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Flow Synthesis and Biological Studies of an Analgesic Adamantane Derivative That Inhibits $P2X_7$-Evoked Glutamate Release

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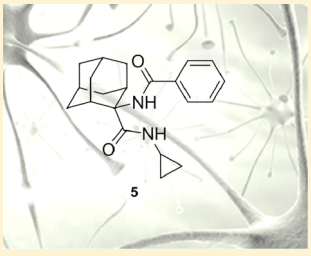
Supporting Information

ABSTRACT: We report the biological evaluation of a class of adamantane derivatives, which were achieved via modified telescoped machine-assisted flow procedure. Among the series of compounds tested in this work, **5** demonstrated outstanding analgesic properties. This compound showed that its action was not mediated through direct interaction with opioid and/or cannabinoid receptors. Moreover, it did not display any significant anti-inflammatory properties. Experiments carried out on rat cerebrocortical purified synaptosomes indicated that **5** inhibits the $P2X_7$-evoked glutamate release, which may contribute to its antinociceptive properties. Nevertheless, further experiments are ongoing to characterize the pharmacological properties and mechanism of action of this molecule.



KEYWORDS: *Adamantane derivatives, 2-aminoadamantane-2-carboxylic acid, analgesic agents, flow chemistry, purinergic receptors*

The ligand-gated ion channel superfamily of receptors is intimately involved in the regulation of important physiological functions. The alteration of these receptors has been associated with a large range of diseases, including cardiac disorders and perception of pain. The pivotal role associated with these receptors is clearly linked to the interest of researchers who are targeting these proteins for the development of new medicines.

The purinergic P2X receptors are trimeric cell surface ion channels gated by extracellular ATP. $P2X_3$, $P2X_{2/3}$, $P2X_4$, and $P2X_7$ receptors have been associated with chronic pain and arthritis, making these receptors potential targets for drug discovery. Particularly interesting is the $P2X_7$ class of receptors. This ligand-gated ion channel is stimulated by extracellular ATP and is present on the surface of hematopoietic lineage, astrocytes, microglia, oligodendrocytes, and Schwann cells, as well as on neurons (primarily glutamatergic neurons) in the central and peripheral nervous system.[1−6] $P2X_7$ can switch from a rapid-gating channel (selective for small cations) conformation to dilated pore conformations (permeable to molecules up to 900 Da).[7] The multiple permeation pathways of $P2X_7$[8] appear to be linked to different modes of glutamate efflux. Presynaptic influx of Ca^{2+} via $P2X_7$ receptors (rapid-gating channel) triggers the vesicular exocytotic release of glutamate.[4,9−11] Additionally, activation of $P2X_7$ can be equally responsible for the non-exocytotic release of glutamate through the receptor-channel, in which the pore apoptotic state and pannexin-1 recruitment are not required for the receptor function and the glutamate efflux.[1,4,12] This receptor seems to modulate the death and survival of neurons and may play different roles in the pathogenesis of disorders involving the dysregulation of the glutamatergic transmission.[2,3,13] Purinergic signaling through $P2X_7$ receptor seems to play primary roles in neuroinflammation that underlie neurodegenerative diseases including multiple sclerosis, Alzheimer's and Huntington's diseases, or amyotrophic lateral sclerosis,[14−19] and $P2X_7$ inhibition has neuroprotective effects in animal models of such diseases.[19−21] A role for the $P2X_7$ receptor in chronic pain is suggested by findings in animal models and by the effectiveness of $P2X_7$ antagonists.[22−28] Recent evidence indicates that the receptor's dilated pore conformation, linked to downstream effects including the release of interleukin-1β and ATP[29−31] from microglia or macrophages, may be involved in allodynia and pain hypersensitivity.[28] These findings are

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expected to strengthen interest in the $P2X_7$ receptor and in the different receptor conformations and permeation properties, as a potential target in chronic pain.

Several groups have reported $P2X_7$ receptor antagonists with a benzamide functionality; among them, AstraZeneca described a class of adamantane containing benzamide molecules (**1**), which showed potent antagonism toward $P2X_7$ receptors.[32] Abbott also reported an adamantane containing $P2X_7$ antagonist (**2**) that exhibited efficacy against neuropathic pain and inflammation in rat models.[33] Noteworthy is that the adamantyl motif is currently found in clinical use for the treatment of neurogenerative diseases, as well as influenza, acne vulgaris and type II diabetes. Further research is in progress for the development of potential agents against iron overload disease, cancer, malaria, and tuberculosis.[34]

We recently described the machine-assisted synthesis of an unnatural amino acid (**3**) along with the generation of some adamantane-containing molecules (**4**–**8**) (Figure 1).[35] The biological properties displayed by similar molecules prompted us to investigate their antinociceptive properties.

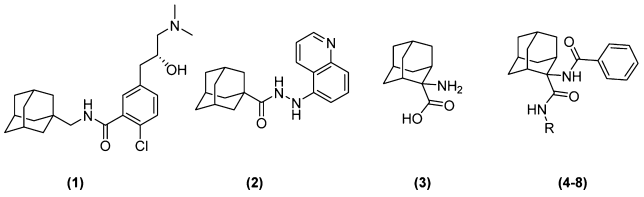


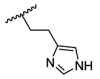
Figure 1. Structures of $P2X_7$ antagonists developed by AstraZeneca (**1**) and Abbott (**2**); structures of 2-aminoadamantane-1-carboxylic acid (**3**) and adamantane containing molecules tested in this work (**4**–**8**).

The synthesis of a small collection of adamantane benzamide compounds is briefly described in Scheme 1. The oxazoline (**9**) can easily be obtained in 2 steps from commercially available 2-adamantanone.[35] Again using flow chemistry, compound **9** was subjected to ozonolysis followed by reaction with different nitrogen nucleophiles to afford **4**–**8** in good yields and purity without the need for prior isolation of azalactone (**10**).

The analgesic profiles of the analogues are depicted in Table 1; BBG, a known antagonist of $P2X_7$ receptors in the nanomolar range,[36] is also included for comparison purposes. It has previously been reported that BBG exhibits therapeutic effects in animal models for neurodegenerative diseases[19,37,38] and reduces motor neuron damage in spinal cord injury or in amyotrophic lateral sclerosis, by reducing microglia and/or astrocyte activation and neutrophil infiltration.[39−41] It also blocks the $P2X_7$ dilated pore formation, which is a key feature in chronic pain.[28] More interestingly, biological studies confirmed that the biological activity of BBG is mainly due to its inhibitory activity on $P2X_7$ receptors.[19,41] However, it has been shown that, at concentrations that may be reached after repetitive dosage,[19,39,41] BBG can also affect the functioning of voltage-dependent Na^+ channels,[42] and therefore, this may play a significant role in determining its biology.

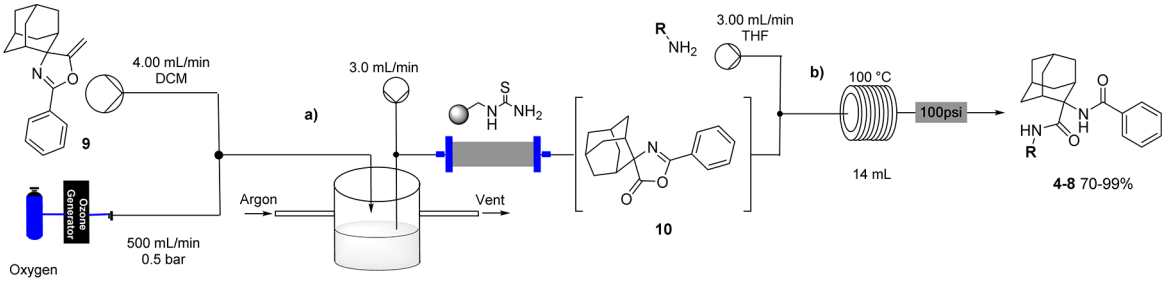
Compounds **4** and **8** displayed no analgesic properties, whereas **6** and **7** exhibited a moderate analgesic effect at higher dosage (60 mg kg^{-1}). Pleasingly, the antinociceptive effect of **5** was noticeable even at a low dose of 3 mg kg^{-1}, and it also

Table 1. Mouse Abdominal Constriction Test (Acetic Acid 0.6%)[a]

	R	n. mice	Dose mg kg^{-1}	n. writhes 30 min	n. writhes 60 min
	control	6	-	32.7 ± 1.9	31.4 ± 2.5
4		8	20	25.8 ± 3.0	-
		8	40	26.7 ± 2.5	-
		8	60	32.7 ± 1.9	-
5		9	3	22.5 ± 3.1[b]	-
		8	10	17.2 ± 2.5[c]	23.9 ± 2.8[c]
		8	20	18.0 ± 2.3[c]	21.3 ± 2.2[c]
		8	30	18.6 ± 2.2[c]	-
6		8	10	33.4 ± 2.5	-
		8	20	21.9 ± 2.8[c]	-
		8	60	18.9 ± 3.4[c]	-
7		6	20	28.8 ± 2.3	-
		8	40	16.3 ± 2.4[c]	-
		8	60	17.7 ± 3.9[c]	-
8		7	20	30.3 ± 3.3	-
		8	40	27.2 ± 2.5	-
		8	60	25.8 ± 3.1	-
	BBG	6	10	26.6 ± 2.8	25.5 ± 2.6
		8	40	19.3 ± 3.3[b]	22.3 ± 3.1[c]
		8	60	20.6 ± 2.2[c]	21.6 ± 3.5[c]

[a]All drugs were administrated per os 30 min before test. [b]$P < 0.05$ versus vehicle-treated mice (control). [c]$P < 0.01$ versus vehicle-treated mice (control).

Scheme 1. Telescoped Process for the Synthesis of 4–8: (a) Ozonolysis Step; (b) Amide Synthesis Step (Oxazolinone Aminolysis)



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displayed better analgesic profiles than BBG at the same dosage. Further tests were then carried out to pinpoint the molecular target(s) of **5**.

We began by investigating whether the outstanding analgesic profile of **5** could be induced via opioid and/or cannabinoid receptors. As seen in Table 2, pretreatment with an opioid receptor antagonist (Naxolone)[43] or a cannabinoid 1 (CB-1) receptor antagonist (SR-141716A)[44] did not revert the antinoception of **5**, showing that its action was not mediated through direct interaction with opioid or CB-1 receptors. We then turned our attention to the effect of **5** on carrageenan induced hyperalgesia and paw edema on rat models (Table 3). Here again, as previously seen in Table 1, **5** and BBG displayed similar activity although **5** was more effective at lower dosage. Similar pharmacological profiles were also obtained, and both compounds were cleared out after 90 min. A slight decrease of the edema volume upon treatment after 30 min showed a low anti-inflammatory activity; however, neither COX-1 nor COX-2 were inhibited by **5** (data not shown), highlighting the fact that the analgesic activity is not associated with any anti-inflammatory properties.

Table 2. Lack of Effect by Opioid (Naxolone) and Cannabinoid CB-1 (SR-141716A) Receptor Antagonists on Analgesia Induced by 5 in the Mouse Abdominal Constriction Test (Acetic Acid 0.6%)[a]

treatment	no. mice	dose mg kg^{-1}	no. writhes 30 min	no. writhes 60 min
control	12		30.4 ± 2.9	33.1 ± 2.2
5	12	10 p.o.	16.5 ± 2.3[b]	19.3 ± 3.1[b]
Naxolone	8	1 i.p.	28.3 ± 3.2	31.9 ± 2.5
SR-141716A	8	1.5 i.p.	33.7 ± 3.6	32.6 ± 2.4
Naxolone + **5**	8	1.0 + 10	15.1 ± 1.9[b]	20.7 ± 2.7[b]
SR-141716A + **5**	8	1.5 + 10	17.8 ± 2.5[b]	18.8 ± 2.0[b]

[a]Naxolone and SR-141716A were administered 5 min before **5**. [b]$P < 0.01$ versus vehicle-treated mice (control).

Finally, experiments were carried out on rat cerebrocortical purified synaptosomes (Chart 1) in order to provide direct evidence for interference with functioning native receptors. In fact, isolated purified nerve terminals prepared from rodent brain regions are helpful models allowing pharmacological characterization of the receptors located on the plasma membrane of the nerve terminals and revealing direct effects of molecules at the receptors themselves. Indeed, release monitoring from a superfused synaptosomal monolayer,[45] by removing any released compound and minimizing metabolism, avoids receptor biophase and prevents indirect effects, enabling nude receptors to be exposed. Under these experimental conditions, only targets located on glutamatergic nerve terminals (when monitoring glutamate release) are selectively acted upon, allowing the pharmacological characterization of release-regulating presynaptic receptors and allowing to assess if a substance, added to the superfusion medium, could interfere with the receptor activation.

In this set of experiments, the rat cerebrocortical synaptosomes were stimulated by BzATP, a preferential agonist of $P2X_7$ receptors,[4,7] inducing an increase of aspartate efflux (Chart 1a). By using this model of functional native rat $P2X_7$ receptor, we found that, unlike BzATP, **5** did not activate the receptor (Chart 1a), but behaved as an inhibitor of responses mediated by $P2X_7$ receptor activation (in our case, activation by BzATP) in the micromolar range (IC_{50} value = 7.7 μM; Chart 1b). It has been previously reported that the activity of BzATP stimulates both Ca^{2+}-dependent and Ca^{2+}-independent mechanisms of glutamate release.[4,12,46] In our case, the BzATP-evoked aspartate release was significantly reduced by compound **5** (Chart 1c). Inhibitory effects were also detected when BBG ($P2X_7$ receptor antagonist)[4,36,47] or A-438079[24,25] (selective and competitive $P2X_7$ receptor antagonist) were administered, although their effects were more pronounced than compound **5**. We then turned our attention to whether this inhibitory effect was mediated by extracellular levels of Ca^{2+}. While BBG and A-438079 could still exert some inhibition under extra cellular Ca^{2+}-free conditions, compound **5** was ineffective (Chart 1c). Moreover, its ineffectiveness on the glutamate release in the presence of Rose Bengal[48] (Chart 1c) confirmed that **5** only inhibited the glutamate release dependent on the $P2X_7$ function as a rapid gated Ca^{2+} channel, while it did not affect the function of the receptor as a permeation pathway for glutamate. Finally, the ineffectiveness of **5**, as well as of BBG, on the $P2X_7$-independent stimulus (K^+ depolarization, Chart 1d) represent a valid evidence that the inhibition exerted by **5** (or BBG) on the BzATP-evoked response may be related to a triggering of $P2X_7$ receptor, rather than to nonspecific effects.[4]

Overall, these findings indicated that **5** behaves as an inhibitor of Ca^{2+}-dependent exocytotic [^{3}H]D-aspartate efflux and interferes with the rapid gating channel coupled to vesicular release. The ability of **5** to distinguish among the $P2X_7$ multiple permeation pathways would merit further investigation.

On-going research toward the development of novel and more efficient approaches for pain relief and treatment in

Table 3. Effect of 5 and BBG on Hyperalgesia and Paw Edema Induced by Carrageenan in the Rat Paw-Pressure Test[a]

			paw pressure (g)					
				after treatment				edema volume (mL)
pretreatment i.pl.	treatment p.o.	dose mg kg^{-1}	before treatment	15 min	30 min	60 min	90 min	30 min
saline	control		61.2 ± 2.5	65.6 ± 3.4	58.9 ± 3.8	61.4 ± 4.1	63.6 ± 4.0	1.44 ± 0.05
carrageenan	control		36.1 ± 3.3	32.8 ± 2.9	30.6 ± 3.2	31.8 ± 2.2	34.8 ± 3.1	2.71 ± 0.08
carrageenan	5	3	34.2 ± 2.4	39.1 ± 3.5	40.8 ± 3.7	35.6 ± 2.8	36.9 ± 3.3	2.53 ± 0.05
carrageenan	5	10	32.5 ± 3.2	44.2 ± 3.2[b]	52.7 ± 3.8[c]	43.4 ± 3.9[b]	38.2 ± 2.5	2.06 ± 0.08[c]
carrageenan	5	20	35.7 ± 3.1	48.4 ± 3.4[c]	53.9 ± 4.6[c]	48.3 ± 4.2[c]	33.6 ± 3.0	2.10 ± 0.09[c]
carrageenan	BBG	10	35.5 ± 2.1	38.7 ± 2.5	43.1 ± 2.2[b]	38.5 ± 2.9	37.1 ± 1.9	2.63 ± 0.07
carrageenan	BBG	40	30.9 ± 1.3	46.3 ± 1.8[c]	50.8 ± 2.5[c]	45.6 ± 1.8[c]	40.2 ± 2.1	2.17 ± 0.08[c]
carrageenan	BBG	60	33.1 ± 1.8	47.2 ± 2.3[c]	49.6 ± 2.8[c]	43.8 ± 2.2[c]	36.2 ± 2.4	2.21 ± 0.08[c]

[a]Carrageenan 100 μL, 1% i.pl. 2 h before test. [b]$P < 0.05$. There were 5 rats per group. [c]$P < 0.01$. There were 5 rats per group.

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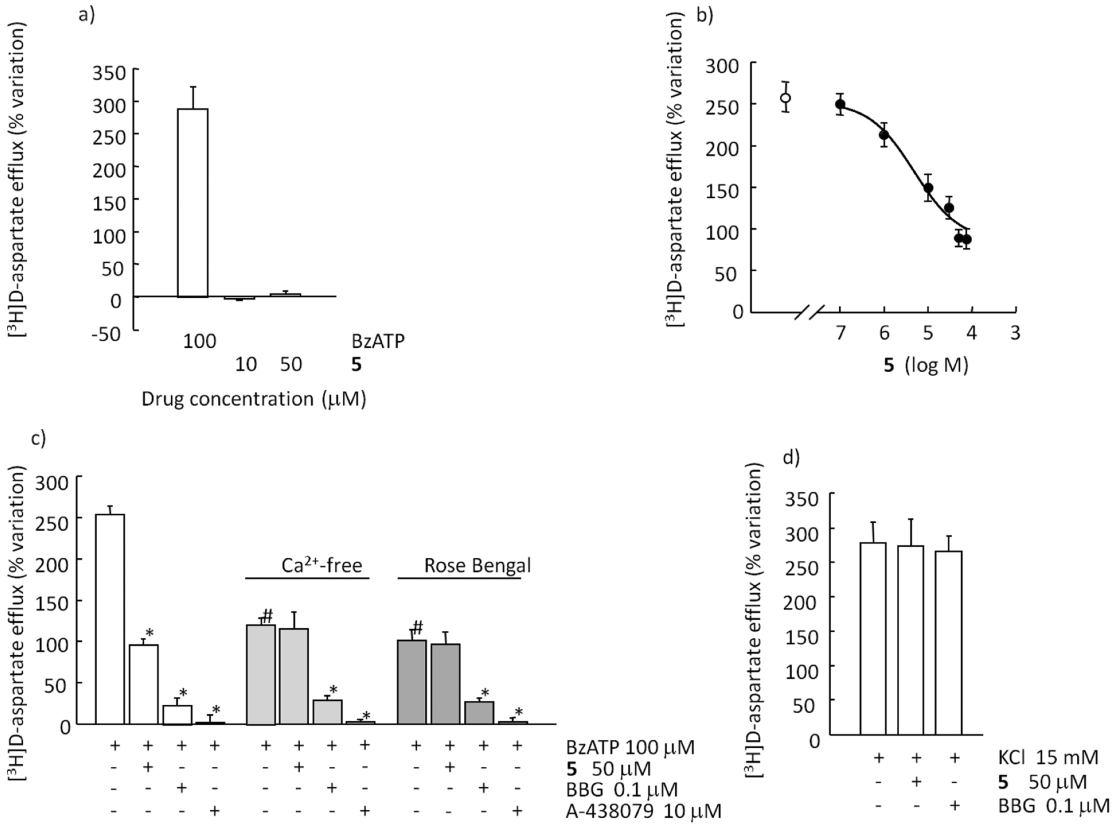
Chart 1. Action of 5 at Presynaptic $P2X_7$ Receptors[a]



[a](a) Ineffectiveness of **5** as a $P2X_7$ receptor agonist. The preferential $P2X_7$ agonist BzATP evoked [^{3}H]D-aspartate efflux from superfused cerebrocortical synaptosomes, while **5** was ineffective. (b) Inhibition of the response to BzATP by **5**: log concentration−response relationship for **5** on the BzATP-evoked [^{3}H]D-aspartate efflux. Effect of 100 μM BzATP alone (○) or in the presence of **5** (●). (c) Effect of **5**, BBG, or the selective $P2X_7$ antagonist A-438079 on the BzATP-evoked [^{3}H]D-aspartate efflux, in the presence or absence of extracellular Ca^{2+} or after preincubation with the inhibitor of vesicular glutamate transporter Rose Bengal. (d) Ineffectiveness of **5** or BBG on the response to K^+ depolarization. BzATP (or KCl) was added for 120 s during superfusion; compound **5**, BBG, or A-438079 was added 8 min before BzATP (or K^+ depolarization). Ca^{2+}-free EGTA (0.5 mM)-containing medium was added 18 min before BzATP; Rose Bengal (0.5 μM) was preincubated (30 min at 37 °C) before and during vesicle loading with [^{3}H]D-aspartate. Data are mean ± SEM of 3−12 independent experiments in triplicate. *P < 0.05 vs BzATP alone; #P < 0.05 vs BzATP in standard medium.

chronic pain is of considerable importance. Several classes of drugs are already effective at controlling particular types of pain; however, side-effects such as addictive issues or long-term toxicity profiles hamper their use, especially during chronic treatments.

Among the series of compounds tested in this work, **5** demonstrated outstanding in vivo analgesic properties in the mouse abdominal constriction test. Further investigations showed that its action was not mediated through direct interaction with opioid and/or cannabinoid receptors. Compound **5** also did not display any significant anti-inflammatory properties. Experiments carried out on rat cerebrocortical purified synaptosomes indicated that **5** behaved as an inhibitor of $P2X_7$-mediated responses. These preliminary findings also point out that **5** would interfere only with Ca^{2+}-dependent exocytotic glutamate release. $P2X_7$ receptors have been proven to be a potential valid molecular targets for neuropathic pain and neurodegenerative diseases;[22−28,37] however, the in vitro data suggest that an additional pathway may be involved. The mechanistic features of **5** will be explored in more details in our laboratories.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic procedures and full characterization of compounds **4**−**8**, material and methods for the mouse abdominal constriction test, hyperalgesia and paw edema induced by carrageenan in the rat paw-pressure test, and rat cerebrocortical purified synaptosomes experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

A-438079, 3-(5-(2,3-dichlorophenyl)-1*H*-tetrazol-1-yl)-methyl pyridine; ATP, adenosine triphosphate; BBG, brilliant blue G;

BzATP, benzoyl benzoyl adenosine triphosphate; CB-1, cannabinoid 1 receptor

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