.32 (m, 5H, H-$4'_A$, H-$4'_B$, H-$5'_A$, H-$5'_B$, H-$5''_A$), 4.11 (m, 1H, H-$5''_B$), 3.80 (s, 6H, CH_{3A}, CH_{3B}), 0.43 (m, 3H, BH_3) ppm. ^{31}P NMR (240 MHz, D_2O) δ: 84.05 (m, 1P, P_α-BH_3), -11.09 (d, J = 18.3 Hz, 1P, P_γ), -22.55 (dd, J = 31.4, J = 18.3 Hz, 1P, P_β) ppm. HR MALDI (negative) calcd for $C_{20}H_{31}B_1N_4O_{21}P_3$ 767.078 found 767.079. Purity data obtained on an analytical column: retention time: 7.01 min (97.31% purity) using solvent system I isocratic elution of 94:6 A:B over 10 min followed by a gradient from 94:6 to 85:15 over 2 min at a flow rate of 1 ml/min. Retention time: 5.08 min (95.92% purity) isocratic elution of 97.5:2.5 A:B over 8 min followed by a gradient from 97.5:2.5 to 85:15 over 2 min at a flow rate of 1 ml/min.

Example 5. Synthesis of di-(5-OMe)-uridine 5''5''-P^1,P^3,β-boranotriphosphate, 17

[0087] The tri-*n*-butylammonium-tri-*n*-octylammonium 5-OMe-uridine mono phosphate salt (401.9 mg, 0.45 mmol) was dissolved in dry DMF (2 ml), and added to a flamed-dried, nitrogen-flushed two-necked round bottom flask containing CDI (364.5 mg, 2.25 mmol, 5 eq). The reaction was stirred at RT. After 2 h TLC (isopropanol: 25% NH_4OH: H_2O 11:2:7) showed the presence of a less polar producr (R_f=0.62) and the complete disappearance of the starting material (R_f=0.35). MeOH (0.09 ml, 2.25 mmol, 5 eq) was added in order to destroy CDI leftovers, and after 10 min, a solution of BPi (523.7 mg, 1.125 mmol, 2.5 eq) in dry DMF (0.5 ml) and $MgCl_2$ (342.7 mg, 3.6 mmol, 8 eq) were added. The solution was stirred at RT and TLC monitoring after 24 hours showed the presence of more polar products and the complete disappearance of the intermediate. The solution was freeze-dried after the addition of water. The semisolid obtained after freeze-drying was chromatographed on an activated Sephadex DEAE-A25 column. The resin was washed with deionized water and loaded with the crude reaction residue dissolved in a minimal volume of water. The separation was monitored by UV detection (ISCO, UA-6) at 280 nm. A buffer gradient of 0-0.2 M NH_4HCO_3 (200 ml of each solution) followed by a second buffer gradient of 0.2-0.4 M NH_4HCO_3 (300 ml of each solution) were applied. The different fractions were pooled and freeze-dried three times to yield a white solid. Final purification of the relevant fractions was carried out on an HPLC system, using a semi-preparative reverse-phase column, under the conditions described bellow. The purity of the nucleotides was evaluated on an analytical reverse-phase column system, in two solvent systems as described below. Finally, aqueous solutions of the products were passed through a Dowex 50WX8-200 ion-exchange Na^+-form resin column and the products were eluted with deionized water to obtain the corresponding sodium salts after freeze-drying.

Purification of 17

[0088] The purification of analogue **17** was accomplished using a semipreparative reverse-phase Gemini 5μ column and isocratic elution with 96:4 (A) 100 mM TEAA, pH 7:(B) CH_3CN at a flow rate of 5 ml/min. The fractions containing the purified analogues (R_t=6.13 min) were collected and freeze-dried. Excess buffer was removed by repeated freeze-drying cycles, with the solid residue dissolved each time in deionized water. Analogue **17** was obtained at 8% overall yield (30.8 mg) after LC separation.

Characterization of 17

[0089] ^1H NMR (D_2O; 600 MHz): δ 7.32 (s, 1H, H-6), 6.02 (m, 2H, H-1'), 4.41 (m, 4H, H-2', H-3'), 4.22 (m, 6H, H-4', H-5', H-5"), 3.80 (s, 6H, CH_3), 0.50 (m, 3H, BH_3) ppm. ^{31}P NMR (240 MHz, D_2O) δ: 80.43 (m, 1P, P_α-BH_3), -7.16 (d, J = 29.48 Hz, 1P, P_β) ppm. HR MALDI (negative) calcd for $C_{20}H_{31}B_1N_4O_{21}P_3$ 767.078 found 767.079. Purity data obtained on an analytical column: retention time: 6.09 min (96.33% purity) using solvent system I isocratic elution of 96:4 A:B over 10 min followed by a gradient from 96:4 to 85:15 over 2 min at a flow rate of 1 ml/min. Retention time: 5.34 min (96.12% purity) isocratic elution of 99.5:0.5 A:B over 8 min followed by a gradient from 99.5:0.5 to 85:15 over 2 min at a flow rate of 1 ml/min.

Example 6: Chemical stability of UDP, 11, 12 and 13A

[0090] The stability of UDP, **11**, **12** and **13A** in a buffer solution (pH 1.4) was evaluated by ^{31}P NMR at 37°C for monitoring possible dephosphorylation products (the signal of the phosphate hydrolysis products is at ~0 ppm, while that of the borano phosphate analogues is at ~85 ppm). NMR spectra were recorded on a Bruker DMX-600 spectrometer with a ^{31}P NMR probe (isotope frequency of 240 MHz) using 85% H_3PO_4 as an external reference.

[0091] Sodium salts of UDP, **11**, **12** and **13A** were dissolved in 0.45 ml of KCl/HCl buffer (pH 1.4) and D_2O (0.05 ml) was added. The final pH was adjusted to pH 1.4. pH measurements were performed with an Metrohm pH electrode and a Metrohm 827 pH meter. Spectra were recorded at 15 min, 1 h, or 24 h time intervals at 37°C. For experiments that were several days long, the solution was kept in an oil bath at 37°C and spectra were recorded at ca. 24 h time intervals. The phosphate ester hydrolysis rate was determined by measuring the change of the integration of one of the phosphates signals of the starting material with time.

[0092] The phosphate ester hydrolysis rate of **13A** was determined by measuring the changes in the integration of one of the phosphate signals with time, and fitted a pseudo-first-order reaction model, as shown in **Fig. 1**. In particular, during the hydrolysis of **13A**, we first observed a signal of inorganic phosphate emerging at 0.62 ppm, together with a signal for the remaining boranophosphate of 5-OMe-UMP(α-B) at 96.57 ppm. At the same time, the signals for P_β (-10.69 ppm) and P_α (86.55 ppm) of the **13A** decreased, indicating that the terminal phosphate was rapidly lost under these conditions. Next, two additional signals appeared at 7.31 and 4.42 ppm indicating the formation of the 5-OMe-uridine-H-phosphonate and inorganic H-phosphonate moieties, respectively. Rate constant of $1.31 \times 10^{-5}\ s^{-1}$ ($t_{1/2} = 16.9$ h) was established for **13** as compared with $6.66\times10^{-7}\ s^{-1}$ ($t_{1/2} = \sim12$ days), $1.14x10^{-5}\ s^{-1}$ ($t_{1/2} = 16.8$ h) and $6.25x10^{-7}\ s^{-1}$ ($t_{1/2} = \sim13$ days) for UDP, **11** and **12**, respectively.

Example 7: Resistance of UDP, 12 and 13A to degradation by NPP1 and NPP3

[0093] In this study, the hydrolysis rate of analogue **13A** as compared to UDP and analogue **12**, by human NPP1 and NPP3 after incubation at 37°C in appropriate buffer, was determined.

[0094] 56.15 µg or 57.78 µg of human NPP1 or NPP3 extract, respectively, was added to 0.575 ml the incubation mixture (1 mM $CaCl_3$, 200 mM NaCl, 10 mM KCl and 100 mM Tris, pH 8.5) and preincubated at 37°C for 3 min. Reaction was initiated by the addition of 0.015 ml of 4 mM of UDP, **12** or **13A**. The reaction was stopped after incubation of 2 h or 3 h with NPP1 or NPP3, respectively, by transferring a 0.1 ml aliquot from the reaction mixture to 0.350 ml ice-cold 1 M perchloric acid. These samples were centrifuged for 1 min at 10000xg. Supernatants were neutralized with 1 M KOH in 4°C and centrifuged 1 min at 10000xg. The reaction mixture was filtered and freeze-dried. The hydrolysis rates of the analogues UDP, **12** and **13A** by NPP1 or NPP3 were determined by measuring the change in the integration of the HPLC peaks for each analogue over time *vs.* control. The percentage of compound degradation was calculated *vs.* control, to take into consideration the degradation of the compounds due to the addition of acid to stop the enzymatic reaction. Therefore, each of the samples was compared to a control which was transferred to acid, but to which no enzyme was added. The percentage of degradation was calculated from the area under the curve of the nucleoside monophosphate peak, after subtraction of the control, which is the amount of the nucleoside monophosphate peak formed due to chemical acidic hydrolysis.

Table 1: hydrolysis percentage of UDP, **12** and **13A** by NPP1 and NPP3

Analogue	**Hydrolysis percentage***	
	NPP1	**NPP3**
UDP	50%	45%
12	49%	36%
13A	**15%**	**28%**

[*] After incubation at 37°C in buffer (1 mM $CaCl_3$, 200 mM NaCl, 10 mM KCl and 100 mM Tris, pH 8.5) for 2 or 3 h, with NPP1 or NPP3, respectively. Values represent mean ± S.D. of two experiments ($p<0.05$).

[0095] As summarized in **Table 1**, in the presence of NPP1, UDP was 50% hydrolyzed to UMP after 2 h of incubation with the enzyme. Analogue **12** was similarly hydrolyzed to 5-OMe-UMP (49%). Yet, analogue **13A** was only 15% hydrolyzed after the same incubation time. Analogue **13A** exhibited relative stability also with regard to hydrolysis by NPP3 as compared to analogues UDP and **12**, undergoing 28%, 45% and 36% hydrolysis, respectively, to the corresponding nucleoside 5'-monophosphates after incubation of 3 h.

Example 8: Stability of UDP, 11, 12 and 13A in human blood serum

[0096] Blood serum contains dephosphorylating enzymes and therefore provides a good model system for estimation of the *in vivo* stability of nucleotide analogues. In this study, the half-life of analogue **13A** in human blood serum as compared to that of UDP, **11** and **12**, was determined.

[0097] The assay mixture containing 0.1 mg each analogue in deionized water (4.5 μl), human blood serum (180 μl) and RPMI-1640 medium (540 μl) (Eliahu *et al.*, 2010a) was incubated at 37°C for 0-24 h. At 0.5-12 h intervals, each sample was heated to 80°C for 30 min, treated with CM Sephadex (1-2 mg), shaken for 2 h and centrifuged for 6 min (12000 rpm), and the aqueous layer was collected and extracted with chloroform (2x500 μl). The aqueous layer was freeze-dried and then dissolved in deionized water (100 μl). Samples were loaded onto an activated Starta X-AW weak anion exchange cartridge, washed with H_2O (1 ml) and eluted with MeOH:H_2O (1:1, 1 ml) followed by NH_4OH:MeOH:H_2O (2:25:73, 1 ml), and then freeze-dried. The resulting residue was analyzed by HPLC on a Gemini analytical column (5μ C-18 557 110A; 150 mm x 4.60 mm), using gradient elution with solvent system I (for UDP and **12** - A:B 96:4 over 10 min) or II (for uridine-5'-*O*-(α-borano diphosphate) and **13A** - A:B 97.5:2.5 over 10 min) at a flow rate of 1 ml/min. The hydrolysis rates of the analogues UDP, uridine-5'-*O*-(α-borano diphosphate), **12** and **13A** with blood serum were determined by measuring the change in the integration of the HPLC peaks for each analogue over time *vs.* control. The percentage of compound degradation was calculated *vs.* control, to take into consideration the degradation of the compounds due to the work up following the enzymatic reaction. Therefore, each of the samples was compared to a control which was put through the same workup, but to which no serum was added. The percentage of degradation was calculated from the area under the curve of the nucleoside monophosphate peak, after subtraction of the control, which is the amount of the nucleoside monophosphate peak formed due to chemical hydrolysis.

[0098] As shown in **Figs. 2A-2B**, UDP was hydrolyzed to UMP, followed by further degradation to uridine, with a half-life of 2.4 h (**2A**). Yet, analogue **12** was hydrolyzed to the corresponding nucleoside 5'-monophosphate and nucleoside with a half-life of 11.9 h, and **11** displayed a half-life of 21 h. **13A** was hydrolyzed to 5-OMe-UMP(α-B) and then to 5-OMe-uridine with a half-life of 17 h (**2B**).

Example 9: Activity of analogues 13-17 at $P2Y_{2/4/6}$-receptors

[0099] As described in Ginsburg-Shmuel *et al.* (2010), both 5-methoxyuridine triphosphate (5-OMe-UTP) and 5-methoxyuridine diphosphate (5-OMe-UDP), **12**, were found to be agonists of the $P2Y_6$-receptors with EC_{50} of 0.9 and 0.08 μM, respectively.

[00100] In this study, analogues **13-17** were tested for their potency and selectivity at $P2Y_{2,4,6}$-receptors based on measurements of increases in intracellular calcium concentrations in 1321N1 astrocytoma cells transfected with the respective plasmid, as described in Materials and Methods, and the results are summarized in **Table 2**.

Table 2: Potencies (EC_{50} in μM) of analogues **13-17** at the $P2Y_{2/4/6}$-receptors in 1321N1 astrocytoma cells

Receptor subtype	**13**		**14**		**15**	**16**		**17**	**UDP**	**UTP**
	A	**B**	**A**	**B**		**A**	**B**			
$P2Y_2$-R	n.d.r	n.d.r	n.d.r	n.d.r	n.d.r	n.d.r	n.d.r	n.d.r	-	0.14
$P2Y_4$-R	n.d.r	n.d.r	n.d.r	n.d.r	n.d.r	n.d.r	n.d.r	n.d.r	-	0.9
$P2Y_6$-R	**0.008±** 0.003	4.3± 1.9	11.23 ±0.63	20.11 ±0.70	> 10[a]	**0.06±** 0.02	2.2± 0.5	0.2± 0.04	0.15± 0.03	-

n.d.r. - no detectable response for nucleotide concentrations of up to 100 μM.

[a] - for nucleotide concentrations of up to 100 μM it was not possible to calculate an EC_{50} value, since the plateau of the rise of $[Ca^{2+}]_i$ was not reached.

[00101] As shown, **13A** was a potent and selective agonist at the $P2Y_6$ receptor, when expressed in 1321N1 astrocytoma cells and was more potent than the standard agonist UDP. The introduction of a chiral center in 5-OMe-UDP by BH_3^- substitution of the non-bridging oxygen at Pα reveals stereo-selectivity preference of the receptor for the A-isomer over the B-isomer. The A-isomer of the mono-nucleotide derivative **13A** (R_p isomer) was the most potent agonist among the tested nucleotides with EC_{50}=0.008 μM, and was more than 500-fold more potent than the corresponding B-isomer **13B** (EC_{50}=4.3 μM, **Fig. 3A**) and 19-fold more potent than the endogenous agonist UDP (EC_{50}=0.15 μM). For the di-nucleotide derivatives of 5-OMe-UDP, **16A** and **16B**, a similar stereo-selectivity could be observed, although less pronounced, wherein the potency of the A-isomer (R_p isomer) (EC_{50}=0.06 μM) was similar to that of UDP (**Fig. 3B**) but only about 37-fold higher than that of the corresponding B-isomer (EC_{50}=2.2 μM).

[00102] The preference of $P2Y_6$-R for R_p isomer has not been observed before. However, we have already described diastereoselective properties of $P2Y_1$- and $P2Y_{11}$-receptors which displayed preference for the R_p and S_p isomers of borano-phosphate and phosphorothioate adenine nucleotides, respectively (Major *et al.*, 2004; Ecke *et al.*, 2006). Based on our computational studies of $P2Y_1$-R, we previously suggested that a hard Mg^{2+} ion, which is bound to the nucleotide inside the receptor, binds preferentially the hard P_α oxygen atom in ATP-α-B analogues, rather than the borane group. Thus, in the ATP-α-B (S_p) isomers, the P_α oxygen is not in a position to coordinate the Mg^{2+} ion, and in this case the coordination occurs probably via $P_{\beta,\gamma}$. Hence, this isomer loses a tight interaction, possibly with Mg^{2+} ion, resulting eventually in its higher EC_{50} values (Major *et al.*, 2004). Similarly, we assume that **13B** is less potent than **13A** due to loss of binding interactions of $P2Y_6$-R with P_α of **13B**.

[00103] Although borano-substitution enhanced dramatically the potency of **12** at $P2Y_6$-R, the corresponding triphosphate mono-nucleotide, **14**, was hardly active at the $P2Y_6$-R, due to the preference of the receptor for three phosphate negative charges (Jacobson *et al.*, 2009; Shaver *et al.*, 2005). Likewise, at the $P2Y_2$-R, **14** was completely inactive, even though it is similar to the receptor's endogenous agonist, UTP. The inactivity of **14** as opposed to the activity of 5-OMe-UTP at

$P2Y_2$-R (EC_{50}=2 μM, compared to UTP, EC_{50}=0.1 μM) (Ginsburg-Shmuel *et al.*, 2010), implies that $P2Y_2$-R does not tolerate the P_α-borano group. This observation was already made before for ATP(α-B) which elicited a very weak response at the $P2Y_2$ receptor, as compared to the endogenous ligands ATP and UTP (Tulapurkar *et al.*, 2004).

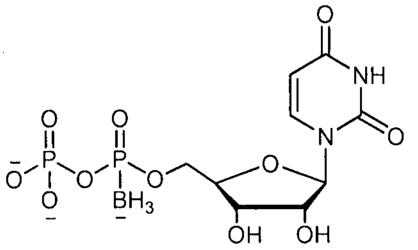
[00104] Borano-dinucleotide derivative **16A** is about 9-times less potent than its mono-nucleotide counterpart **13A**. Analogue **17**, a P_β-borano dinucleotide derivative of 5-OMe-UDP is equipotent to UDP (EC_{50}=0.2 μM) (**Fig. 3D**). Apparently, the decrease in activity of **16A** *vs.* **13A** is due to the requirement for a terminal phosphate for molecular recognition by $P2Y_6$-R. Even so, **16A** is still more active than **15** or **17** since it is structurally more similar to **13A**. The least active $P2Y_6$-R agonist is **15** which is the dinucleotide bearing two methoxy substitutions, one on each of the uracil rings. Surprisingly, this analogue was less active at $P2Y_6$-R than the previously reported dinucleotide triphosphate, Up_3U. Apparently, the double 5-OMe substitution in dinucleotide **15** noticeably reduced the potency at the $P2Y_6$ receptor (**Fig. 3C**). A plateau for the intracellular calcium response could not be reached for nucleotide concentrations of up to 100 μM. Nevertheless, similarly to the mono-nucleotides, the beneficial effect of the borano group in enhancing potency at the $P2Y_6$-R is evident: all borano-bearing nucleotides, either at P_α or P_β were far more active than their non-borano counterparts - **13A** *vs.* **12**, and **16A** and **17** *vs.* **15**.

[00105] All the tested nucleotides were inactive at the $P2Y_2$-receptor and $P2Y_4$-receptor in 1321N1 cells and at 1321N1 wild type cells, demonstrating specificity.

Example 10: Analogues 12 and 13A reduce IOP in normotense rabbits

[00106] The reduction of intraocular pressure (IOP) in normotense rabbits by the various 5-methoxyuridine nucleotide and dinucleotide derivatives was compared to that of UDP, which is the endogenous $P2Y_6$-receptor ligand, as a control, and to that of certain marketed drugs, and as shown in **Fig. 4**, analogue **12** reduced IOP by 31%, and analogue **13A** reduced IOP by 45%, more than any marketed drug, e.g., Xalatan®, Trusopt®, and Pilocarpine. The duration of action was about 4 h.

APPENDIX



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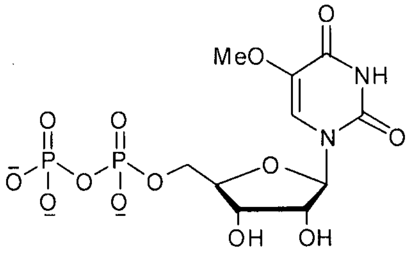

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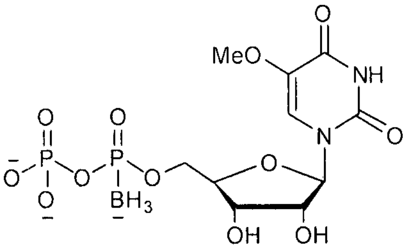
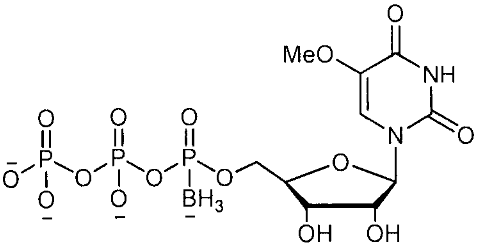
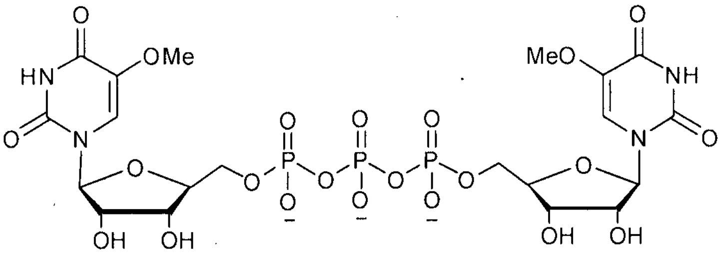


13A/B

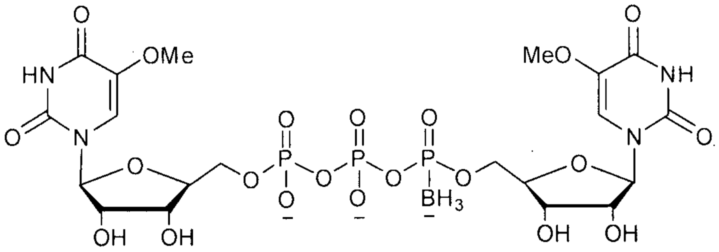


14A/B

15



16A/B



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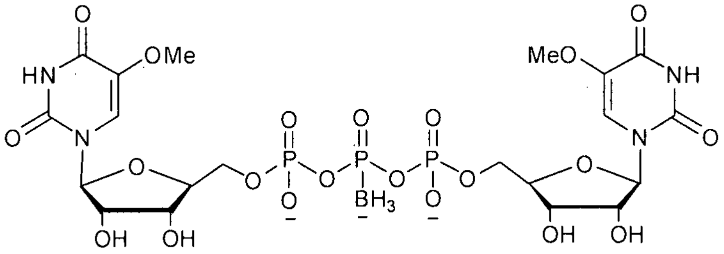
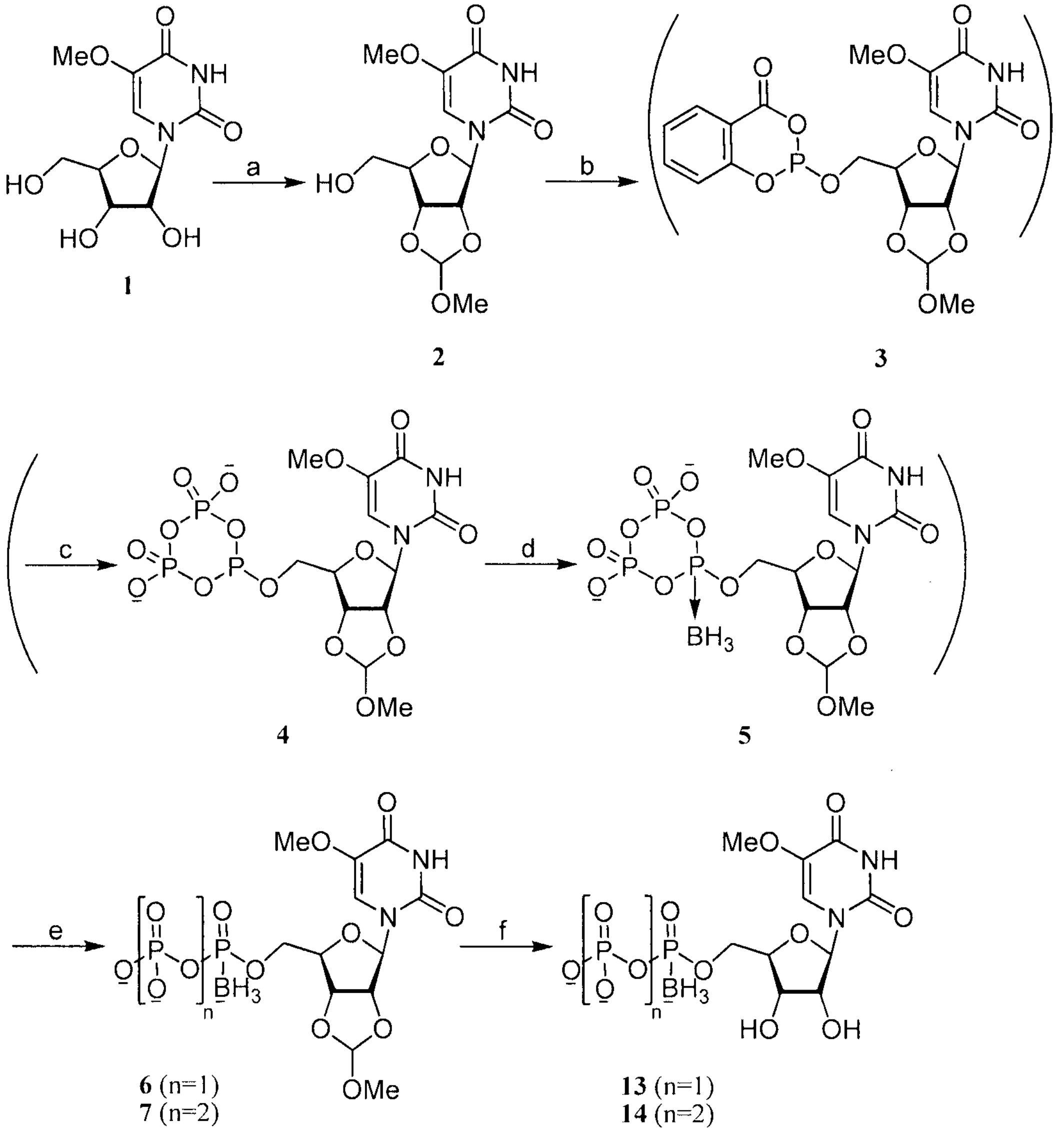


Scheme 1: Synthesis of analogues **13** and **14**



Reaction conditions

a) (1) $HC(OMe)_3$, *p*-TsOH, RT, overnight; and (2) Dowex (weak base), RT, 3 h, 96.3%; **b**) 2-Cl-1,3,2-benzdioxaphosphorin-4-one, dry DMF, dry dioxane, RT, 10 min; **c**) 1 M $P_2O_7H_2^{2-}(Bu_3N^+H)_2$ in dry DMF, Bu_3N, RT, 5 min; and **d**) 2 M $BH_3{\cdot}SMe_2$ in THF, RT, 15 min; **e**) ethylenediamine, RT, 10 min; and **f**) (1) 10% HCl, pH 2.3, RT, 3 h; and (2) 24% NH_4OH, pH 9, RT, 45 min. Compounds **6** and **7** were obtained as a mixture. Compounds **13** and **14** were obtained in a yield of 50.9% and 9%, respectively, each as a mixture of two diastereoisomers.

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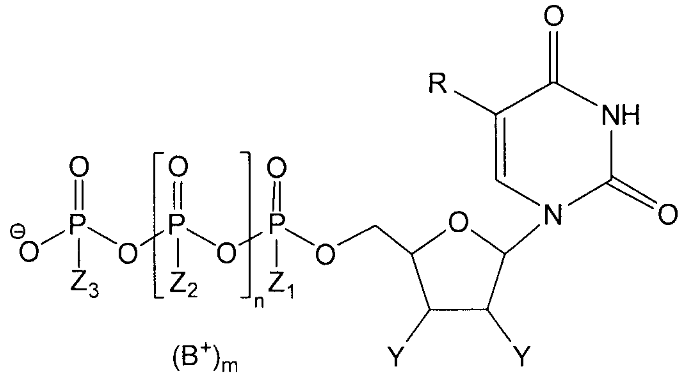
CLAIMS

1. A compound of the general formula I:

I



or a diastereoisomer or mixture of diastereoisomers thereof,

wherein

R is -O-(C_1-C_8)alkyl, or -S-(C_1-C_8)alkyl;

Y each independently is H, or -OH;

Z_1, Z_2 and Z_3 each independently is O^-, or BH_3^-;

n is 0 or 1;

m is 3 or 4; and

B^+ represents a pharmaceutically acceptable cation,

but excluding the compounds wherein Z_1, Z_2, if present, and Z_3 are each O^-.

2. The compound of claim 1, wherein R is -O-(C_1-C_8)alkyl, preferably -O-(C_1-C_4)alkyl, more preferably $-OCH_3$ or $-OC_2H_5$.

3. The compound of claim 1, wherein R is -S-(C_1-C_8)alkyl, preferably -S-(C_1-C_4)alkyl, more preferably $-SCH_3$ or $-SC_2H_5$.

4. The compound of claim 1, wherein n is 0, and at least one of Z_1 and Z_3 is BH_3^-; or n is 1, and at least one of Z_1 to Z_3 is BH_3^-.

5. The compound of claim 4, wherein n is 0, and (i) Z_1 is BH_3^-, and Z_3 is O^-; (ii) Z_3 is BH_3^-, and Z_1 is O^-; or (iii) Z_1 and Z_3 are BH_3^-.

6. The compound of claim 4, wherein n is 1, and (i) Z_1 is BH_3^-, and Z_2 and Z_3 are O^-; (ii) Z_2 is BH_3^-, and Z_1 and Z_3 are O^-; (iii) Z_3 is BH_3^-, and Z_1 and Z_2 are O^-;

(iv) Z_1 and Z_2 are BH_3^-, and Z_3 is O^-; (v) Z_1 and Z_3 are BH_3^-, and Z_2 is O^-; (vi) Z_2 and Z_3 are BH_3^-, and Z_1 is O^-; or (vii) Z_1 to Z_3 are BH_3^-.

7. The compound of any one of claims 1 to 6, wherein R is -O-(C_1-C_4)alkyl, preferably $-OCH_3$ or $-OC_2H_5$, n is 0, and (i) Z_1 is BH_3^-, and Z_3 is O^-; (ii) Z_1 is O^-, and Z_3 is BH_3^-; or (iii) Z_1 and Z_3 are BH_3^-.

8. The compound of claim 7, wherein R is $-OCH_3$, Y each is -OH, n is 0, Z_1 is BH_3^-, and Z_3 is O^- (herein identified compound **13**).

9. The compound of claim 8, characterized by being the isomer with a retention time (R_t) of 8.97 min when separated from a mixture of diastereoisomers using a semi-preparative reverse-phase Gemini 5μ column (C-18 110A, 250×10 mm, 5 micron), and isocratic elution [100 mM triethylammonium acetate, pH 7: CH_3CN, 94:6] with flow rate of 5 ml/min (herein identified compound **13A**).

10. The compound of claim 7, wherein R is $-OCH_3$, Y each is -OH, n is 1, Z_1 is BH_3^-, and Z_2 and Z_3 is O^- (herein identified compound **14**).

11. The compound of any one of claims 1 to 6, wherein R is -O-(C_1-C_4)alkyl, preferably $-OCH_3$ or $-OC_2H_5$, n is 1, and (i) Z_1 is BH_3^-, and Z_2 and Z_3 is O^-; (ii) Z_2 is BH_3^-, and Z_1 and Z_3 are O^-; (iii) Z_3 is BH_3^-, and Z_1 and Z_2 are O^-; (iv) Z_1 and Z_2 are BH_3^-, and Z_3 is O^-; (v) Z_1 and Z_3 are BH_3^-, and Z_2 is O^-; (vi) Z_2 and Z_3 are BH_3^-, and Z_1 is O^-; or (vii) Z_1 to Z_3 are BH_3^-.

12. The compound of any one of claims 1 to 11, wherein B is a cation of an alkali metal, NH_4^+, an organic cation of the formula R_4N^+ wherein each one of the Rs independently is H or C_1-C_{22}, preferably C_1-C_6, alkyl, a cationic lipid or a mixture of cationic lipids.

13. A pharmaceutical composition comprising a compound of the general formula I as claimed in claim 1, or a diastereoisomer or mixture of diastereoisomers thereof, and a pharmaceutically acceptable carrier or diluent.

14. A pharmaceutical composition for reducing intraocular pressure, comprising a compound of the general formula I in claim 1, or a diastereoisomer or mixture of diastereoisomers thereof, and a pharmaceutically acceptable carrier or diluent.

15. The pharmaceutical composition of claim 14, comprising compound **13**, preferably **13A**, or **14**, or a compound of the general formula I wherein R is $-OCH_3$, n is 0, Y each is -OH, and Z_1 and Z_3 are O^- (compound **12**).

16. The pharmaceutical composition of claim 14, for prevention or treatment of intraocular hypertension and/or glaucoma.

17. The pharmaceutical composition of claim 16, wherein the glaucoma is primary open angle glaucoma, normal pressure glaucoma, acute angle closure glaucoma, absolute glaucoma chronic glaucoma, congenital glaucoma, juvenile glaucoma, narrow angle glaucoma, chronic open angle glaucoma, or simplex glaucoma.

18. The pharmaceutical composition of any one of claims 14 to 17, formulated as ophthalmic drops, emulsion, suspension, gel, ointment, or a membranous ocular eye patch.

19. A compound of the general formula I in claim 1, or a diastereoisomer or mixture of diastereoisomers thereof, for use in reducing intraocular pressure.

20. Use of a compound of the general formula I in claim 1, or a diastereoisomer or mixture of diastereoisomers thereof, for the preparation of a pharmaceutical composition for reducing intraocular pressure.

21. A method for reducing intraocular pressure in an individual in need thereof comprising administering to said individual a therapeutically effective amount of a compound of the general formula I in claim 1, or a diastereomer or mixture of diastereoisomers thereof.

Fig. 1

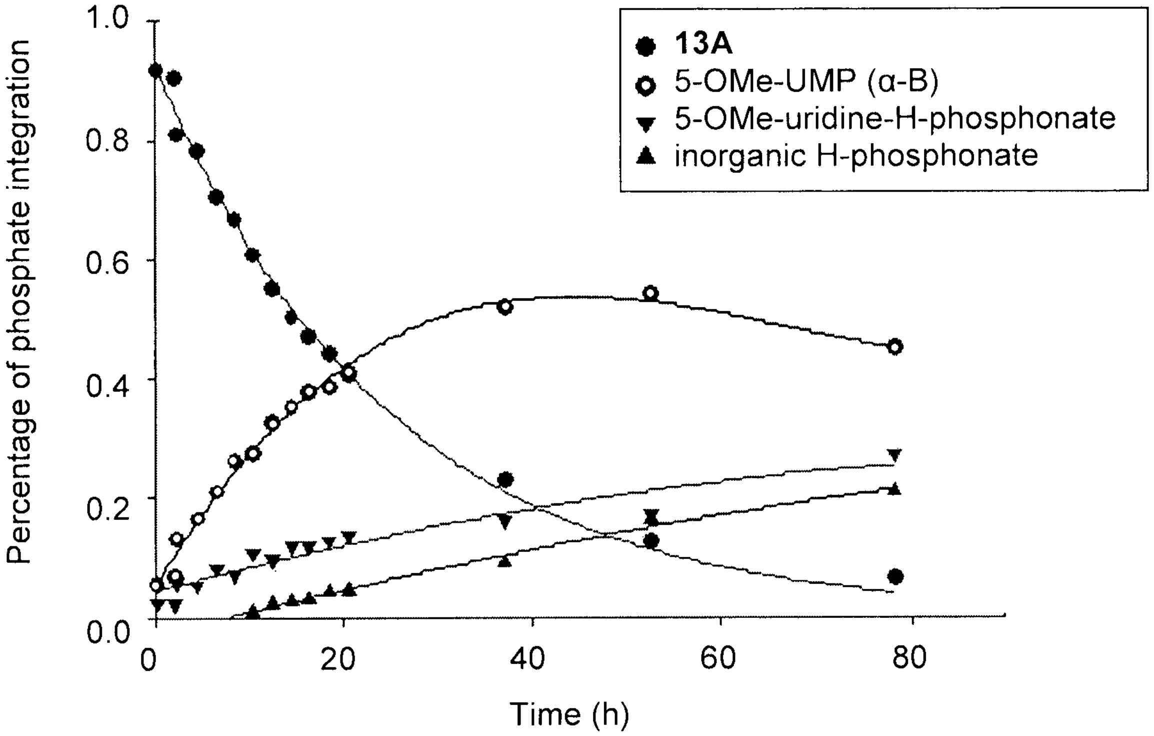

Percentage of phosphate integration
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
Time (h)
● 13A
○ 5-OMe-UMP (α-B)
▼ 5-OMe-uridine-H-phosphonate
▲ inorganic H-phosphonate

Fig. 2A

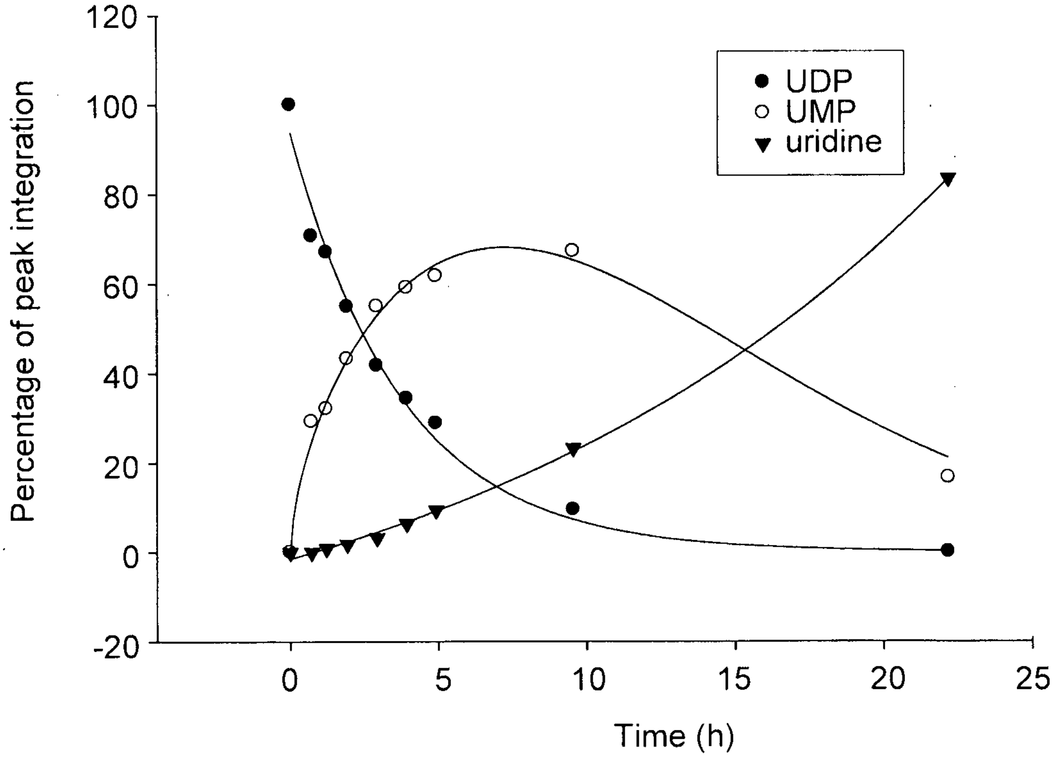

Percentage of peak integration
120
100
80
60
40
20
0
-20
0
5
10
15
20
25
Time (h)
● UDP
○ UMP
▼ uridine

Fig. 2B

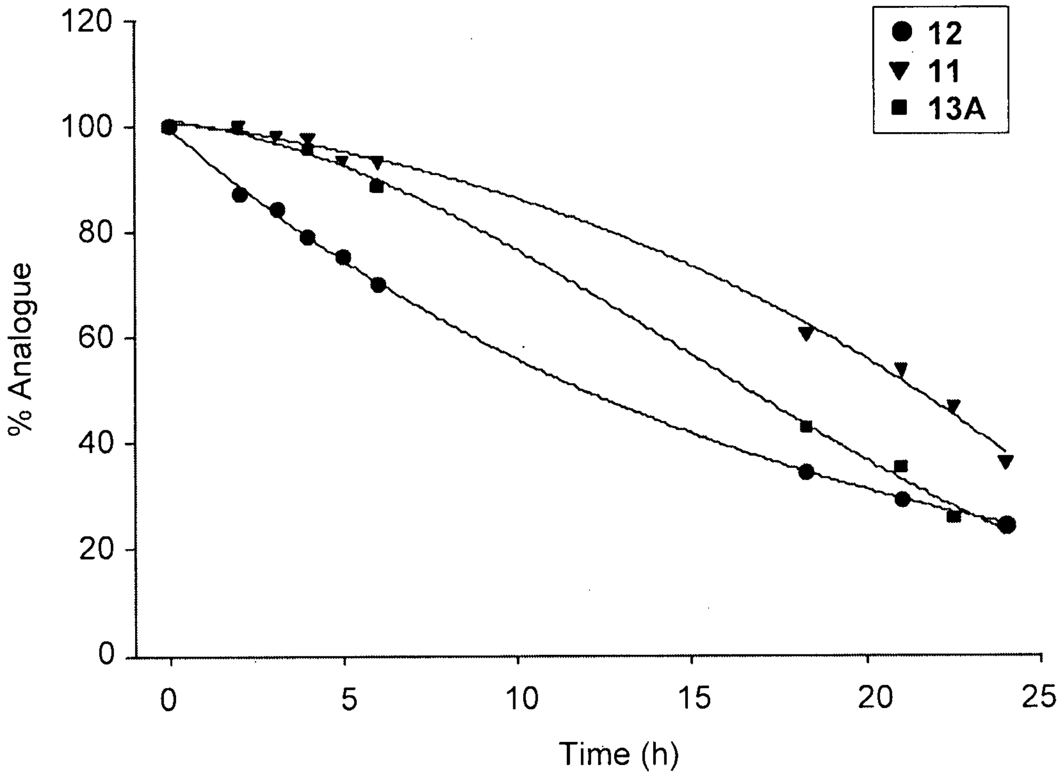

12
11
13A
% Analogue
Time (h)
0
20
40
60
80
100
120
0
5
10
15
20
25

Fig. 3A

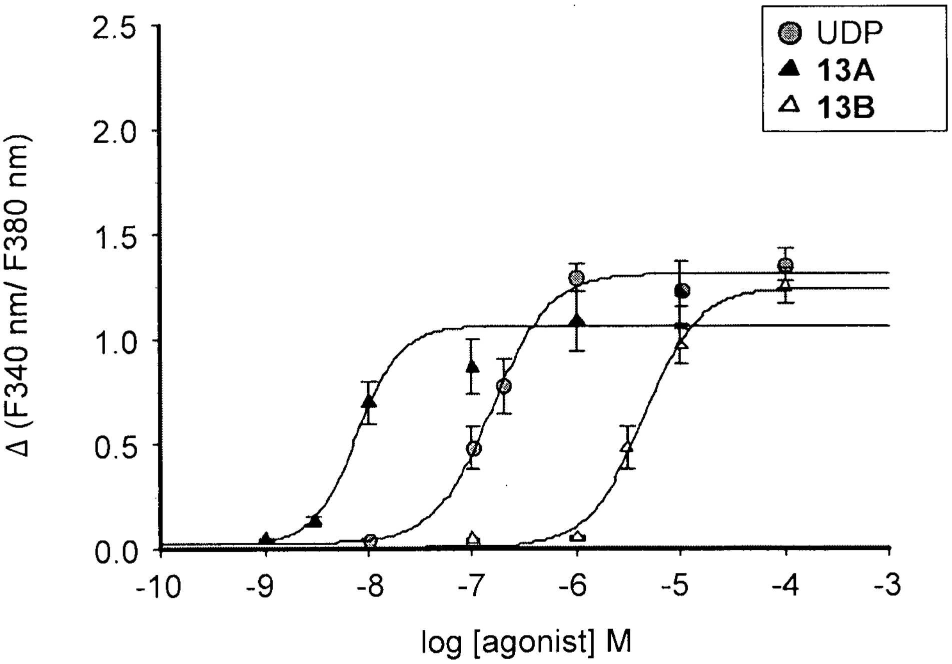

UDP
13A
13B
Δ (F340 nm/ F380 nm)
log [agonist] M
0.0
0.5
1.0
1.5
2.0
2.5
-10
-9
-8
-7
-6
-5
-4
-3

Fig. 3B

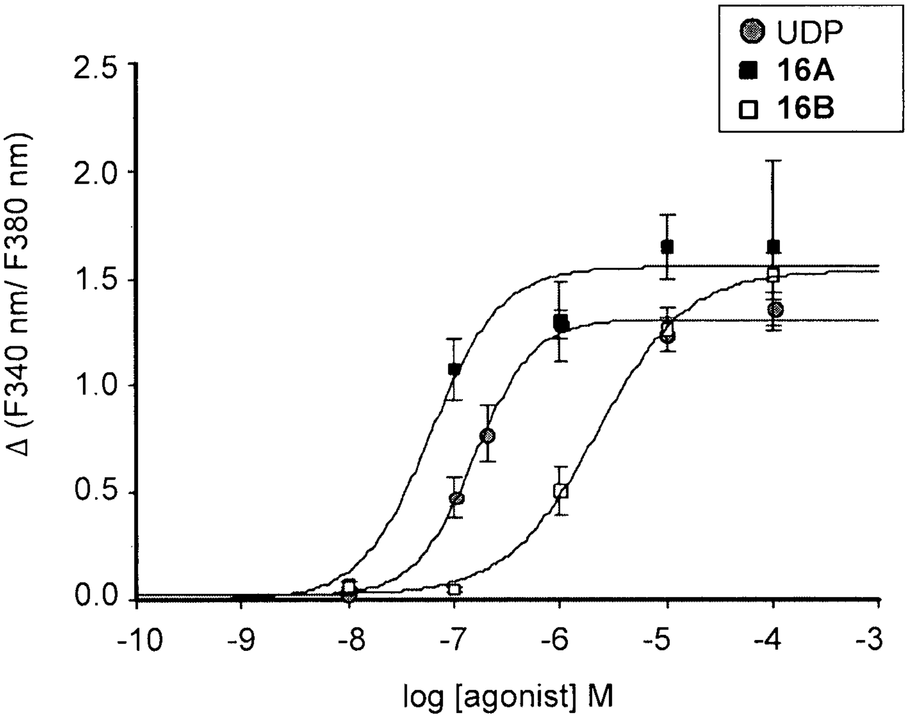

UDP
16A
16B
Δ (F340 nm/ F380 nm)
2.5
2.0
1.5
1.0
0.5
0.0
-10
-9
-8
-7
-6
-5
-4
-3
log [agonist] M

Fig. 3C

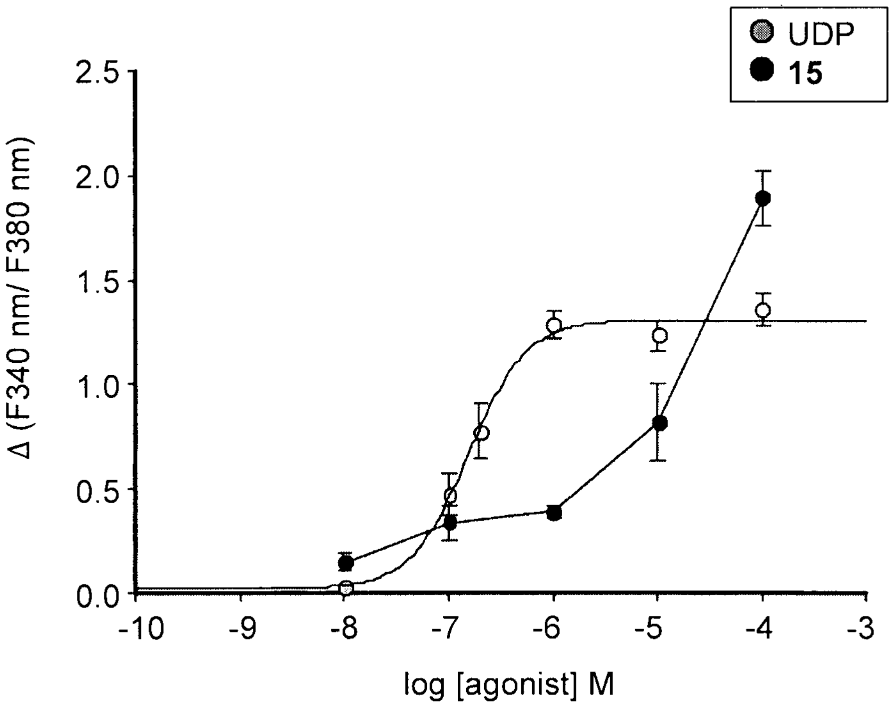

UDP
15
Δ (F340 nm/ F380 nm)
2.5
2.0
1.5
1.0
0.5
0.0
-10
-9
-8
-7
-6
-5
-4
-3
log [agonist] M

Fig. 3D

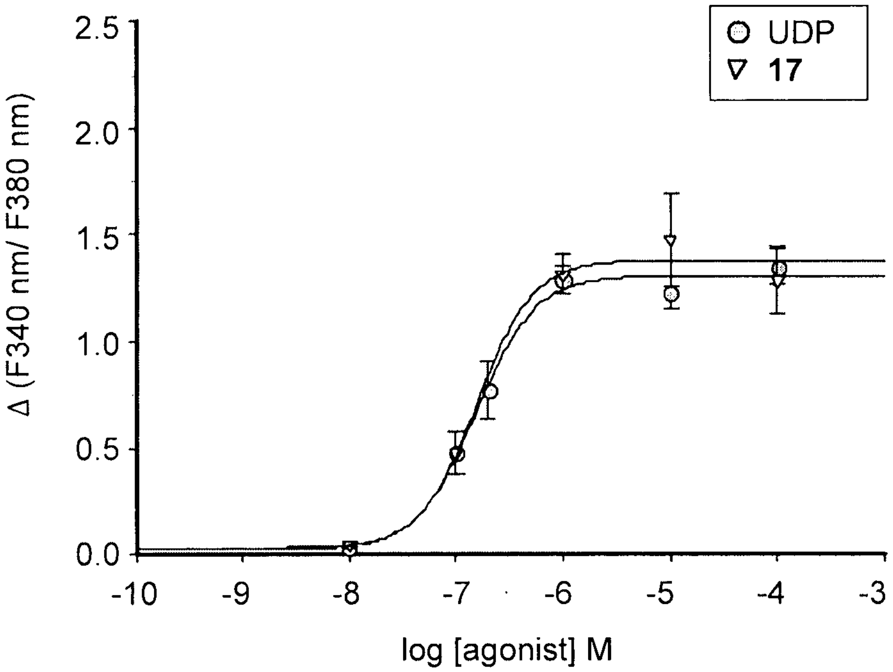

UDP
17
Δ (F340 nm/ F380 nm)
2.5
2.0
1.5
1.0
0.5
0.0
-10
-9
-8
-7
-6
-5
-4
-3
log [agonist] M

Fig. 4A

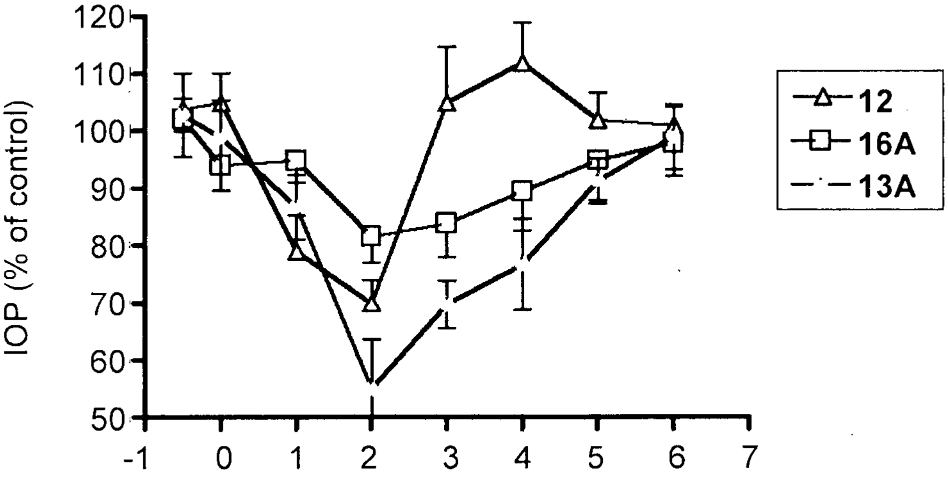

12
16A
13A
IOP (% of control)
120
110
100
90
80
70
60
50
-1
0
1
2
3
4
5
6
7
Time (h)

Fig. 4B

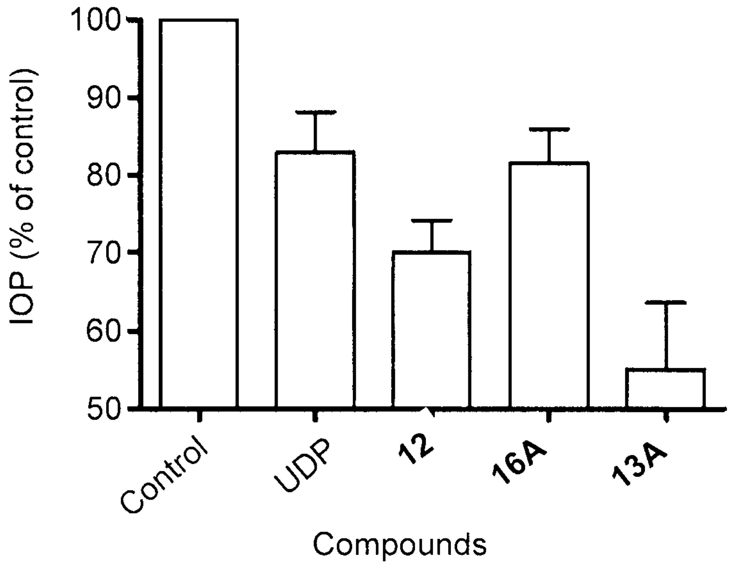
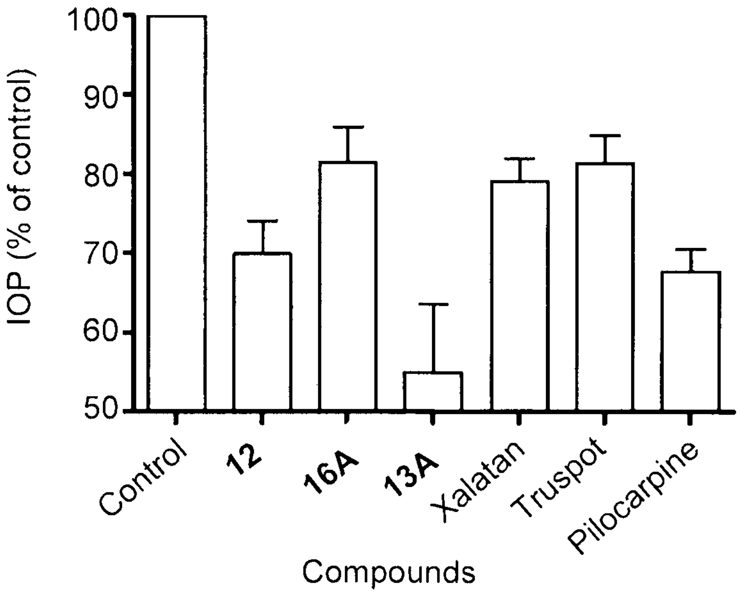

IOP (% of control)
100
90
80
70
60
50
Control
UDP
12
16A
13A
Compounds

Fig. 4C


IOP (% of control)
100
90
80
70
60
50
Control
12
16A
13A
Xalatan
Truspot
Pilocarpine
Compounds

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2011/000913

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07F9/6558 A61P27/06 A61K31/665
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07F A61P A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TAMAR GINSBURG-SHMUEL ET AL: "5-OMe-UDP is a Potent and Selective P2Y 6 -Receptor Agonist", JOURNAL OF MEDICINAL CHEMISTRY, vol. 53, no. 4, 25 February 2010 (2010-02-25), pages 1673-1685, XP55020028, ISSN: 0022-2623, DOI: 10.1021/jm901450d cited in the application compounds 19,21 abstract -----	1-21
Y	WO 2009/066298 A1 (UNIV BAR ILAN [IL]; FISCHER BILHA [IL]; ELYAHU SHAY [IL]) 28 May 2009 (2009-05-28) page 23, line 26 claim 1 -----	1-21

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search 23 February 2012	Date of mailing of the international search report 05/03/2012
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Eberhard, Michael

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IL2011/000913

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009066298 A1	28-05-2009	CN 101925610 A	22-12-2010
		EP 2231688 A1	29-09-2010
		JP 2011504489 A	10-02-2011
		US 2010256086 A1	07-10-2010
		WO 2009066298 A1	28-05-2009