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Discovery and synthesis of a novel and selective drug-like $P2X_1$ antagonist

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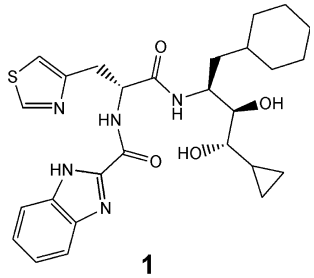
Abstract—Although there is extensive literature to indicate that many different types of P2 purinoceptors are present in the lower urinary tract, the physiological role of these receptors in micturition is still uncertain. In part, this uncertainty has been caused by a lack of P2 subtype selective ligands. In this paper we report the discovery, gram scale synthesis, and binding results for **1**, the first potent, drug-like, selective $P2X_1$ receptor antagonist described. Compound **1** was shown to be more than 30-fold selective over other purinergic receptor subtypes.

1. Introduction

P2X receptors are plasma membrane, ligand gated ion channels that open in response to the binding of extracellular ATP. These purinergic receptors are abundantly distributed in the body, and functional responses are seen in neurons, glia, epithelia, bone, muscle, and hemopoietic tissues.[1] Although reports indicate that many different types of P2 receptors are present in the lower urinary tract, the role of these receptors in micturition is still uncertain.[2,3] In recent years considerable effort has been dedicated to identifying subtype selective P2X receptor ligands.[3,4] However, the study of the P2X receptors continues to be hampered by the lack of drug-like, potent and selective antagonists.



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In this study, we report the discovery and preparation of compound **1** and its in vitro affinity profile as a $P2X_1$ antagonist. High-throughput screening of the Roche compound library resulted in the discovery of **1** (a compound originally prepared as a potential rennin inhibitor but shown to be inactive[5]) as a validated hit with $P2X_1$ affinity. For further biological characterization a synthetic route was required to provide **1** in substantial quantities.

The synthetic approach developed to **1** is presented in Schemes 1 and 2.

Synthesis of **1** was initiated with the preparation of large quantities of the chiral α-hydroxy-β-aminoacid **2** as a key starting material (following the synthesis described in the literature[6]) (Scheme 1). Thus, addition of an excess of cyclopropyllithium[7] to the carboxylic acid **2** gave the cyclopropyl ketone **3** in 94% yield (Scheme 1). Stereoselective reduction[6]of ketone **3** with triacetoxyborohydride produced the corresponding (*S*)-hydroxy compound in quantitative yield. Treatment of the crude product from the previous step with KOH in MeOH/water afforded the crucial chiral aminodiol compound **4** (52% over two steps). Coupling reaction between aminodiol **4** and the commercially available N-boc-protected aminoacid **5** using PyBOP,[8] furnished the amide **6** (Scheme 2). TFA induced deprotection of **6** gave the corresponding α-aminoamide **7** in 98% yield. Finally, the coupling reaction between this aminoacid **7** with

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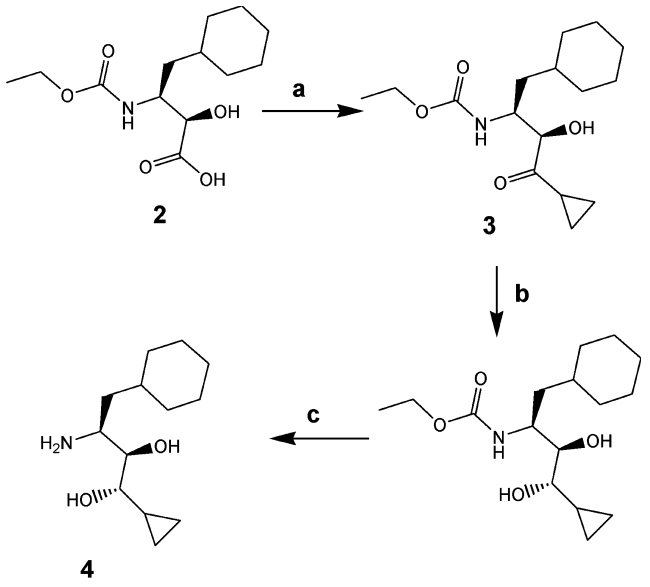


Scheme 1. Reagents and conditions: (a) cyclopropyllithium, toluene/THF/LiBr, 94%; (b) triacetoxyborohydride, heptane/MeCN; (c) KOH, MeOH/water, 52% over the two steps.

2-imidazolylcarboxylic acid afforded the final product **1** (51% yield).

Biological evaluation in vitro of **1** shows high affinity for $P2X_1$ receptor and remarkable selectivity (more than 30-fold) over other purinergic receptor subtypes (Table 1).

In summary we have described the discovery that compound **1** is a selective $P2X_1$ antagonist. In addition, we have reported a new practical synthesis of this material, which allowed us to prepare **1** in large quantities for its biological characterization.

Table 1. Shows the (**1**) IC_{50}s from $[CA^{2+}]_i$ measurements obtained with FLIPR[a]

Receptor	IC_{50}, μM
$hP2X_1$	3
$hP2X_2$	>100
$hP2X_3$	>100
$hP2_{2/3}$	>100

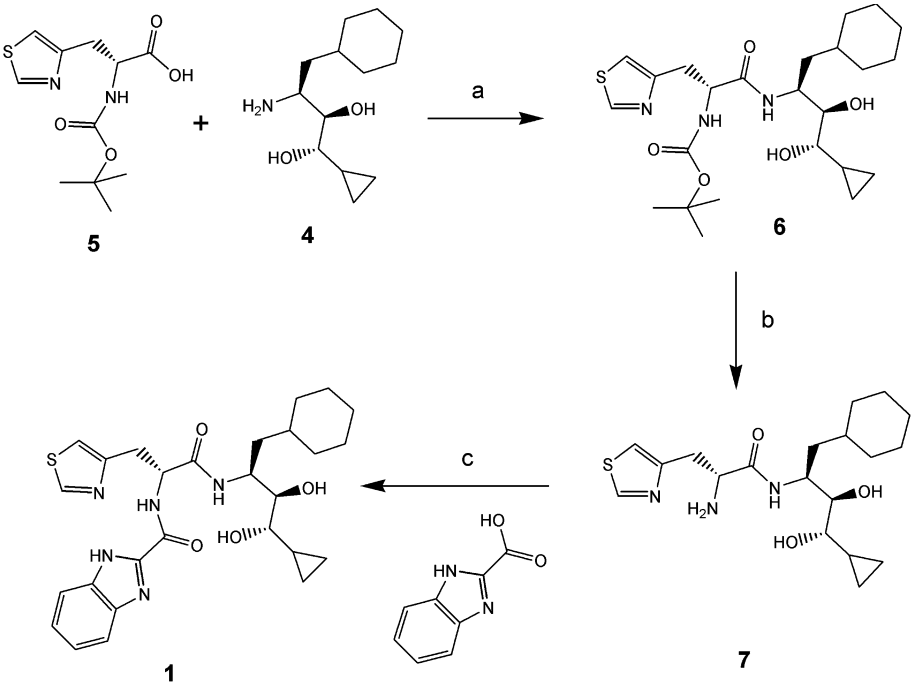
[a] Flurometric imaging plate reader.

2. Experimental

The ^1H NMR and ^{13}C NMR spectra were measured on a Bruker Avance DPX-300 NMR or on a Bruker AMX-300 NMR spectrometer, operating at a proton (^1H) frequency of 300.13 MHz and carbon (^{13}C) frequency of 75.43 MHz. The mass spectra were recorded on a Finnigan-MAT 8230 or on a VG ZabSpec-QFPD spectrometer.

2.1. (1-(*S*)-Cyclohexylmethyl-3-cyclopropyl-2-(*R*)-hydroxy-3-oxo-propyl)-carbamic acid ethyl ester (3)

Lithium bromide (2.22 g, 25.58 mmol) and a solution of 4-cyclohexyl-3-(*S*)-ethoxycarbonylamino-2-(*R*)-hydroxybutyric acid[6] (**2**) (2.69 g, 9.84 mmol) in toluene were combined and stirred under a nitrogen atmosphere. The mixture was cooled to 5 °C and 10 mL of THF was added. The mixture was stirred for 30 min at this temperature, and then cooled to −40 °C. A solution of cyclopropyllithium[7] (0.88 M in ethyl ether, 4.6 mL, 40.54 mmol) was slowly added over 30 min while maintaining the temperature at −40 °C. The reaction was stirred for 15 min at this temperature and then stirred at 5 °C for 2 h. The reaction was quenched at 5 °C by addition of a saturated aqueous solution of ammonium chloride. After the layers were separated, the



Scheme 2. Reagents and conditions: (a) PyBOP, Et_3N, DMF, 77%; (b) TFA, dichloromethane, 98%; (c) PyBOP, Et_3N, DMF, 51%.

aqueous layer was extracted with dichloromethane (4 × 40 mL). The combined organics were dried ($MgSO_4$) and concentrated under vacuum. The crude product was used in the next step without further purification. Yield 2.76 g (94%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.73 (br s, 1H, D_2O), 4.69 (d, 1H, J = 10.0 Hz), 4.48 (m, 1H), 4.32 (s, 1H), 4.05 (q, 2H, J = 6.9 Hz), 2.10 (m, 1H), 1.91 (d, 1H, J = 12.5 Hz), 1.76–1.51 (m, 8H), 1.28 (m, 1H), 1.92–1.30 (m, 7H), 1.20 (t, 3H, J = 6.9 Hz). ^{13}C NMR (75.47 MHz, $CDCl_3$): δ 211.02, 156.04, 78.56, 60.86, 49.57, 40.80, 34.17, 33.51, 33.10, 26.51, 26.27, 26.14, 17.44, 14.56, 13.02, 12.44. MS *m/z*: 298 $[M+H]^+$.

2.2. 3-(*S*)-Amino-4-cyclohexyl-1-cyclopropyl-butane-1-(*S*),2-(*R*)-diol (4)

Sodium triacetoxyborohydride (1.92 g, 9.09 mmol) was suspended in heptane (5 mL), and (1-(*S*)-cyclohexylmethyl-3-cyclopropyl-2-(*R*)-hydroxy-3-oxo-propyl)-carbamic acid ethyl ester (**3**) (2.76 g, 9.28 mmol) dissolved in acetonitrile (5 mL) was added at room temperature. The reaction mixture was stirred for 4 h at 20–24 °C, then cooled to 5–10 °C, and quenched with 9% aqueous sodium bicarbonate (18 mL). The product was extracted with dichloromethane (4 × 30 mL). Combined organics were dried ($MgSO_4$) and concentrated under vacuum. To the crude product dissolved in a mixture of methanol/water (7:3) (25 mL) was added 45% aqueous potassium hydroxide (4.6 g), and the reaction solution was heated at reflux for 18 h. The reaction solution was cooled to 10 °C and heptane (7 mL) was added. After bringing to reflux, again, the solution was cooled in an ice bath, and the resultant solid was collected. The solid product was washed with a mixture of methanol/water (6:1) (10 mL) and water (2 × 15 mL) and vacuum dried to obtain 1.09 g of off-white crystalline solid **4** (52% yield), mp 139.1–140.9 °C (Lit.[5] mp 141–142 °C). Calculated for $C_{13}H_{25}NO_2$: 0.3 mol H_2O; C, 67.09; H, 11.09; N, 6.02. Found: C, 66.92; H, 10.69; N, 6.03. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.77 (br s, 1H, D_2O), 3.44 (m, 1H), 3.26 (m, 1H), 3.15 (dd, 1H, J = 3.6, 7.6 Hz), 1.80–1.60 (m, 5H), 1.50–1.10 (m, 6H), 1.05–0.80 (m, 3H), 0.63–0.36 (m, 3H), 0.33–0.22 (m, 1H). ^{13}C NMR (75.47 MHz, $CDCl_3$): δ 78.92, 74.34, 48.62, 43.53, 34.05, 33.95, 32.91, 26.55, 26.36, 26.20, 13.96, 2.39, 1.79. MS *m/z*: 228 $[M+H]^+$.

2.3. [1-(*R*)-(1-(*S*)-Cyclohexylmethyl-3-cyclopropyl-2-(*R*),3-(*S*)-dihydroxy-propylcarbamoyl)-thiazol-4-yl-ethyl]-carbamic acid *tert*-butyl ester (6)

To a mixture of the amine **4** (1 g, 4.3 mmol), Boc-D-aminoacid **5** (1.19 g, 4.3 mmol) (Synthetech Inc.) and triethylamine (1.3 g, 12.9 mmol) in DMF (50 ml) was added PyBOP[8] (2.74 g, 5.2 mmol) (Calbiochem–Novabiochem Corporation) at room temperature. The reaction mixture was stirred for 8 h at the same temperature. The mixture was then poured into water (200 ml), and the product was extracted with EtOAc (3 × 100 ml). The combined organic layers were washed with water (4 × 100 ml), dried (Na_2SO_4), and evaporated under vacuum. The crude product was purified by column chromatography (SiO_2, Hex/EtOAc, 1:1) to provide 1.62 g (77% yield) of the title compound, mp 133–135 °C. Calculated for $C_{24}H_{39}N_3O_5S$: 0.5 mol H_2O; C, 59.85; H, 8.16; N, 8.72. Found: C, 58.85; H, 8.03; N, 8.81. ^{1}H NMR (300 MHz, DMSO): δ 9.0 (d, 1H, J = 1.8 Hz), 7.38 (d, 1H, J = 8.5 Hz, D_2O), 7.34 (d, 1H, J = 1.8 Hz), 7.02 (d, 1H, J = 8.1 Hz, D_2O), 4.76 (br d, 1H, D_2O), 4.40 (br s, 1H, D_2O), 4.31 (m, 1H), 4.09 (m, 1H), 2.81–3.21 (m, 4H) 0.70–1.75 (m, 14H), 1.34 (s, 9H), 0.14–0.36 (m, 4H). ^{13}C NMR (75.47 MHz, DMSO): δ 171.95, 155.10, 153.45, 153.30, 115.38, 78.23, 76.04, 70.89, 54.34, 46.87, 45.70 33.40, 33.05, 32.10, 28.07, 26.11, 25.78, 25.63, 13.71, 8.62, 1.95, 0.14. MS *m/z*: 482 $[M+H]^+$.

2.4. 2-(*R*)-Amino-*N*-(1-(*S*)-cyclohexylmethyl-3-cyclopropyl-2-(*R*),3-(*S*)-dihydroxy-propyl)amino]-3-thiazol-4-yl-propionamide (7)

To a solution of Boc-aminodiol **6** (1.8 g, 4.7 mmol) in dichloromethane (100 ml) was added TFA (10 ml) at 0 °C. The reaction mixture was stirred for 4 h at room temperature. Then the mixture was poured into a saturated aqueous solution of $NaHCO_3$ (300 ml) and the product was extracted with dichloromethane (3 × 200 ml). Organic extracts were dried (Na_2SO_4) and evaporated under vacuum. The crude product was used in the next step without any further purification (1.4 g, 98% yield). MS *m/z*: 382 $[M+H]^+$.

2.5. 1*H*-Benzoimidazole-2-carboxylic acid [1-(*R*)-(1-(*S*)-cyclohexylmethyl-3-cyclopropyl-2-(*R*),3(*S*)-dihydroxy-propylcarbamoyl)-2-thiazol-4-yl-ethyl]-amide (1)

To a mixture of aminothiazole **7** (1.5 g, 4.1 mmol), 1*H*-benzimidazole-2-carboxylic acid (0.73 g, 4.5 mmol) (Maybridge) and triethylamine (1.25 g, 12.3 mmol) in DMF (50 ml) was added PyBOP (2.57 g, 4.9 mmol) at room temperature. The reaction mixture was stirred for 8 h at the same temperature. After that the reaction was poured into water (300 ml) and the product crystallized out from the mixture. The product was collected by filtration and crystallized from ether to give 1.1 g of pure product (51% yield) as a white solid. $[\alpha]_D$ −39.0 (c = 0.5 g/mL, MeOH). ^{1}H NMR (300 MHz, DMSO): δ 13.29 (s, 1H, D_2O), 9.92 (d, 1H, J = 1.9 Hz), 8.92 (d, 1H, J = 8.5, D_2O), 7.53–7.50 (br d, 1H, J = 7.2 Hz), 7.75–7.57 (m, 2H), 7.40 (d, 1H, J = 1.9 Hz), 7.34–7.24 (m, 2H), 4.98–4.90 (m, 1H), 4.81 (d, 1H, J = 6.3 Hz, D_2O) 4.42 (d, 1H, J = 4.7 Hz, D_2O), 4.18–4.08 (m, 1H), 3.44 (m, 1H), 3.33–315 (m, 2H) 3.16–3.11 (m, 1H), 2.92–2.86 (m, 1H), 1.75–0.77 (m, 14H), 0.33–0.17 (m, 4H). ^{13}C NMR (75.47 MHz, DMSO): δ 170.86, 158.39, 153.85, 153.11, 145.11, 142.50, 134.63, 124.37, 122.77, 120.07, 115.80, 112.68, 76.07, 70.92, 53.13, 47.24, 39.08, 33.65, 33.51, 32.27, 26.22, 25.88, 25.78, 13.88, 2.10, 0.22, 0.10. HRMS calcd for $C_{27}H_{36}N_5O_4S$ $(M+H)^+$ 526.2488, found 526.2481.

2.6. Intracellular calcium measurements (FLIPR)

Transfected CHO-K1 cells (expressing $hP2X_1$ or $hP2X_3$) and 1321N1 astrocytoma cells (expressing $hP2X_2$ or

$hP2X_{2/3}$) were centrifuged and resuspended in nutrient media at 2.5×10^5 cells/ml. The cells were aliquoted into black-walled, clear bottom, 96-well FLIPR plates at a density of 50,000 cells/well and incubated overnight in 7% CO_2 at 37 °C. After overnight incubation, the cells were washed in calcium- and magnesium-free Hank's balanced salt solution supplemented with 10 mM HEPES, 2 mM $CaCl_2$ and 2.5 mM probenecid (FLIPR buffer); the cells were then allowed to incorporate a calcium-sensitive fluorescent dye (2 μM Fluo-3 AM; Molecular Probes, Eugene, OR) at 37 °C for 1 h in 100 μL FLIPR buffer. RO0437626 was dissolved in DMSO at 10 mM and serially diluted with FLIPR buffer to 4× concentrations ranging from 400 μM to 40 nM. The cells were washed four times with FLIPR buffer, leaving 75 μL in each well, and 25 μL of the 4X solutions of RO0437626 or vehicle added to each well in order to attain final concentrations ranging from 100 μM to 10 nM. The cells were allowed to equilibrate with antagonist at room temperature for 20 min before a baseline fluorescence measurement was obtained (excitation 488 nm, emission 510–570 nm) and the reactions started with the addition of α,β-meATP (0.1 μM for $hP2X_1$, 1 μM for $hP2X_3$, 5 μM for $hP2X_{2/3}$; final concentrations) or ATP (3 μM for $hP2X_2$; final concentration). Fluorescence was measured for 2 min at 1–20 s intervals, with readings taken until either a plateau phase was reached ($hP2X_2$ and $hP2X_{2/3}$) or the fluorescence returned to baseline ($hP2X_1$ and $hP2X_3$). Finally, a calcium ionophore, ionomycin, was added to each well (5 μM final concentration) to establish cell viability and maximum fluorescence of dye-bound cytosolic calcium. IC_{50}s were determined by fitting the data using the four parameter logistic equation utilized by GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

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