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Synthesis and P2Y Receptor Activity of a Series of Uridine Dinucleoside 5′-Polyphosphates

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Abstract—A series of dinucleoside 5-polyphosphates Up_nU ($n=2$–7) was synthesized. Their relative potencies as agonists at the G-protein-coupled receptors $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ were determined by intracellular calcium measurements using fluorescent imaging techniques. The correlation of phosphate chain length to activities at these receptors is discussed.

Introduction

Diadenosine 5′-polyphosphates (Ap_nA, $n=2$–6) have received a great deal of attention over the last decade, with their release from platelets and chromaffin cells inspiring many studies on the potential biological activity of these molecules.[1] Interest in uridine nucleotides as extracellular signalling molecules increased with the discovery that uridine 5′-triphosphate (UTP) was an agonist at the $P2Y_2$ receptor, with a potency comparable to the natural purine agonist for the receptor, adenosine 5′-triphosphate (ATP). Stimulation of this receptor results in activation of mucosal hydration and mucociliary clearance mechanisms in the body.[2–5] While a variety of dipurine polyphosphates have been reported[6] and the series Ap_nA ($n=2$–6) and Gp_nG ($n=2$–5) are commercially available,[7] several of the pyrimidine dinucleotides Up_nU ($n=2$–7) have not been described.[8] Here we report syntheses of this latter series of compounds of which three ($n=5$, 6, and 7) are new and determine their relative potencies as agonists at the $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ G-protein-coupled receptors. One member of this family of dinucleotides, P^1, P^4-di(uridine 5′-)tetraphosphate (Up_4U, INS365), exhibits comparable potency with UTP as an agonist at the $P2Y_2$ and $P2Y_4$ receptors and is currently in clinical development for the treatment of dry eye[9] and chronic lung diseases, such as cystic fibrosis.[10]

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Synthesis

As a result of our ongoing interest in dinucleoside 5′-polyphosphates, we have developed a variety of coupling protocols to prepare them from the corresponding nucleotides. In general, these procedures rely on converting the water soluble salts of nucleotides into organic soluble trialkylammonium salts, which allows the phosphate couplings to be carried out under anhydrous conditions in DMF or DMSO. The present method starts with the conversion of uridine monophosphate (UMP, **1**), uridine diphosphate (UDP, **3**), or UTP (**5**) from their sodium salts into their NBu_3 salts by standard procedures,[11] followed by activation with either CDI or DCC in DMF. The activated intermediates were not isolated, but were directly condensed with another nucleotide (also as the NBu_3 salt).

Compound **1** was treated with CDI to give the phosphorimidazolidate **2** as the activated species,[12] and this was reacted with either **1** or **3** to give diuridine 5′-diphosphate (Up_2U, **7**) or diuridine 5′-triphosphate (Up_3U, **8**), respectively (Scheme 1). Diuridine 5′-tetraphosphate (**9**) was synthesized by activation of **5** with DCC, which gave the known cyclic uridine 5′-trimetaphosphate (**6**),[13] followed by condensation with **1**. Alternately, **3** could be activated with CDI to give the corresponding imidazolidate (**4**), which was condensed with **3** to give **9**. Diuridine 5′-pentaphosphate (Up_5U, **10**) was prepared by conversion of **5** to **6** with DCC, followed by ring opening of the cyclic 5′-trimetaphosphate with **3**. In the course of the synthesis of **10**, diuridine

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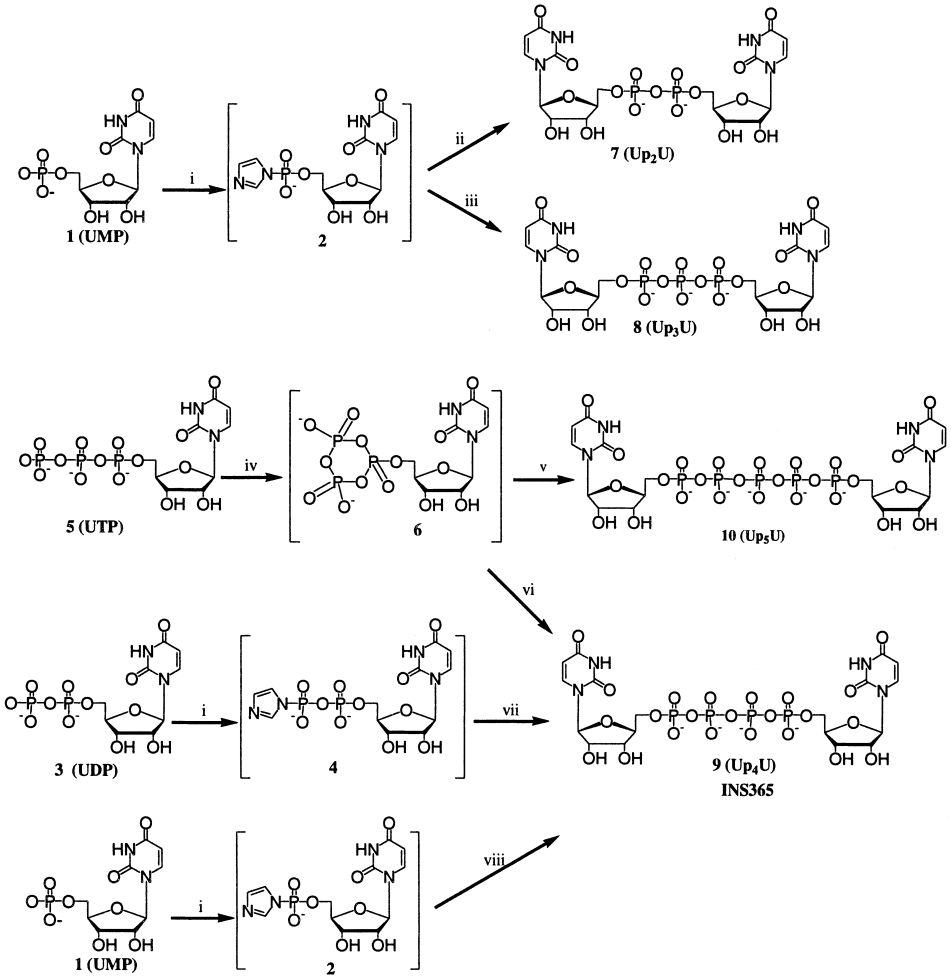
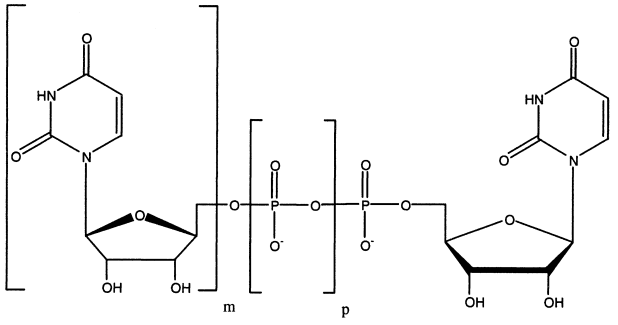


Scheme 1. Reagents and conditions: (i) CDI, NBu_3, DMF; (ii) **1**, DMF, 50 °C, 63%; (iii) **3**, DMF, 50 °C, 50%; (iv) DCC, NBu_3, DMF, rt, quantative; (v) **3**, DMF, 40 °C, 8.5% as compound **10**; (vi) **1**, DMF, 40 °C, 32%; (vii) **3**, DMF, 50 °C, 25%; (viii) triethylammoniumpyrophosphate, pyridine, 30 °C.

Table 1. Correlation of P2Y agonist activity with chain length in Up_nU derivatives versus UTP and UDP



Compound	m	p	EC_{50} (μM)			
			$P2Y_1$	$P2Y_2$	$P2Y_4$	$P2Y_6$
5 (UTP·3Na)	0	2	ia[a]	0.03	0.1	>10
3 (UDP·2Na)	0	1	ia	>10	ia	0.1
7 (Up_2U·2NH_4)	1	1	ia	ia	ia	>30
8 (Up_3U·3NH_4)	1	2	ia	22.0	>100	0.2
9 (Up_4U·4Na)	1	3	ia	0.10	0.4	20.0
10 (Up_5U·5NH_4)	1	4	ia	5.6	>20	>30
11 (Up_6U·6NH_4)	1	5	ia	9.8	>30	>30
12 (Up_7U·7NH_4)	1	6	ia	8.4	>30	>100

[a]ia, inactive at 100 μM.

5′-hexaphosphate (Up_6U, **11**) and diuridine 5′-heptaphosphate (Up_7U, **12**) were generated as minor byproducts. Compound **11** arose from the condensation of **6** with **5**, as a result of a small amount of hydrolysis of **6** in the reaction mixture. Compound **12** was likely generated from the reaction between **6** and uridine 5′-tetraphosphate (Up_4), which is present as a minor impurity in **5**. All compounds were purified[14] using either gel filtration chromatography or ion exchange HPLC, and were characterized by ^1H NMR, ^{31}P NMR, and HRMS.[15]

Biological Activity

The functional activities of test compounds were assessed by measuring changes in the levels of cytosolic calcium in 1321N astrocytoma cells infected with a retrovirus encoding the human $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors. For assay, the cells were plated in black wall/clear bottom cell culture plates (cat. # 3904, Corning Inc., Corning, NY) and grown to confluence. On the day of assay, the growth medium was aspirated and replaced with a solution of Fluo-3, AM (2.5 μM final concentration (Molecular Probes, Eugene, OR)) in an

assay buffer consisting of (mM): KCl (10.0), NaCl (118), $CaCl_2$ (2.5), $MgCl_2$ (1.0), HEPES (20), glucose (10) pH 7.4. After a 60 min incubation with Fluo-3, AM at 25 °C, cells were washed free of dye (Columbus Plate Washer, TECAN U.S., Inc., Research Triangle Park, NC). Receptor/cell activation was measured by monitoring changes in fluorescence intensity (an indicator of cytosolic calcium mobilization) using the FLIPR™ (Molecular Devices Corp., Sunnyvale, CA). Data (relative fluorescence units) were modeled using PRISM™ (San Diego, CA). EC_{50} values (Table 1) were estimated from the fitted curve functions.

Results and Discussion

While seemingly simple and relatively mild, the phosphate coupling reactions depicted in Scheme 1 often give rise to byproducts that are difficult to remove. Consequently, we found that multiple chromatographic purifications on either Sephadex DEAE A-25 or PRP-X100 HPLC were required to obtain compounds with adequate purity for biological testing.[14] In general, the yields were inversely proportional to phosphate chain length, in keeping with the larger number of unwanted side reactions possible between the starting materials, their degradation products and any excess of coupling reagents in the more complex reaction mixtures. For example, the reaction between **2** and **1** or **3** proceeded with greater than 90% HPLC conversion to **7** or **8**, and gave isolated yields of 63 and 50%, respectively. At the other extreme, compound **10** was obtained in only 20% crude chromatographic purity, contaminated with the closely-eluting byproducts **9**, **11**, and **12**. Following chromatography on Sephadex DEAE A-25, an 8.5% yield of **10** was obtained, with a purity of 87% (remainder 0.7% **9** and 12% **11**). Compounds **11** and **12** were isolated from the same reaction by HPLC, with a final purity of 94% (remainder 2.5% **10** and 3.3% **12**) and 91% (remainder 1.4% **10** and 7.2% **11**), respectively. Compound **9** was prepared by several routes, with the method employing DCC giving the greatest conversion by HPLC (>50%) and highest isolated yield (32%). Use of CDI to couple **3** gave a somewhat lower isolated yield (25%), while the coupling of two molecules of intermediate **2** with inorganic pyrophosphate[16] gave a low yield of **9** which was tainted with Up_4 following purification on Sephadex. Of the six diuridine 5-polyphosphates tested, only **7**, **8**, and **9** were obtained with purity greater than 99%.

None of the compounds tested exhibited activity at the $P2Y_1$ receptor. The activities at the $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors were dependent upon the length of the phosphate chains. The rank-order of potency at the $P2Y_2$ and $P2Y_4$ receptors were similar with **9** being the most potent compound. The potencies of all compounds in this series were approximately 4-fold lower at the $P2Y_4$ receptor as compared with the $P2Y_2$ receptor. Only **8** exhibited significant activity at the $P2Y_6$ receptor. These results are consistent with the activities observed with UTP and UDP, where the nucleotides' activities at the $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors were also dependent upon phosphate chain length. In the dinucleotide series, when the phosphate chain was extended by one, the activity paralleled that of the natural nucleotide agonist for a given receptor. Thus, compound **8** mirrored the activity of UDP on $P2Y_2$ and $P2Y_6$, whereas compound **9** mimicked that of UTP on $P2Y_2$ and $P2Y_4$. Compounds **10**, **11**, and **12** showed some selectivity for $P2Y_2$, with **10** being the most potent of the three. However, this apparent increase in potency might be attributable to the small amount of **9** that was present in **10**.

In conclusion, we have described the synthesis and biological activities of a series of diuridine polyphosphate compounds. No activity was observed at the $P2Y_1$ receptor with any of the compounds. The $P2Y_2$ and $P2Y_4$ receptors exhibited a marked preference for **9** whereas the $P2Y_6$ receptor exhibited a marked preference for **8**.

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14. Gel filtration: Pharmacia Sephadex DEAE A-25, 40120 μm, gradient from water to 1 M NH_4HCO_3; HPLC: Hamilton PRP-X100 column, 250 4.1 mm, 10 μm, gradient from water to 90% 1 M NH_4HCO_3/10% ACN over 30 min, 2 mL/min, monitor at 260 nm. All reported yields in Scheme 1 are for isolated compounds.
15. Selected spectroscopic data: Compound **9** (4Na salt), ^{1}H NMR (300 MHz, D_2O) δ 7.81 (d, J = 8.1 Hz, 1H), 5.86 (d, J = 5.4 Hz, 1H), 5.84 (d, J = 8.1 Hz, 1H), 4.27 (m, 1H), 4.25 (m, 1H), 4.14 (m, 1H), 4.11 (m, 2H); ^{31}P NMR (D_2O, H_3PO_4 std) δ −22.32 (m, 2P), −10.75 (m, 2P). HRMS (FAB) m/z $C_{18}H_{22}N_4O_{23}P_4Na_4$ $(M-Na)^-$; calcd: 854.9318; found: 854.9268. Compound **7** (NH_4 salt): ^{31}P NMR (D_2O) δ −10.10 (s), HRMS (FAB) m/z $C_{18}H_{23}N_4O_{17}P_2$ $(M-H)^-$; calcd: 629.0533; found: 629.0552. Compound **8** (NH_4 salt): ^{31}P NMR (D_2O) δ −10.31 (d, J = 19.2 Hz, 2P), −21.87 (t, J = 18.95 Hz, 1P). HRMS (FAB) m/z $C_{18}H_{24}N_4O_{20}P_3$ $(M-H)^-$; calcd: 709.0197; found: 709.0191. Compound **10** (NH_4 salt): ^{31}P NMR (D_2O) δ −10.30 (d, 2P), −22.14 (m, 3P), HRMS (FAB) m/z $C_{18}H_{26}N_4O_{26}P_5$ $(M-H)^-$; calcd: 868.9523; found: 868.9561. Compound **11** (NH_4 salt): ^{31}P NMR (D_2O) δ −10.12 (d, 2P), −21.76 (m, 4P). HRMS (FAB) m/z $C_{18}H_{27}N_4O_{29}P_6$ $(M-H)^-$; calcd: 948.9187; found: 948.9199. Compound **12** (Na salt): ^{31}P NMR (D_2O) δ −10.18 (d, 2P), −21.72 (m, 5P). HRMS (FAB) m/z $C_{18}H_{29}N_4O_{32}P_7$ $(M-2H+Na)^-$; calcd: 1050.8670; found: 1050.8629.
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