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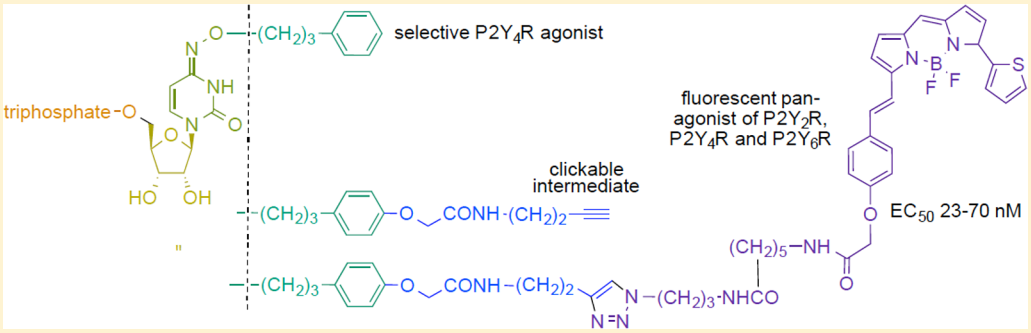
4-Alkyloxyimino Derivatives of Uridine-5′-triphosphate: Distal Modification of Potent Agonists as a Strategy for Molecular Probes of $P2Y_2$, $P2Y_4$, and $P2Y_6$ Receptors

P. Suresh Jayasekara,[†] Matthew O. Barrett,[‡] Christopher B. Ball,[‡] Kyle A. Brown,[‡] Eva Hammes,[†] Ramachandran Balasubramanian,[†] T. Kendall Harden,[‡] and Kenneth A. Jacobson*[,†]

[†]Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892 United States

[‡]Department of Pharmacology, University of North Carolina, School of Medicine, Chapel Hill, North Carolina 27599 United States

Supporting Information



ABSTRACT: Extended N^4-(3-arylpropyl)oxy derivatives of uridine-5′-triphosphate were synthesized and potently stimulated phospholipase C stimulation in astrocytoma cells expressing G protein-coupled human (h) P2Y receptors (P2YRs) activated by UTP ($P2Y_{2/4}R$) or UDP ($P2Y_6R$). The potent $P2Y_4R$-selective N^4-(3-phenylpropyl)oxy agonist was phenyl ring-substituted or replaced with terminal heterocyclic or naphthyl rings with retention of P2YR potency. This broad tolerance for steric bulk in a distal region was not observed for dinucleoside tetraphosphate agonists with both nucleobases substituted. The potent N^4-(3-(4-methoxyphenyl)-propyl)oxy analogue **19** (EC_{50}: $P2Y_2R$, 47 nM; $P2Y_4R$, 23 nM) was functionalized for chain extension using click tethering of fluorophores as prosthetic groups. The BODIPY 630/650 conjugate **28** (MRS4162) exhibited EC_{50} values of 70, 66, and 23 nM at the $hP2Y_{2/4/6}Rs$, respectively, and specifically labeled cells expressing the $P2Y_6R$. Thus, an extended N^4-(3-arylpropyl)oxy group accessed a structurally permissive region on three G_q-coupled P2YRs, and potency and selectivity were modulated by distal structural changes. This freedom of substitution was utilized to design of a pan-agonist fluorescent probe of a subset of uracil nucleotide-activated hP2YRs.

INTRODUCTION

The P2Y receptors (P2YRs) are G protein-coupled receptors (GPCRs) that are activated by extracellular nucleotides.[1] The widely expressed[2] UTP-activated ($P2Y_2R$ and $P2Y_4R$) and UDP-activated ($P2Y_6R$) receptors belong to the subfamily of $P2Y_1$-like receptors that promote inositol lipid signaling through G_q-mediated activation of phospholipase C-β (PLC-β). Few selective agonists and antagonists[3] are available for pharmacological differentiation of these uracil nucleotide-regulated receptors, and $P2Y_2R$, $P2Y_4R$, and $P2Y_6R$ exhibit much overlap in affinities for pyrimidine nucleotides. This study was initiated to synthesize nucleoside 5′-triphosphate derivatives as potent agonist probes of the $P2Y_4R$, including chain derivatization for incorporation of bulky reporter groups. The message for this P2YR subtype is expressed at high levels in intestine, pituitary, and brain, with lower levels observed in adipose tissue, prostate, spleen, lymphocytes, skeletal muscle, and lung.[2,3] The $P2Y_4R$ has been shown to regulate cardiovascular development,[4] and activation of this signaling protein results in intestinal chloride secretion,[5] inhibition of contraction of the mouse ileum longitudinal muscle, and K^+ secretion in the luminal membrane of the distal colon.[6,7] Postsynaptic $P2Y_4Rs$ are expressed on neurons of the rat hypothalamus that release neuropeptides.[8] Thus, ligands of the $P2Y_4R$ are of potential interest for pharmacotherapies directed at intestinal function, angiogenesis, cardiac remodeling, postischemic revascularization, CNS function, and inflammation.[4,9]

Uridine-5′-triphosphate (UTP, **1**) is the endogenous $P2Y_4R$ agonist (EC_{50} = 80 nM),[1,10] while adenosine-5′-triphosphate (ATP) antagonizes the human homologue of this receptor and

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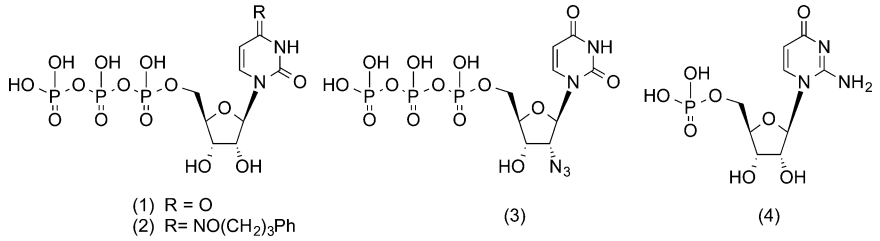
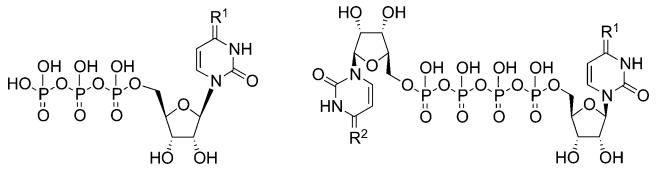
Chart 1. Structures of Pyrimidine Nucleotides As Prototypical Agonist Ligands for Studying the P2Y$_4$R[a]



[a]EC_{50} values for activation of PLC via the human P2Y$_4$R are: **2**, 23 nM; 2′-azido-2′-deoxy-UTP **3**, 1100 nM; *iso*-CMP **4**, 4980 nM.[11−13]

Table 1. Potency of a Series of Pyrimidine Nucleotide Derivatives at Three Subtypes of hP2YRs



2, 5 – 28 **29 – 31**

no.	structure, R^1 =	potency, EC_{50} (nM) P2Y$_2$[a]	P2Y$_4$[a]	P2Y$_6$[a]
	5′-Triphosphates			
5[b]	=N-O-CH_3	28 ± 4	25 ± 3	130 ± 21
6[b]	=N-O-CH_2CH_3	817 ± 93	210 ± 79	877 ± 50
7	=N-O-$(CH_3)_3CO_2H$	299 ± 56	470 ± 130	697 ± 167
8[b]	=N-O-CH_2Ph	620 ± 75	97 ± 14	230 ± 37
9[b]	=N-O-$(CH_2)_2$Ph	1200 ± 270	73 ± 17	1210 ± 220
2[b]	=N-O-$(CH_2)_3$Ph	640 ± 147	23 ± 4	740 ± 29
10	=N-O-$(CH_2)_4$Ph	214 ± 11	54 ± 14	251 ± 110
11	=N-O-$(CH_2)_2$O-Ph	3400 ± 880	430 ± 93	1780 ± 430
12	=N-O-$(CH_2)_3$-3-F-Ph	190 ± 28	100 ± 22	539 ± 28
13	=N-O-$(CH_2)_3$-4-F-Ph	547 ± 125	105 ± 27	1210 ± 290
14	=N-O-$(CH_2)_3$-4-Br-Ph	350 ± 70	141 ± 60	225 ± 39
15	=N-O-$(CH_2)_3$-3-I-Ph	325 ± 100	114 ± 13	299 ± 10
16	=N-O-$(CH_2)_3$-4-I-Ph	187 ± 61	91 ± 15	90 ± 13
17	=N-O-$(CH_2)_3$-3-NO_2-Ph	457 ± 11	95 ± 25	9700 ± 1500
18	=N-O-$(CH_2)_3$-4-NO_2-Ph	458 ± 145	84 ± 24	1330 ± 290
19[c]	=N-O-$(CH_2)_3$-4-CH_3O-Ph	47 ± 2	23 ± 8	277 ± 75
20	=N-O-$(CH_2)_3$-3-pyridyl	327 ± 77	300 ± 230	2950 ± 1020
21	=N-O-$(CH_2)_3$-4-pyridyl	285 ± 20	250 ± 160	4280 ± 1330
22	=N-O-$(CH_2)_3$-3-thienyl	103 ± 7	20 ± 2	164 ± 48
23	=N-O-$(CH_2)_3$C≡CH	168 ± 30	410 ± 28	3000 ± 1500
24	=N-O-$(CH_2)_3$-(2-naphthyl)	139 ± 29	128 ± 26	90 ± 12
25	=N-O-$(CH_2)_2$-C(CN)$(Ph)_2$	934 ± 92	47 ± 13	177 ± 21
26	=N-O-$(CH_2)_3$-Ph-(4-O-CH_2CONH-$(CH_2)_2$C≡CH	109 ± 33	40 ± 14	183 ± 42
27	Alexa Fluor 488 derivative	577 ± 146	200 ± 42	334 ± 36
28	BODIPY 630/650 derivative	66 ± 17	70 ± 21	23 ± 7

	R^1 =	R^2 =	potency, EC_{50} (nM) P2Y$_2$[a]	P2Y$_4$[a]	P2Y$_6$[a]
	Dinucleoside Tetraphosphates				
29[b,c]	=O	=O	210 ± 30	130 ± 10	1160 ± 420
30	=N-O-$(CH_3)_3$Ph	=O	389 ± 114	93 ± 19	332 ± 80
31	=N-O-$(CH_3)_3$Ph	=N-O-$(CH_3)_3$Ph	>10000	1330 ± 100	>10000

[a]Functional assays were conducted with 1321N1 astrocytoma cells expressing recombinant hP2Y$_2$, P2Y$_4$, or P2Y$_6$Rs. Values are expressed as the mean ± SEM. [b]Data from Maruoka et al.[10] [c]**29**, Up_4U.

activates the rat homologue.[11] Structure−activity relationship (SAR) studies of the P2Y$_4$R have been performed through preparation of modified analogues in conjunction with molecular modeling. Two analogues of UTP, N^4-(3-

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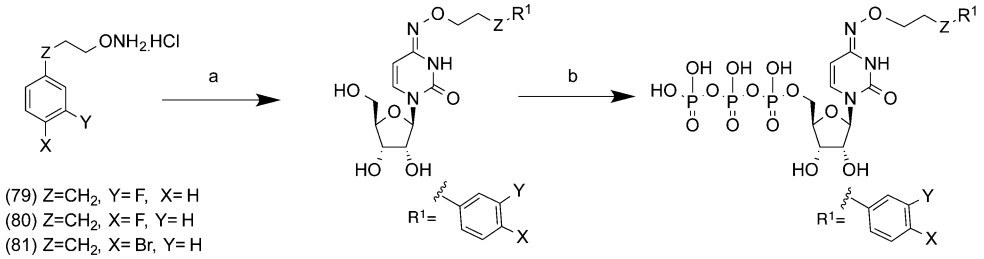
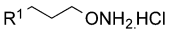
Scheme 1. Synthesis of Various 4-Alkoxyiminopyrimidine Ribonucleoside 5′-Triphosphates[a]



(79) Z=CH_2, Y= F, X= H
(80) Z=CH_2, X= F, Y= H
(81) Z=CH_2, X= Br, Y= H
(82) Z=CH_2, Y= I, X= H
(83) Z=CH_2, X= I, Y= H
(84) Z=CH_2, Y= NO_2, X= H
(85) Z=CH_2, X= NO_2, Y= H
(86) Z=CH_2, X=OMe, Y=H
(87) Z=CH_2CH_2, X=H, Y=H
(88) =O, X=H, Y=H
(89) Z= CH(Ph)CN, X=H, Y=H
(91) Z=CH_2, X= $OCH_2CO_2{}^tBu$, Y= H



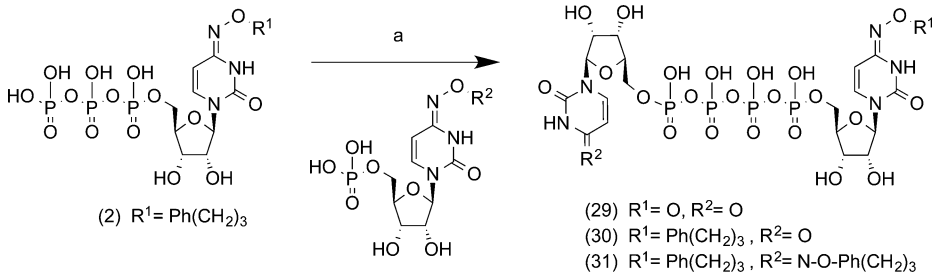
(90) R^1= 2-naphthyl
(92) R^1= 3-thienyl
(93) R^1= C≡CH
(94) R^1= CO_2Et
(95) R^1= 3-pyridyl
(96) R^1= 4-pyridyl

(97) Z=CH_2CH_2, X=H, Y=H
(98) Z=O, X=H, Y=H
(99) Y=F, X=H, Z=CH_2
(100) X= F, Y=H, Z=CH_2
(101) X=Br, Y=H, Z=CH_2
(102) Y=I, X=H, Z=CH_2
(103) X=I, Y=H, Z=CH_2
(104) Y=NO_2, X=H, Z=CH_2
(105) X=NO_2, Y=H, Z=CH_2
(106) X=OMe, Y=H, Z=CH_2
(107) R^1=3-pyridyl, Z=CH_2
(108) R^1=4-pyridyl, Z=CH_2
(109) R^1=3-thienyl, Z=CH_2
(110) R^1=C≡CH , Z=CH_2
(111) R^1= 2-naphthyl, Z=CH_2
(112) R^1= H, Z= C(Ph)CN
c (113) R^1= CO_2Et, Z=CH_2
(114) R^1= CO_2H, Z=CH_2
c, d (115) Z=CH_2, X= $OCH_2CO_2{}^tBu$, Y= H
(116) Z=CH_2, X= $OCH_2CONH(CH_2)_2C{\equiv}CH$, Y= H

(10) Z=CH_2CH_2, X=H, Y=H
(11) Z=O, X=H, Y=H
(12) Y=F, X=H, Z=CH_2
(13) X= F, Y=H, Z=CH_2
(14) X=Br, Y=H, Z=CH_2
(15) Y=I, X=H, Z=CH_2
(16) X=I, Y=H, Z=CH_2
(17) Y=NO_2, X=H, Z=CH_2
(18) X=NO_2, Y=H, Z=CH_2
(19) X=OMe, Y=H, Z=CH_2
(20) R^1=3-pyridyl, Z=CH_2
(21) R^1=4-pyridyl, Z=CH_2
(22) R^1=3-thienyl, Z=CH_2
(23) R^1=C≡CH, Z=CH_2
(24) R^1= 2-naphthyl, Z=CH_2
(25) R^1= H, Z= C(Ph)CN
(7) R^1= CO_2H, Z=CH_2
(26) Z=CH_2, X= $OCH_2CONH(CH_2)C{\equiv}CH$, Y= H

[a]Reagents and conditions: (a) cytidine, pyridine, 110 °C; (b) proton sponge, $POCl_3$, $PO(OMe)_3$, tributylammonium pyrophosphate, DMF; (c) 1 N NaOH, THF, 50 °C; (d) PyBOP, DIEA, but-4-yn-1-amine.

Scheme 2. Synthesis of 4-Alkoxyiminopyrimidine Dinucleoside Tetraphosphates[a]



(2) R^1= $Ph(CH_2)_3$

(29) R^1= O, R^2= O
(30) R^1= $Ph(CH_2)_3$, R^2= O
(31) R^1= $Ph(CH_2)_3$, R^2= N-O-$Ph(CH_2)_3$

[a]Reagents: (a) DIC, $MgCl_2$, DMF.

phenylpropyloxy)cytidine-5′-triphosphate, **2**, and 2′-azido-2′-deoxy-UTP, **3**, and one 5′-monophosphate derivative, iso-CMP **4**,[10,12,13] have demonstrated (Chart 1) low to moderate selectivity for this subtype, but highly potent and selective $P2Y_4R$ agonists have not been identified to date.[10−16] The 4-benzyloxyiminopyrimidine group was identified as a structurally permissive site for synthesis of functionalized congeners,[17] leading to high affinity molecular probes for the closely related UDP-activated $P2Y_6R$.[14] Because of the structural similarity of the $P2Y_4R$ and $P2Y_6R$, we focused on the same site for derivatization of analogues of **1** that included an elongated alkyl chain, i.e., a N^4-phenylpropyloxy group, which we hypothesized would be more suitable for conferring $P2Y_4R$ selectivity.[10] One goal of these studies was to generate an analogue(s) with tolerance for steric bulk when bound to the receptor, potentially allowing attachment of a fluorophore or other reporter group to serve as a tracer for the receptor on live cells. This approach has been validated for adenosine receptors and other GPCRs.[18,19]

RESULTS

We considered a 4-arylalkyloxyimino group in the pyrimidine nucleobase as an approach to expand the range of potent agonists of $P2Y_4R$ and related P2YRs. In summary, we have modified the 4-(3-phenylpropyloxy)imino group of **2** with various ring substitutions and chain elongations and evaluated the new nucleotide derivatives in functional assays of the $hP2Y_4R$, $P2Y_6R$, and $P2Y_2R$. Because dinucleoside tetraphosphates are also known to activate the $P2Y_4R$,[10] we included several derivatives in the dinucleotide series to test their compatibility with 4-alkyloxyimino modifications.

Novel nucleotide derivatives **7**, **10**−**28**, and **30**−**31** (Table 1) were prepared and purified to homogeneity. Two series of nucleotide derivatives were chosen: 5′-triphosphates related to UTP and dinucleotides related to Up_4U **29**, which have

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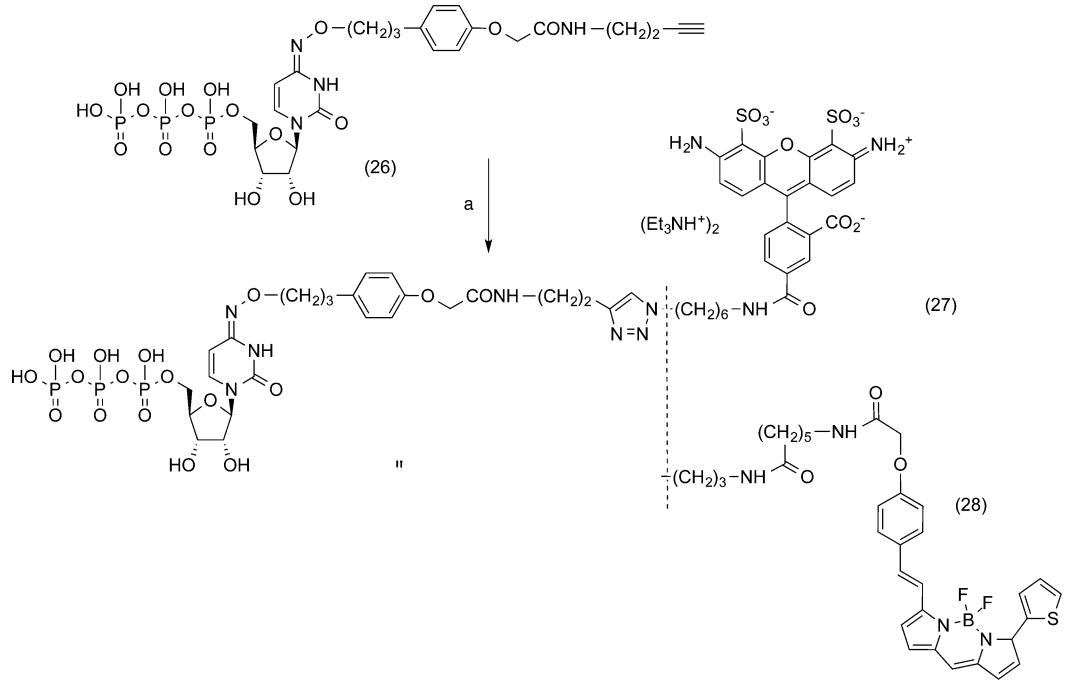
Scheme 3. Synthesis of Fluorescent 4-Alkoxyiminopyrimidine Ribonucleoside 5′-Triphosphates 27 and 28 by Click Reaction with Azido-Dyes[a]



[a]Reagents and conditions: (a) azide (e.g., **124**, 1 equiv) and alkyne (1.4 equiv) precursors, ^{t}BuOH/water, TBTA, sodium ascorbate, cupric sulfate.

relatively high potency at both $P2Y_2R$ and $P2Y_4R$. The synthetic routes to these groups of structurally modified pyrimidine nucleotides include: UTP analogues containing a N^4-alkoxy group (Scheme 1), modified Up_4U dinucleotide analogues (Scheme 2), and UTP analogues containing Alexa Fluor 488 and BODIPY 630/650 fluorescent dyes[19,27] (Scheme 3).

The synthesis of N^4-alkoxy cytidines **10**–**26** from cytidine was performed using corresponding alkoxyamine hydrochlorides. Various alkoxy groups including phenyl, pyridyl, thienyl, naphthyl, and alkyl ester derivatives were chosen. These alkoxyamine derivatives were synthesized from the corresponding 3-arylpropyl bromides using Gabriel synthesis or a variation thereof using *N*,*N*′-di-*tert*-butoxycarbonylhydroxylamine as the source of the hydroxylamine moiety.[22,25] The 3-alkylpropyl bromides **56**–**62** and **67** were synthesized from the corresponding benzyl bromides by homologation in four steps (Scheme 4A).[23] The 3-arylpropyl bromides **73**, **76**, and **78** were synthesized from the corresponding alcohols and converted to the alkyoxyimino derivatives (Scheme 4B).[22] (3-Thien-3-yl)propyl bromide **71** was synthesized from the corresponding α,β-unsaturated carboxylic acid (Scheme S1, Supporting Information). The resulting N^4-alkoxy-cytidines were phosphorylated by standard methods[10] to give the desired N^4-alkoxy-cytidine 5′-triphosphates **10**–**26** (Scheme 1). In each case, the unprotected nucleoside was first treated with phosphorus oxychloride and the reaction mixture was treated immediately with bis(tri-*n*-butylammonium) pyrophosphate to produce the 5′-triphosphate. The synthesis of dinucleoside tetraphosphates, including an asymmetrically substituted analogue **30**, was performed using *N*,*N*′-diisopropylcarbodiimide (DIC) as a coupling reagent for condensing a 5′-monophosphate and 5′-triphosphate in the presence of magnesium chloride (Scheme 2), which dramatically increased the reactivity compared to carbodiimide alone.[24] The fluorescent Alexa Fluor 488 5′-triphosphate **27** and BODIPY 5′-triphosphate **28** derivatives were synthesized from alkyne nucleotide precursor **26** and an azide derivative (e.g., a BODIPY 630/650 azide **124** for product **28**, Scheme S2, Supporting Information) using copper-catalyzed [2 + 3] cycloaddition click reactions with azides (Scheme 3).[20]

The newly synthesized analogues were tested for potency against the $P2Y_2R$, $P2Y_4R$, and $P2Y_6R$.[13−15] Similar N^4-3-phenylalkyloxy derivatives were shown to be only weakly active at the $P2Y_{14}R$;[10] thus, this subtype was not part of the initial screen. The potencies of nine known reference compounds (**2**, **5**–**6**, **8**–**9**, and **29**) were included for comparison.[10,14] Standard assays of potency for activation of PLC were performed as described[26] in 1321N1 human astrocytoma cell lines stably expressing either the $P2Y_2R$, $P2Y_4R$, or $P2Y_6R$ (1321N1-P2YR cells).

Among substituted phenyl groups, 3-F **12**, 4-I **16**, and 4-MeO **19** provided relatively high potency at the three $P2Y_2$, $P2Y_4$, and $P2Y_6$Rs. Thus, we decided to elongate functionalized congeners based on *p*-substituted ethers related to **19**. Other aryl moieties that appeared to enhance potency were thien-3-yl **22** and naphth-2-yl **23**, indicating a tolerance of steric bulk in this region. The optimal chain length at the $P2Y_4R$ was reached, with the *n*-propyl analogue **2** not substituted with an ether in the chain (cf. **11**). In contrast, the longer *n*-butyl homologue **10** displayed increased potency at the $P2Y_2R$ and $P2Y_6R$. High potency at the $P2Y_4R$ was achieved with a terminal alkyne at the N^4-linked chain for tethering sterically bulky groups by click chemistry (**26**, EC_{50} = 40 nM, Figure 1). However, potency at $P2Y_2R$ and $P2Y_6R$ also was largely retained in this ether- and amide-linked alkyne, with EC_{50} values of 109 and 183 nM, respectively.

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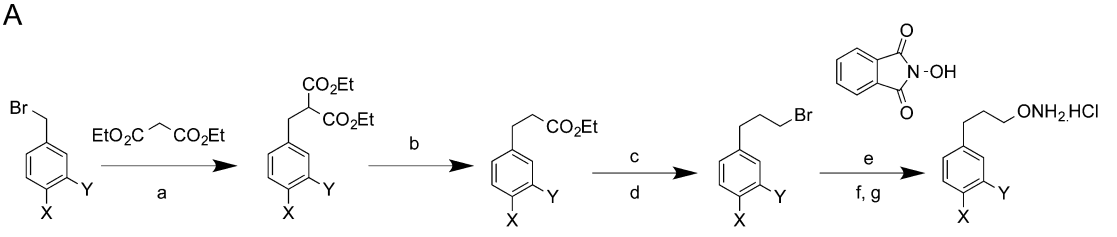
Scheme 4. Synthesis of *N*-Alkoxyamines: (A) 3-Phenylpropyl and 3-(naphth-2-yl)propyl Intermediates Prepared by the Gabriel Synthesis.[25] (B) *N*-Alkoxyamine Intermediates Prepared Using *N,N′*-di-*tert*-Butoxycarbonylhydroxylamine as Described[a]

A



(32) Y= F, X= H
(33) X= F, Y= H
(34) X= Br, Y= H
(35) Y= I, X= H
(36) X= I, Y= H
(37) Y= NO_2, X= H
(38) X= NO_2, Y= H
(39) X,Y = fused phenyl

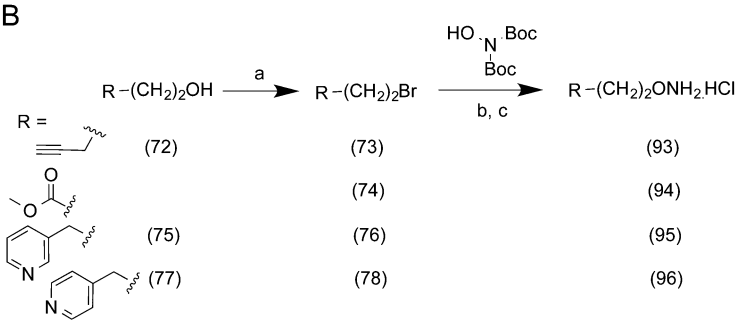
(40) Y= F, X= H
(41) X= F, Y= H
(42) X= Br, Y= H
(43) Y= I, X= H
(44) X= I, Y= H
(45) Y= NO_2, X= H
(46) X= NO_2, Y= H
(47) X,Y = fused phenyl

(48) Y= F, X= H
(49) X= F, Y= H
(50) X= Br, Y= H
(51) Y= I, X= H
(52) X= I, Y= H
(53) Y= NO_2, X= H
(54) X= NO_2, Y= H
(55) X,Y = fused phenyl

(56) Y= F, X= H
(57) X= F, Y= H
(58) X= Br, Y= H
(59) Y= I, X= H
(60) X= I, Y= H
(61) Y= NO_2, X= H
(62) X= NO_2, Y= H
(63) X=OMe, Y=H
(64) $Ph(CH_2)_4Br$
(65) $PhO(CH_2)_2Br$
(66) $(Ph)_2CNCH(CH_2)_2Br$
(67) X,Y = fused phenyl

(79) Y= F, X= H
(80) X= F, Y= H
(81) X= Br, Y= H
(82) Y= I, X= H
(83) X= I, Y= H
(84) Y= NO_2, X= H
(85) X= NO_2, Y= H
(86) X=OMe, Y=H
(87) $Ph(CH_2)_4$
(88) $PhO(CH_2)_2$
(89) $(Ph)_2CNCH(CH_2)_2$
(90) X,Y = fused phenyl

B



[a]Reagents and conditions: (A) (a) diethyl malonate, NaH, THF; (b) NaCl, DMSO; (c) DiBal-H; (d) CBr_4, PPh_3; (e) Et_3N, DMF, 100°C; (f) $NH_2NH_2{\cdot}H_2O$; (g) 1 N HCl. Protected 3-(4-carboxymethyloxy-phenyl)propyl (**91**) and 3-(thien-3-yl)propyl (**92**). *N*-alkoxyamine intermediates were prepared by analogous methods (Scheme S1, Supporting information): (a) K_2CO_3, CH_3CN, reflux; (b) CBr_4, PPh_3; (c) Et_3N, DMF, 100 °C; (d) $NH_2NH_2{\cdot}H_2O$; (e) 10% Pd/C, H_2, MeOH; (f) $LiAlH_4$, THF; (g) Br_2, PPh_3, 2,6-lutidine.[22] (B) (a) HBr in acetic acid, 50 °C; (b) DBU, CH_3CN, 50 °C; (c) 1 N HCl, CH_2Cl_2.

The fluorescent dye, Alexa Fluor 488, is a suitable fluorophore for flow cytometry (FCM) binding assays and live cell imaging.[14,18] This fluorophore was incorporated in conjugate **27**, but only an intermediate potency was observed with this molecule at the three P2YRs. An alternative BODIPY 630/650 fluorophore was incorporated in conjugate **28**. Curiously, in comparison to the precursor molecule **26**, the BODIPY conjugate exhibited enhanced potency at the $P2Y_2R$ (EC_{50} = 66 nM) and $P2Y_6R$ (EC_{50} = 23 nM) but slightly decreased potency at the $P2Y_4R$. Thus, **28** is a high affinity pan-agonist fluorescent probe with nearly equivalent potency at the $hP2Y_2R$, $hP2Y_4R$, and $hP2Y_6R$.

A preliminary feasibility study was carried out to determine if **28** could serve as a fluorescent P2YR probe. These experiments were with $P2Y_6R$-expressing 1321N1 cells because **28** is most potent at this P2YR subtype (Figure 2). Fluorescent binding of **28** was compared by FCM, using several known $P2Y_6R$ ligands to compete for cell labeling. Incubation of cells with 100 nM **28** for 30 min at 37 °C in the presence of the ecto-nucleotidase inhibitor α,β-methylene-adenosine 5′-diphosphate **125** (100 μM)[33,34] resulted in significant cell labeling (Figure 2). The level of cell-associated fluorescence was not reduced by coincubation of **28** with 10 μM N^6-methyl-2-deoxyadenosine 3,5′-bisphosphate (**117**, MRS2179), a $P2Y_1R$-selective antagonist that is inactive at $P2Y_2$, $P2Y_4$, and $P2Y_6Rs$.[28] However, significant decreases in mean fluorescence intensity were observed after preincubation with $P2Y_6R$ ligands, including the $P2Y_6R$-selective noncompetitive antagonist *N,N″*-1,4-butanediyl-bis[*N′*-(3-isothiocyanatophenyl)]thiourea (**118**, MRS2578, 10 μM) and the competitive antagonist suramin (**119**, 10 μM).[29,30] Although **119** is a nonselective P2X and P2Y antagonist, we included it in this assay because it has been shown to effectively block the $P2Y_6R$.[26] The potent $P2Y_6R$-selective dinucleotide agonist P^1-(uridine 5′-)-P^4-(N^4-methoxycytidine 5′-)triphosphate (**120**, MRS2957, 10 μM)[14] produced the largest decrease in cell-associated fluorescence. Fluorescence microscopy comparing binding to 1321N1-$P2Y_6R$ cells in the presence and absence of **118** (Figure 3) indicated that 1 μM **28** specifically labeled cells expressing the receptor.

DISCUSSION

We have identified a functionalization approach that provides versatile nucleotide analogues for further derivatization of high potency $P2Y_2R$, $P2Y_4R$, and $P2Y_6R$ agonists. The initial approach was based on a $P2Y_4R$-selective agonist, and many of the new derivatives tend toward selectivity at this subtype. However, upon elongation of a functionalized chain at the N^4

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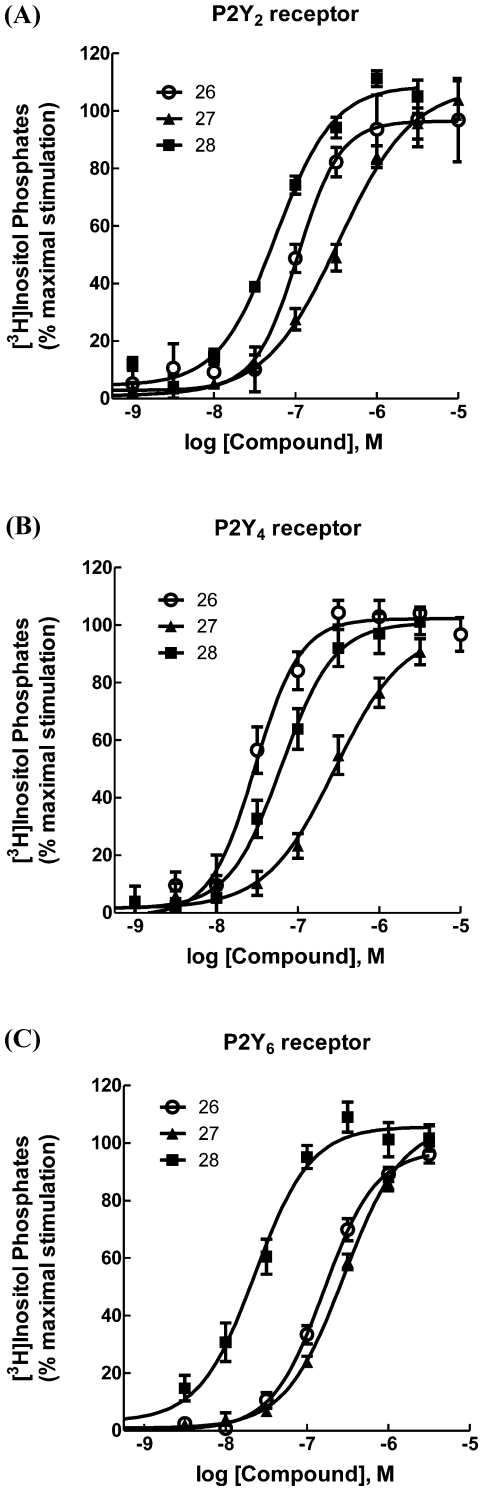

Figure 1. Comparison of potencies of alkyne **26** and fluorescent analogues **27** and **28** in activation of PLC via the human $P2Y_2R$ (A), $P2Y_4R$ (B), and $P2Y_6R$ (C) in stably transfected 1321N1 human astrocytoma cells.

position, significant potency also was observed at the related $P2Y_2R$ and $P2Y_6R$ subtypes. For example, compound **19** was particularly potent at $P2Y_2$ and $P2Y_4Rs$.

The derivatization strategy was aimed to clarify where to modify the pyrimidine nucleotides to preserve or increase potency at any or all of the relevant P2YRs to allow the attachment of sterically large fluorophores[27] and other reporter

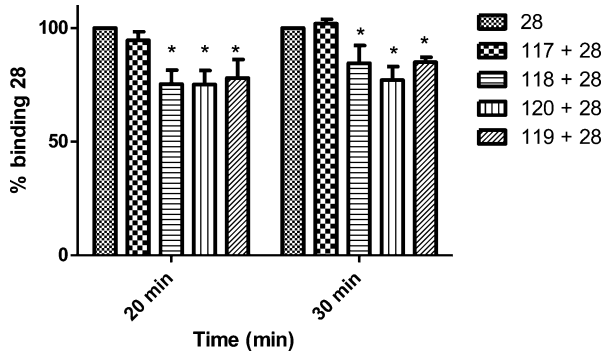

Figure 2. Fluorescence ligand binding experiments using FCM in stably transfected 1321N1-$P2Y_6R$ cells with **28** after preincubation at 37 °C with known $P2Y_6R$ agonist or $P2Y_6R$ antagonists. Each column shows the brightness of each compound using **28** set at 100% and after correcting the MFI values for autofluorescence. Results are expressed as mean ± SEM (n = 4). Binding of **28** at 20 and 30 min to the $P2Y_6R$ was significantly blocked after preincubation with selective antagonist **118**, selective agonist **120**, and nonselective antagonist **119**. No significant difference in MFI was observed for $P2Y_1R$ antagonist **117**. *, $p < 0.05$, when compared to cells treated with only **28**. Average of MFI after 20 min incubation of **28**: 107.0 ± 30.2; after 30 min incubation of **28**: 222.8 ± 72.1.

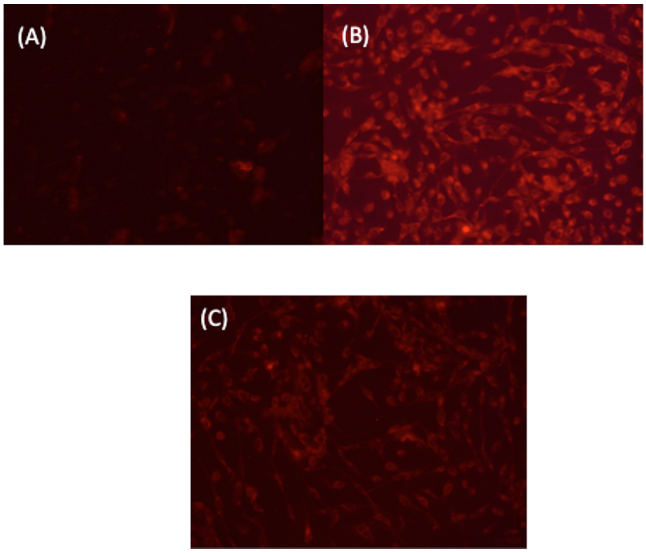

Figure 3. Fluorescent micrographs using a Keyence fluorescent microscope (BZ-9000) of $P2Y_6R$-expressing 1321N1 astrocytoma cells exposed to the fluorescent agonist **28** (1 μM, 30 min incubation at 37 °C). (A) Control cells in the absence of **14**. (B) Incubation with 1 μM **14** at 37 °C for 30 min in medium. (C) Incubation with 1 μM **14** at 37 °C for 30 min in cells pretreated with noncompetitive antagonist **118** (10 μM).

groups. Questions to be answered were: If the pseudo-oxime chain of **2** proved to be a suitable attachment point, how long should the alkyl linker (i.e., propyl in **2**) be for optimal potency (cf. **8**, **9**, **11**, **25**)? Were ring-substitutions (cf. **12**–**19**) or replacement of the terminal phenyl moiety with heteroaryl or other group (cf. **7**, **20**–**24**) desirable for enhancing potency? What position for attachment of an elongated chain on the phenyl (cf. **12**–**19**) or other aryl group was best suited for high potency? Thus, model compounds of various intermediate substructures were prepared and tested before the final target fluorescent conjugates were designed. Briefly, we chose the *p*-

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position for extension of an alkyl ether chain on the 4-(3-phenylpropyloxy)imino group of **2**, which was click-linked[20] through a terminal alkyne, i.e., **26**, to an azide-bearing fluorophore. Other possible approaches could have been through chain elongation at the 2, 3, or 5 positions of a uracil ring.[13,21]

Modifications of the distal phenyl ring of a 3-phenylpropyloxyimino group, shown previously to be optimal for $P2Y_4R$ potency, allowed a bulky fluorescent reporter group to be introduced, but the $P2Y_4R$ selectivity was compromised. Nevertheless, these molecules also provide high affinity leads for further modified agonists that could exhibit higher selectivity at one of these receptors. Possible directions for structural modifications of pyrimidine nucleotides conducive to $P2Y_4R$ selectivity might include ribose and nucleobase modifications such as appear in compounds **3** and **4**.

This discovery provides an optimal new route for generation of fluorescent probes for facile pooled quantification of these three P2YRs in living cells. In cells expressing a single P2YR subtype, such as our astrocytoma cells, there is no ambiguity. Following the development of standard assay conditions, the potent fluorescent ligand **28** could potentially be used for high-throughput screening of drug libraries with $P2Y_2R$-, $P2Y_4R$-, or $P2Y_6R$-expressing cells. Radioligands are not available for any of these receptors. Our previously reported fluorescent nucleotide P2YR probe, a conjugate of Alexa Fluor 488, tethered from a cytosine nucleobase in the diphosphate series, was selective for the $P2Y_6R$.[14] The current work adds a second viable fluorescent probes for studying the $P2Y_6R$. Although we did not directly examine its binding to $P2Y_2R$- and $P2Y_4R$-expressing cells, compound **28** adds the potential capability of fluorescent characterization of these subtypes for which no high affinity assay probes are available. Assay development for routine application of this fluorescent probe presents a separate experimental challenge that will be addressed in future work.

The high potency of **28** at the $hP2Y_2R$, $hP2Y_4R$, and $hP2Y_6R$ suggests the existence of energetically favorable interactions with groups on the conformationally flexible extracellular regions of $P2Y_2R$ and $P2Y_6R$. The modeling of these extracellular regions is difficult because of their flexibility and the lack of a close structural template. Further characterization of the location of the fluorescent label within the cells as well as delineation of the saturability, reversibility, kinetics, and other measurements will be required before this probe molecule can be used routinely for FCM labeling of cells expressing P2YRs.

CONCLUSIONS

A series of N^4-(3-arylpropyloxy) derivatives of uridine-5′-triphosphate were synthesized and found to display high potency as agonists of the initial target G protein-coupled $hP2Y_4R$ and also $hP2Y_2R$ and $hP2Y_6R$. The phenyl group could be substituted with heterocyclic rings or a naphthyl ring with retention of P2YR affinity, indicating a broad tolerance for steric bulk in this distal region of the nucleotide. Thus, an extended N^4-(3-arylpropyloxy) group accessed a structurally permissive region on various G_q-coupled P2YRs and was modulated by distal structural changes to alter selectivity. This freedom of substitution can be utilized for the design of pan-agonist affinity probes of the uracil nucleotide-activated, G_q-coupled $P2Y_{2/4/6}$ subset of receptors.

EXPERIMENTAL SECTION

Chemical Synthesis. ^1H NMR spectra were obtained with a Varian Mercury 400 spectrometers using D_2O, MeOD, $CDCl_3$, or DMSO-d_6 as a solvent. The chemical shifts are expressed as relative ppm from HOD (4.80 ppm). ^{31}P NMR spectra were recorded at room temperature (rt) on Varian Mercury 400 (162.10 MHz) spectrometers; orthophosphoric acid (85%) was used as an external standard. In several cases, the signal of the terminal phosphate moiety was not visible due to high dilution. High-resolution mass measurements were performed using a Micromass/Waters LCT Premier Electrospray time of flight (TOF) mass spectrometer coupled with a Waters HPLC system, unless noted. Alexa Fluor 488 azide was from Invitrogen (Carlsbad, CA). All other reagents were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO).

Purification of the nucleotide analogues for biological testing was carried out on (diethylamino)ethyl (DEAE)-A25 Sephadex columns with a linear gradient (0.01−0.5 M) of 0.5 M ammonium bicarbonate as the mobile phase. Then these nucleotides were additionally purified by HPLC with a Luna 5 μ RP-C18(2) semipreparative column (250 mm × 10.0 mm; Phenomenex, Torrance, CA) and using the following conditions: flow rate of 2 mL/min; 10 mM triethylammonium acetate (TEAA)-CH_3CN from 100:0 to 95:5 (system B) (or up to 99:1 to 50:50 (system C)) in 30 min (and isolated in the triethylammonium salt form). All other compounds (cytidine derivatives) were purified from a silica column chromatography ($CHCl_3$:MeOH-gradient from 100:0 to 70:30). Purity of the compounds submitted for bioassay was checked using a Hewlett−Packard 1100 HPLC equipped with a Zorbax SB-Aq 5 μm analytical column (50 mm × 4.6 mm; Agilent Technologies Inc., Palo Alto, CA). Mobile phase: linear gradient solvent system; 5 mM TBAP (tetrabutylammonium dihydrogenphosphate)-CH_3CN from 80:20 to 40:60 in 13 min; the flow rate was 0.5 mL/min (system A). Peaks were detected by UV absorption with a diode array detector at 230, 254, and 280 nm. All nucleotide derivatives tested for biological activity showed >98% purity by HPLC analysis (detection at 254 nm).

General Procedure for the Preparation of Nucleoside Triphosphates (7, 10−26). A solution of the nucleoside **97−112**, **114** (73.0 μmol), and 1,8-bis(dimethylamino)naphthalene (Proton Sponge, 24.0 mg, 0.11 mmol) in trimethyl phosphate (0.4 mL) was stirred for 10 min at 0 °C.[15] Then, phosphorus oxychloride (13.0 μL, 0.13 mmol) was added dropwise, and the reaction mixture was stirred for 2 h at 0 °C. A solution of tributylammonium pyrophosphate (0.80 mL, 0.44 mmol) and tributylamine (69.0 μL, 0.29 mmol) in *N*,*N*-dimethylformamide (DMF, 1 mL) was added, and stirring was continued at 0 °C for additional 10 min. Then 0.2 M Triethylammonium bicarbonate solution (1.5 mL) was added, and the clear solution was stirred at rt for 1 h. After removal of solvents, the residue was purified by Sephadex-DEAE A-25 resin and preparative HPLC.

***N*^{4}-(3-((4-But-3-yn-1-aminocarbonylmethyleneoxy)phenyl)-propyloxy)cytidine-5′-triphosphate Triethylammonium Salt (26).** Compound **26** (7.0 mg, 6.9 μmol, 10%) was obtained as a white solid using N^4-(3-((4-but-3-yn-1-aminocarbonylmethyleneoxy)-phenyl)propyloxy)cytidine **116** (35.5 mg, 70.1 μmol). ^1H NMR (D_2O) δ 7.27−7.19 (m, 3H), 6.94 (d, J = 8.6, 2H), 5.94 (d, J = 6.4 Hz, 1H), 5.77 (d, J = 8.2, 1H), 4.60 (s, 2H), 4.44−4.40 (m, 1H), 4.36 (t, J = 5.9, 1H), 4.26−4.14 (m, 3H), 4.06 (t, J = 6.0, 2H), 3.44 (t, J = 6.6, 2H), 2.75−2.63 (m, 2H), 2.44 (dt, J = 6.6, J = 2.7, 2H), 2.33 (t, J = 2.6, 1H), 2.07−1.91 (m, 2H). HRMS-EI found: 741.0974 $(M - H^+)^-$. $C_{24}H_{32}N_4O_{17}P_3$ requires 741.0970; analytical HPLC (system A: 6.54 min).

***N*^{4}-(3-((4-(2-Ethylamino)carbonylmethyleneoxy-(Alexa Fluor 488-1*H*-1,2,3-Triazol-4-yl))phenyl)propyloxy)cytidine-5′-triphosphate Triethylammonium Salt (27).** To a mixture of Alexa Fluor 488 azide (Alexa Fluor 488 5-carboxamido-(6-azidohexanyl), bis(triethylammonium salt)), 5-isomer, 0.5 mg, 0.8 μmol, 1 equiv), and alkyne **26** (0.8 mg, 0.8 μmol, 1.4 equiv) in a 0.20 mL of (1:1) mixture of ^{t}BuOH and water, were added tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl] amine (TBTA, 0.1 mg) and freshly prepared 1 M aqueous sodium ascorbate solution (1.6 μmol, 1.6 μL, 2 equiv) followed by

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7.5% aqueous copper sulfate pentahydrate solution (4.0 μL, 1.2 μmol, 1.5 equiv). The reaction mixture was stirred overnight at rt, solvent was evaporated, and the residue was purified by semipreparative HPLC as described above to obtain **27** (1.64 mg, 34% yield) as a yellow/orange solid. HRMS-EI found: 1399.2106 $(M - H^+)^-$. $C_{51}H_{58}N_{10}O_{27}S_2P_3$ requires 1399.2104.

N^4-(3-((4-O-CH_2CONH-(CH_2)$_2$(BODIPY-1*H*-1,2,3-Triazol-4-yl))-phenyl)propyloxy)cytidine-5′-triphosphate Triethylammonium Salt (28). To a mixture of BODIPY 630/650 azide (**124**, Supporting Information, 1.0 mg, 1.0 μmol, 1 equiv) and alkyne **26** (2.0 mg, 1.8 μmol, 1.2 equiv) in a 200 μL of (1:1) mixture of ^{t}BuOH and water, and 20 μL of DMF, were added tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl] amine (TBTA, 0.05 mg) and freshly prepared 1 M aqueous sodium ascorbate solution (1.1 μL, 1.6 μmol, 1 equiv) followed by 7.5% aqueous copper sulfate pentahydrate solution (2.5 μL, 0.8 μmol, 0.8 equiv). The bright-blue color reaction mixture was stirred overnight at rt, solvent was evaporated, and the residue was purified by semipreparative HPLC as described above to obtain desired triphosphate **28** as blue-colored solids. HRMS-EI *m/z* (M + ES MS) for **28** found, 1388.3648 $(M + H^+)^+$; calcd for $C_{64}H_{68}N_{11}O_{20}BF_2P_3S$, 1388.3631; analytical HPLC (system A: 7.91 min). Fluorescence absorption and emission spectra indicated max values at 634 and 650 nm, respectively (Supporting Information), as measured in solution in a cuvette using a SpectraMax M5 reader (Molecular Devices, Sunnyvale, CA).

General Synthesis of P^1-(N^4-Phenylpropoxycytidine-5′-)P^4-(uridine-5′-)-tetraphosphate Triethylammonium Salt (30). Uridine 5′-monophosphate disodium salt (4.4 mg, 11.0 μmol, 2 equiv) and N^4-(3-phenylpropoxy)cytidine-5′-triphosphate triethylammonium salt **2** (4.5 mg, 5.4 μmol, 1 equiv) were converted to the tributylammonium salts by treatment with ion-exchange resin (DOWEX 50WX2-200 (H)) and tributylamine. After removal of the water, the obtained uridine monophosphate dibutylammonium salt and the tributylammonium salt of N^4-phenylpropoxycytidine-5′-triphosphate **2** (4.5 mg, 5.5 μmol, 1 equiv) were dried under high vacuum for 1 h in separate vials. DIC (1 μL, 5.5 μmol, 1 equiv) was added to a solution of N^4-(3-phenylpropoxy)cytidine-5′-triphosphate tributylammonium salt **2** in DMF (200 μL). After stirring the reaction mixture at rt for 3 h, a solution of the uridine 5′-monophosphate tributylammonium salt (11.0 μmol) and $MgCl_2$ (1.0 mg, 11.0 μmol, 2 equiv) in DMF (100 μL) were added. The reaction mixture was stirred at rt overnight. After removal of the solvent, the $MgCl_2$ was removed by treatment with ion-exchange resin (DOWEX 50WX2-200 (H)) and ammonia bicarbonate or tri-*n*-butylamine, and the residue was purified by a semipreparative HPLC purification using system C. Compound **30** (1.0 mg, 0.8 μmol, 16%) was obtained as a white solid. ^{1}H NMR (D_2O) δ 7.93 (d, *J* = 7.9 Hz, 1H), 7.35−7.30 (m, *J* = 8.3 Hz, 4H), 7.25−7.21 (m, 2H), 6.02−5.91 (m, 2H), 5.78 (d, *J* = 7.9 Hz, 1H), 4.42−4.33 (m, 4H), 4.30−4.16 (m, 6H), 4.05 (t, *J* = 4.8 Hz, 2H), 2.74 (t, *J* = 8.3 Hz, 2H), 2.05−1.79 (m, 2H). ^{31}P NMR (D_2O) δ −11.51 (br), −23.22 (br). HRMS-EI found: 922.0742 $(M - H^+)^-$. $C_{27}H_{36}N_5O_{23}P_4$ requires 922.0752; analytical HPLC (system A: 7.92 min).

P^1-(N^4-(3-Phenylpropoxy)cytidine-5′-)P^4-(N^4-(3-phenylpropoxy)cytidine-5′-)-tetraphosphate Triethylammonium Salt (31). Compound **31** (0.5 mg, 0.3 μmol, 10%) was obtained as a white solid using N^4-(3-phenylpropoxy)cytidine-5′-triphosphate triethylammonium salt (3.0 mg, 3.0 μmol) and N^4-(3-phenylpropoxy)cytidine-5′-monophosphate diethylammonium salt (3.0 mg, 6.1 μmol) from the previous procedure. ^{1}H NMR (D_2O) δ 7.45−7.10 (m, 12H), 5.95−5.87 (m, 2H), 5.79−5.63 (m, 2H), 4.39−4.29 (m, 4H), 4.27−4.16 (m, 6H), 4.06−4.00 (m, 4H), 2.80−2.65 (m, 4H), 2.05−1.93 (m, 4H). HRMS-EI found: 1055.1593 $(M - H^+)^-$. $C_{36}H_{47}N_6O_{23}P_4$ requires 1055.1643; analytical HPLC (system A: 9.10 min).

Synthesis of compounds **40**−**96** is described in the Supporting Information.

General Procedure for the Synthesis of N^4-Alkoxycytidine analogues (97−110, 113) N^4-(4-Phenylbutyloxy)cytidine (97). A suspension of cytidine (360 mg, 1.5 mmol) and 4-phenylbutoxyamine hydrochloride (**87**, 617 mg, 3.0 mmol, 2 equiv) in pyridine (4 mL) was stirred at 100 °C overnight. The reaction mixture was evaporated, and the residue was evaporated twice with toluene, triturated with chloroform, and filtered. The filtrate was evaporated, and the residue was purified by flash chromatography (chloroform−methanol, gradient of 3−10%) to afford N^4-(4-phenylbutyloxy)-cytidine (**97**) (0.430 g, 1.10 mmol, 73%). ^{1}H NMR (400 MHz, MeOD): 7.52 (d, J_1 = 8.20 Hz, 1H), 7.20−7.17 (m, 2H), 7.15−7.05 (m, 3H), 5.83 (d, J_1 = 4.84 Hz, 1H), 5.63 (d, J_1 = 8.12 Hz, 1H), 4.13 (t, J_1 = 5.20 Hz, 1H), 4.09 (m, 1H), 4.00−3.92 (m, 3H), 3.78 (dd, J_1 = 12.16 Hz, J_1 = 2.72 Hz, 1H), 3.68 (dd, J_1 =12.20 Hz, J_2 = 3.16 Hz, 1H), 2.60 (t, J_1 = 6.88 Hz, 2H), 1.70−1.60 (m, 4H). ^{13}C NMR (400 MHz, MeOD): 149.2, 147.6, 142.0, 137.1, 135.0, 128.0, 127.9, 125.4, 95.0, 88.9, 84.8, 74.4, 73.7, 69.9, 60.9, 35.1, 27.7, 27.3. *m/z* (M + ESI MS) found, 392.1813; calcd for $C_{19}H_{26}N_3O_6$, 392.1816.

N^4-(3-((4-*tert*-Butyloxy-carbonylmethoxy)phenyl)-propyloxy)cytidine (115). Compound **115** (45 mg, 88.5 μmol, 65%) was obtained as a white solid from cytidine (29 mg, 0.12 mmol) and N^4-(3-(4-*tert*-butyloxy-carbonylmethoxy)phenyl)propyloxyamine hydrochloride (**91**) (79 mg, 0.25 mmol) using the above procedure. ^{1}H NMR (400 MHz, MeOD): 7.16 (d, J_1 = 8.24 Hz, 1H), 7.06 (d, J_1 = 8.36 Hz, 2H), 6.76 (d, J_1 = 8.52 Hz, 1H), 5.82 (d, J_1 = 5.20 Hz, 1H), 5.55 (d, J_1 = 8.24 Hz, 1H), 4.46 (s, 2H), 4.13−4.10 (m, 2H), 3.94−3.91 (m, 3H), 3.76 (d, J_1 = 12.08 Hz, 1H), 3.66 (dd, J_1 =12.12 Hz, J_2 =3.00 Hz, 1H), 2.58 (t, J_1 = 7.08 Hz, 2H), 2.01−1.86(m, 2H), 1.42(s, 9H). ^{13}C NMR (400 MHz, MeOD): 169.0, 156.2, 150.1, 144.7, 134.7, 131.4, 129.1, 114.2, 97.6, 88.4, 84.6, 82.0, 73.4, 72.8, 70.3, 65.4, 61.3, 30.8, 30.4, 27.0. *m/z* (M + ESI MS) found, 508.2294; calcd for $C_{24}H_{34}N_3O_9$, 508.2290.

N^4-(3-(((But-3-ynyl-1-amino)-4-methoxycarbonyl)phenyl)-propyloxy)cytidine (116). N^4-(3-((4-*tert*-Butyloxy-carbonylmethoxy)phenyl)propyloxy)cytidine **115** (44 mg, 0.12 mmol) was dissolved in 1.0 mL of THF and treated with 2.0 mL of 1 N NaOH. The reaction mixture was stirred at 50 °C for 2 h. The mixture was cooled to rt and neutralized by adding 1N HCl. After removal of the solvent, N^4-(3-((4-methoxycarbonate)phenyl)-propyloxy)cytidine (31 mg, 68 μmol, 56%) was isolated after column chromatography. ^{1}H NMR (400 MHz, MeOD): 7.17 (d, J_1 = 8.24 Hz, 1H), 7.09 (d, J_1 = 8.44 Hz, 2H), 6.81 (d, J_1 = 8.64 Hz, 1H), 5.82 (d, J_1 = 5.44 Hz, 1H), 5.55 (d, J_1 = 8.24 Hz, 1H), 4.57 (s, 2H), 4.12 (t, J_1 = 5.40 Hz, 1H), 4.08 (m, 1H), 3.95−3.90 (m, 3H), 3.74 (dd, J_1 = 12.08 Hz, J_1 = 2.88 Hz, 1H), 3.66 (dd, J_1 = 12.16 Hz, J_2 = 3.40 Hz, 1H), 2.60 (t, J_1 = 7.40 Hz, 2H), 1.95−1.86 (m, 2H). ^{13}C NMR (400 MHz, MeOD): 156.2, 150.1, 144.7, 134.7, 131.3, 129.0, 114.1, 97.4, 88.3, 84.7, 73.3, 72.7, 70.3, 64.6, 61.3, 30.8, 30.4, 27.0. *m/z* (M + ESI MS) found, 442.1665; calcd for $C_{20}H_{26}N_3O_9$, 452.1664.

The above isolated acid (50 mg, 0.1 mmol), DIEA (0.128 mL, 0.93 mmol, 8.5 equiv), and 6.0 mL of DMF were added to round-bottom flask. Then 4-amino-1-butyne (13.5 μL, 0.16 mmol, 1.5 equiv) and 1.0 mL of DMF were added to the above mixture, which was then stirred for 10 min at rt. PyBOP (57 mg, 0.11 mmol, 1.1 equiv) was added, and the mixture was stirred overnight. Solvent was removed, and **116** (45.1 mg, 89.9 μmol, 80%) was isolated following column chromatography. ^{1}H NMR (400 MHz, MeOD): 7.17 (d, J_1 = 8.28 Hz, 1H), 7.11 (d, J_1 = 8.56 Hz, 2H), 6.86 (d, J_1 = 8.64 Hz, 2H), 5.82 (d, J_1 = 5.44 Hz, 1H) 5.53 (d, J_1 = 8.24 Hz, 1H), 4.43 (s, 2H), 4.15−4.00 (m, 2H), 3.95 (t, J_1 = 6.36 Hz, 2H), 3.91 (q, J_1 = 3.32 Hz, 1H), 3.75 (dd, J_1 = 12.16 Hz, J_1 = 2.88 Hz, 1H), 3.66 (dd, J_1 = 12.12 Hz, J_2 = 3.36 Hz, 1H), 3.38 (t, J_1 = 7.08 Hz, 2H), 2.62 (t, J_1 = 7.40 Hz, 2H), 2.37 (td, J_1 = 7.08 Hz, J_1 = 2.64 Hz, 2H), 2.25 (t, J_1 = 2.64 Hz, lH), 2.01−1.85 (m, 2H). ^{13}C NMR (400 MHz, MeOD): 170.11, 155.98, 150.03, 144.68, 135.27, 131.37, 12 129.5, 123.9, 114.7, 114.5, 112.1, 111.9, 97.4, 88.3, 84.7, 73.3, 72.5, 70.3, 61.3, 31.4, 30.1. *m/z* (M + H ESI MS) found, 503.2139; calcd for $C_{24}H_{31}N_4O_8$, 503.2136.

Procedures for Phospholipase C Assay. Stable cell lines for study of the human (h) $P2Y_2$, $P2Y_4$, and $P2Y_6$Rs were produced by retroviral expression of the individual receptors in 1321N1 human astrocytoma cells, which do not natively express P2YRs.[31] Agonist-stimulated [^{3}H]inositol phosphate accumulation was quantified in cells plated at 20000 cells/well on 96-well plates two days prior to assay.

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The inositol lipid pool of cells was radiolabeled 16 h prior to the assay by incubation with 100 μL of serum-free inositol-free Dulbecco's Modified Eagle's Medium, containing 1.0 μCi of [^{3}H]*myo*-inositol. No changes of medium were made subsequent to the addition of [^{3}H]inositol. Test drugs were added in 25 μL of 100 mM Hepes (*N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid), pH 7.3, in HBSS containing 50 mM LiCl. Incubations were for 30 min at 37 °C and were terminated by aspiration of the drug-containing medium and addition of 90 μL of ice-cold 50 mM formic acid. The samples were neutralized with 30 μL of 150 mM NH_4OH and applied to Dowex AG1-X8 anion exchange columns. Total inositol phosphates were eluted, and radioactivity was measured using a liquid scintillation counter.[26]

Data Analysis. Agonist potencies (EC_{50} values) were determined from concentration−response curves by nonlinear regression analysis using the GraphPad software package Prism (GraphPad, San Diego, CA). Each concentration of drug was tested in triplicate assays, and concentration effect curves for each test drug were repeated in at least three separate experiments with freshly diluted molecule. The results are presented as mean ± SEM from multiple experiments or in the case of concentration effect curves from a single experiment carried out with triplicate assays that were representative of results from multiple experiments.

Cell Cultures for FCM. 1321N1 human astrocytoma cells overexpressing the $P2Y_6R$ were grown in DMEM with 5% FBS, 50 U/mL penicillin/streptomycin, and 2 mM L-glutamine. Cells were grown in 6-well plates (approximately 3 × 10^5 cells/well) and incubated at 37 °C and 5% CO_2 for 24 h. At 80% confluency, the medium was replaced with fresh preheated medium, in addition to α,β-methylene-adenosine 5′-diphosphate (**125**, AMP-CP, 100 μM) and a known agonist or antagonist of the $P2Y_6R$. After a 30 min incubation, **28** was added at different time intervals and the decrease in fluorescence intensity was measured by FCM.

Fluorescent Ligand Binding in 1321N1 Astrocytoma Cells Expressing $P2Y_6R$. Binding of **28** to the $P2Y_6R$ in overexpressing 1321N1 astrocytoma cells was blocked by using known $P2Y_6R$ ligands, such as antagonists **118** and **119** or the agonist **120** (all 10 μM). Ectonucleotidase inhibitor **125** (100 μM) was added to prevent the metabolism of 5′-di and triphosphate derivatives to the 5′-monophosphates.[32−,34] After a 30 min preincubation with the appropriate agonist or antagonist and **125** (100 μM), **28** (100 nM) was added at different time intervals from 20 to 30 min. At the end of incubation, the medium was removed, the cells were washed three times with ice-cold DPBS, and 1 mL of 0.2% EDTA was added to each well to detach the cells from the plate. Cells were then incubated at 37 °C for 5−10 min. After detaching, 1 mL of medium was added to neutralize the EDTA. The cell suspensions were transferred to 5 mL of polystyrene round-bottom BD Falcon tubes (BD, Franklin Lakes, NJ) and centrifuged for 5 min at 23 °C and 400*g*. After the supernatant was discarded, the cells were washed with 3 mL of DBPS and centrifuged again at 23 °C and 400*g* for 5 min. After centrifugation, the supernatant was discarded and the cells suspended in 0.5 mL of DPBS for analysis by FCM.

FCM Analysis. The intensity of fluorescence emission of each sample was measured by using FCM. Cell suspensions were vortexed briefly before analysis on a Becton and Dickinson FACSCalibur flow cytometer (BD, Franklin Lakes, NJ). Samples were maintained in the dark during the analysis to avoid photobleaching. Measured fluorescent intensities (MFIs) were obtained in the FL-4 channel in log mode. Ten thousand events were analyzed per sample. Data were collected and analyzed using Cell Quest Pro software (BD, Franklin Lakes, NJ).

Fluorescent Microscopy Studies. $P2Y_6R$-expressing astrocytoma cells were grown on glass coverslips placed in a 6-well plate. When the cells reached 80% confluence, the medium was replaced with fresh medium, $P2Y_6R$ antagonist **118** (10 μM) was added to the cells, and incubation was continued for 30 min at 37 °C. Then **16** was added to achieve a final concentration of 1 μM, and incubation continued for 30 min at 37 °C. At the end of the incubation, the cells were washed with ice-cold PBS and mounted on a glass slide. The cells were visualized using a Keyence BZ-9000 fluorescent microscope.

Data Analysis. Data analysis was performed with the Prism 5 (GraphPad, San Diego CA) software. The mean autofluorescence of 1321N1 astrocytoma cells was measured in the absence of the fluorescent ligand. The mean fluorescence intensity in the presence of fluorescent ligand was corrected by subtracting the autofluorescence.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Chemical synthesis of intermediates and nucleotides, characterization data including selected NMR and MS spectra, HPLC purification and purity analysis procedures, cell culture, and membrane preparation. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 301-496-9024. Fax: 301-480-8422. E-mail: kajacobs@helix.nih.gov. Address: Bldg. 8A, Rm. B1A-19, NIDDK, National Institutes of Health, Bethesda, Maryland 20892-0810, United States.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

1321N1-P2YR cells, 1321N1 human astrocytoma cells expressing a human P2Y receptor; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DIC, *N*,*N*′-diisopropylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DPBS, Dulbecco's phosphate buffered saline; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide; FCM, flow cytometry; GPCR, G protein-coupled receptor; HBSS, Hank's Balanced Salt Solution; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HPLC, high performance liquid chromatography; MFI, measured fluorescent intensity; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; P2YR, P2Y receptor; PLC, phospholipase C; SAR, structure−activity relationship; TBAP, tetrabutylammonium dihydrogenphosphate; TBTA, tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl] amine; TEAA, triethylammonium acetate; THF, tetrahydrofuran; TLC, thin layer chromatography; UTP, uridine-5′-triphosphate

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