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Design, synthesis, and biological evaluation of LNA nucleosides as adenosine A_3 receptor ligands

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Abstract—We have prepared a series of adenosine analogs based on the bicyclo[2.2.1]heptane scaffold of locked nucleic acid (LNA) and tested them for both agonist and antagonist activity at the adenosine A_3 receptor. The design of these derivatives was based on the known A_3 agonist IB-MECA and related compounds. Modifications thus include the 5′-uronamides and N^6-(3-iodobenzyl) derivatives. In this way we have prepared analogs of known A_3 agonists with the sugar ring restricted in an N-conformation. For comparison we have also prepared 2′-*O*-methyl derivatives of IB-MECA. The LNA nucleosides showed no agonist activity but some of them are potent antagonists. The 2′-*O*-methyl derivative of IB-MECA is an agonist with similar potency as the parent compound.

1. Introduction

The adenosine receptors (AR) are members of the family of G-protein coupled receptors. Four distinct subtypes of ARs are known, the A_1, A_{2A}, A_{2B}, and A_3 subtypes, respectively. Adenosine receptors are widely distributed throughout the body and therefore play a role in a wide range of physiological functions and responses.[1]

In recent years the A_3AR has been the focus of increased interest because of its involvement in several clinical indications. Ligands at the A_3AR, in particular agonists, are therefore of significant therapeutic value.[2,3] For example, in cancer the reference A_3AR agonist IB-MECA, **1**[4] (Fig. 1), has been shown to inhibit tumor growth in different in vivo models[5,6] and is currently in phase II clinical trial for colorectal cancer.[7] Furthermore, A_3AR agonists are believed to have beneficial effects on different kinds of ischemia, lung injury, and arthritis. A_3AR antagonists on the other hand have been suggested as anti-glaucoma, anti-inflammatory agents and for the treatment of renal failure and asthma.[2,3]

Traditionally, agonists for the human A_3AR have been adenosine derivatives.[8] Adenosine itself is an intermediately potent agonist with an EC_{50} value of 290 nM.[9] Through manipulation at different sites of adenosine it is possible to obtain highly potent and selective ligands

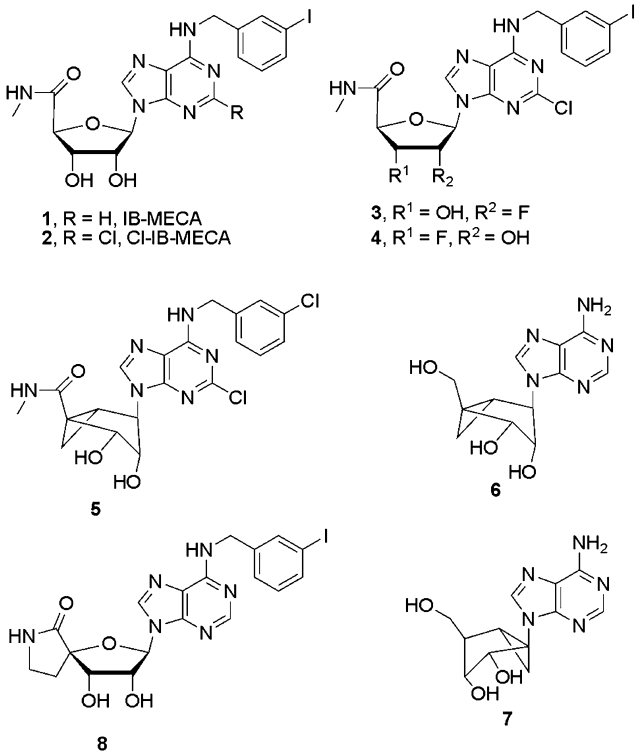

Figure 1.

Keywords: LNA; Amino-LNA; Adenosine A_3 receptor; IB-MECA.
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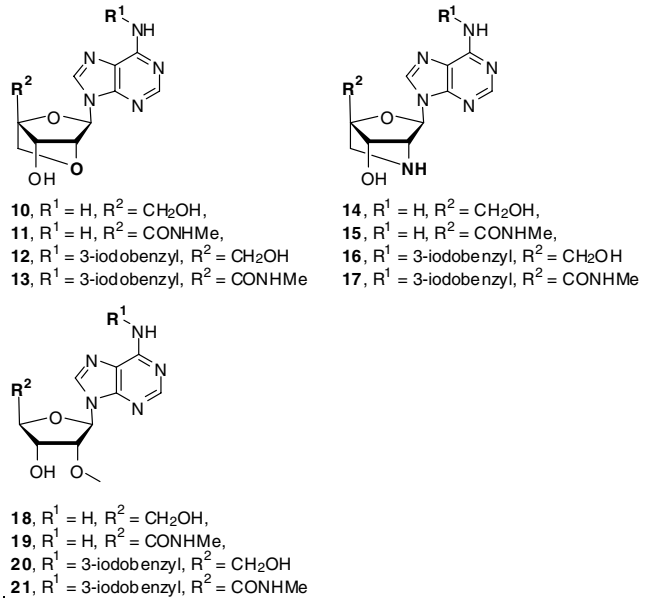
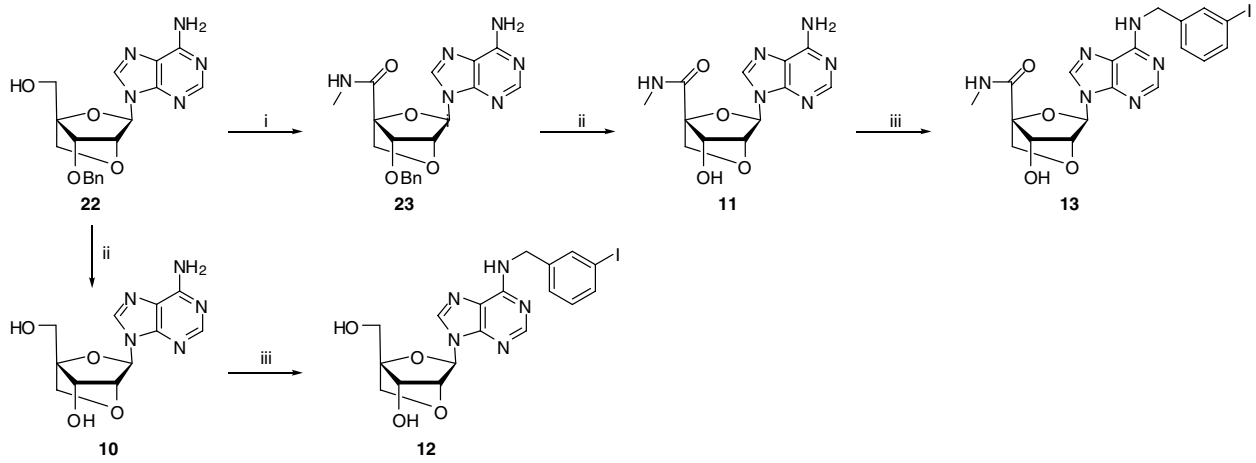


Figure 2.

at the A_3AR, although the boundary between full agonists, partial agonists, and antagonists is highly dependent on the exact combination of modifications.[8] Generally, N^6-alkylated 5′-uronamide derivatives are amongst the most potent ligands reported. Further substitution at the 2-position is tolerated, for example, Cl-IB-MECA **2**,[10] and can lead to increased selectivity but can also convert an agonist into an antagonist. It is generally believed that one or both of the 2′- and 3′-hydroxy groups are required for A_3AR activation though no conclusive study has been made. Both the 2′-deoxy-2′-fluoro and the 3′-deoxy-3′-fluoro derivatives **3**[11,12] and **4**[12,13] of Cl-IB-MECA showed a dramatic decrease in binding affinity toward the A_3AR suggesting a role for the hydroxy groups as hydrogen bond donors, not hydrogen bond acceptors.

The influence of the ribose ring conformation on binding affinity and activation of the A_3AR has also been explored using ligands with conformationally restricted ribose rings. In this way the high-affinity agonist **5** was discovered by replacement of the ribose ring with a bicyclo[3.1.0]hexane scaffold in which the carbocyclic pseudosugar ring is restricted in a North-conformation indicating a conformational preference of the receptor.[14] A direct comparison of an N- and S-conformation could be made after the preparation of **6** and **7** with binding constants (K_i) of 404 and 63,000 nM, respectively.[15] From this study it is apparent that the N-conformation of the sugar ring is preferred at the A_3AR. Interestingly, the spirolactam **8** was shown to be an antagonist with reasonable affinity.[16] This complete loss of agonist activity could be attributed to the conformational restriction of either the sugar ring or the amide group. The hydrogen bonding ability of the latter has been shown to be important for activation of the A_3AR.[17]

In the present work, we wanted to further study the effect of conformationally restricted adenosine analogs by the introduction of locked nucleic acid (LNA[18]) adenosine analogs (Fig. 2). LNA is based on a 2,5-dioxabicyclo[2.2.1]heptane scaffold constraining the sugar ring in an almost complete North-conformation.[19] In addition, we have prepared a series of 2′-amino-LNA adenosine analogs, containing the 2-oxa-5-azabicyclo[2.2.1]heptane scaffold, thereby combining the conformational characteristics of the LNA scaffold and a hydrogen bond donor at the 2′-heteroatom. Although LNA is a bicyclic structure, we will use the conventional nucleoside numbering in order to compare with other nucleosides. Because the 2′-oxygen in LNA is alkylated, we have also prepared a few of the monocyclic 2′-*O*-methyl adenosine derivatives. These compounds will contribute to an understanding of the role of the 2′-OH group as it, with a minimal of structural change, removes the 2′-OH hydrogen which is believed to be crucial in binding to the receptor. For all scaffolds we have prepared the four possible combinations of *N*-methyl-5′-uronamide and N^6-(3-iodobenzyl) modifications (Fig. 2) for a direct comparison with the reference compound IB-MECA (**1**).



Scheme 1. Reagents and conditions: (i) (a) $PhI(OAc)_2$, TEMPO, CH_3CN/H_2O, 40 °C; (b) $SOCl_2$, EtOH, rt; (c) $MeNH_2$, MeOH, rt; (ii) HCO_2NH_4, $Pd(OH)_2/C$, MeOH, reflux; (iii) (a) 3-iodobenzyl bromide, DMF, 70 °C; (b) NH_4OH, MeOH, 50 °C.

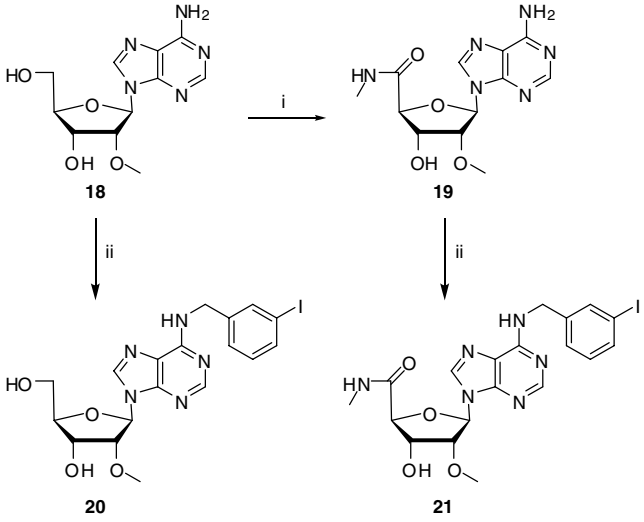
2. Results and discussion

2.1. Chemistry

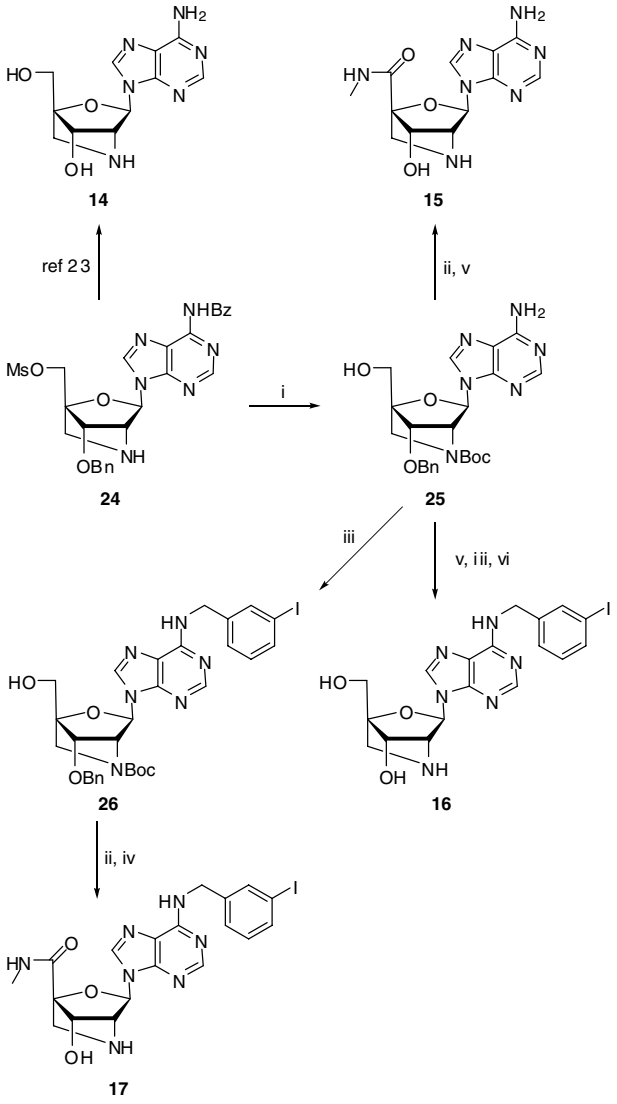
For the preparation of LNA adenosine analogs the 3′-*O*-benzyl derivative **22**[20] was used as starting material (Scheme 1). Treating this compound with diacetoxyiodobenzene and a catalytic amount of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) gave the corresponding 5′-uronic acid which was converted to the *N*-methyl-5′-uronamide**23** using $SOCl_2$/EtOH followed by treatment with excess methylamine. Debenzylation via palladium catalyzed transfer hydrogenation then gave the fully deprotected *N*-methyl-5′-uronamide **11**. Alkylation of N-1 of this compound with 3-iodobenzyl bromide followed by ring-opening/ring-closure under alkaline conditions (Dimroth rearrangement[21] where N-1 becomes the exocyclic N^6) gave the N^6-(3-iodobenzyl)-*N*-methyl-5′-uronamide **13**. Similar N^6-alkylation of LNA adenosine **10** (obtained from **22**[20]) finally gave the N^6-(3-iodobenzyl)-5′-hydroxy nucleoside **12**. We have recently reported the synthesis of 2′-amino-LNA purine nucleosides, including the adenosine **14**.[22] The starting compound **24**[22] was used in this study to prepare a series of 2′-amino-LNA adenosine analogs (Scheme 2). Boc-protection of the 2′-amino group was followed by removal of the 5′-*O*-mesylate via the benzoyl ester to give the key intermediate **25**. Conversion of this intermediate to the 5′-uronamide **15** was accomplished using the same method as for **11**. Because the Boc group is lost during treatment with $SOCl_2$, N^6-alkylation with 3-iodobenzyl bromide was applied to nucleoside **25**to give **26** followed by oxidation to the 5′-uronamide. Again, methanesulfonic acid was used for the final debenzylation giving **17**. For the synthesis of the 5′-hydroxy analog **16** on the other hand, the sequence was simply reversed, starting with catalytic transfer hydrogenation followed by alkylation. Finally, the same synthetic strategy used in the preparation of **11**–**13** was used to prepare the corresponding 2′-*O*-methyl adenosine analogs **19**–**21** (Scheme 3) from commercially available **18**.

2.2. Adenosine A_3 receptor activity

The prepared compounds were all tested for activity at the human adenosine A_3 receptor. AequoScreen™ (Euroscreen, Belgium) cell lines expressing the A_3 human recombinant receptor were used as a functional assay to estimate both agonist and antagonist activity in terms of EC_{50} and IC_{50} values, respectively. The results for all tested compounds are shown in Table 1. In this assay, the reference agonist IB-MECA (**1**) was measured to have an EC_{50} of 2.0 nM, which is in good accordance with literature data.[23]



Scheme 2. Reagents and conditions: (i) (a) $PhI(OAc)_2$, TEMPO, CH_3CN/H_2O, 40 °C; (b) $SOCl_2$, EtOH, rt; (c) $MeNH_2$, MeOH, rt; (ii) (a) 3-iodobenzyl bromide, DMF, 70 °C; (b) NH_4OH, MeOH, 50 °C.



Scheme 3. Reagents and conditions: (i) (a) Boc_2O, Et_3N, DCM, rt; (b) NaOBz, DMSO, 100 °C; (c) NH_3, MeOH, rt; (ii) (a) $PhI(OAc)_2$, TEMPO, CH_3CN/H_2O, 40 °C; (b) $SOCl_2$, EtOH, rt; (c) $MeNH_2$, MeOH, rt; (iii) (a) 3-iodobenzyl bromide, DMF, 70 °C; (b) NH_4OH, MeOH, 50 °C; (iv) MsOH, DCM, rt; (v) HCO_2NH_4, $Pd(OH)_2/C$, MeOH, reflux; (vi) TFA, rt.

The purpose of this study was to investigate the effect of introducing a 2′-*O*,4′-*C*-methylene linkage into a known A_3 receptor agonist. As can be seen from the data for the LNA nucleosides **10**–**13** this modification completely eliminates the agonist activity but leaving a moderate antagonist activity, for example, 150 nM for **12**. Surprisingly, the LNA analog of IB-MECA, **13**, has neither agonist, nor antagonist activity. The introduction of the 2′-*O*,4′-*C*-methylene linkage has two distinct effects: (i) locking the sugar furanose ring in an N-type conformation and (ii) alkylating the 2′-oxygen, thus removing a potential hydrogen bond donor. In order to further investigate this relationship, we prepared a small series of 2′-amino-LNA adenosines **14**–**17**. These compounds effectively preserve the conformational restriction but reintroduce a hydrogen bond donor at the 2′-position. This exchange of the 2′-oxygen with an NH does not restore any agonist activity. The introduction of the 2′-amino group does, however, increase the antagonist activity, especially for the 3-iodobenzyl analogs, resulting in very potent antagonists **16** and **17**. These results indicate that the lack of agonist activity in the LNA nucleoside compounds may not be due to removal of the 2′-hydroxy hydrogen, but is rather caused by restriction of the furanose ring into an unfavorable conformation. In order to further elaborate on this, we prepared a series of 2′-*O*-methyl adenosine derivatives **18**–**21**, thus removing the 2′-OH hydrogen but leaving the conformational flexibility of the furanose ring essentially intact. From the receptor data for these compounds it is clear that they all bind efficiently to the receptor. It also seems that the *N*-methyl-5′-uronamide is required for agonist activity. Notably, in the direct comparison of **21** and **1**, it is apparent that the 2′-*O*-methyl group does not affect the agonist activity. This clearly indicates that the binding of **1** to the receptor does not involve the 2′-OH group as a hydrogen bond donor. This is in obvious contrast to the work of Jeong and co-workers.[11] They concluded that the diminished activity of the corresponding 2′-deoxy-2′-fluoro compound **3** was caused by the removal of the 2′-OH group as a hydrogen bond donor. As an alternative, we would like to propose that the fluorine atom in **3** shifts the conformation of the furanose ring, causing a drop in activity. These results clearly suggest that the lack of agonist activity of the LNA and 2′-amino-LNA adenosine derivatives is caused by the restriction of conformation. This is surprising considering that the carbocyclic nucleoside **5**, in which the pseudosugar ring is restricted into a conformation close to that of the LNA nucleosides, is an extremely potent agonist. The furanose ring of LNA nucleosides has been shown to be locked in an 3′-*endo* conformation[19] (Fig. 3) which is also the preferred conformation of 2′-deoxy-2′-fluoro nucleosides.[24] The pseudofuranose ring of **5** is locked in an 2′-*exo* conformation.[14] The antagonist data for especially the 2′-amino-LNAs **16** and **17** show that the compounds still bind to the receptor, although with lower affinity. Apparently, the 3′-*endo* conformation (LNA and 2′-deoxy-2′-fluoro) does not activate the receptor, while the 2′-*exo* (**6**) conformation does. It must be concluded that the activation of the receptor is highly dependent on the furanose conformation and that only small changes in this conformation have a detrimental effect on the activity. The increased antagonist activity of 2′-amino-LNA adenosines over LNA adenosines is also interesting. Considering the lack of involvement of the 2′-OH hydrogen in the binding of IB-MECA, it is unlikely that the reintroduction of a hydrogen (–O– to –NH–) is responsible for this higher potency. Instead, a potential positive charge on the 2′-nitrogen might influence the binding mode.

In order to investigate the issue of selectivity we also measured the agonist and antagonist effect of the five most potent compounds **16**–**18**, **20**, and **21** against the closely related adenosine A_1 receptor (Table 1). None of the tested compounds showed any activity up to a concentration of 3 μmol and consequently must be

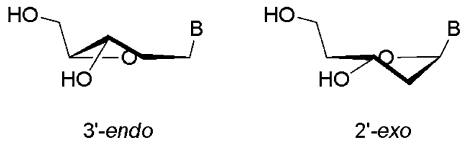


Figure 3.

Table 1. In vitro functional agonist (EC_{50}, E_{MAX}) and antagonist (IC_{50}, I_{MAX}) data for adenosine analogs at the human adenosine A_3 and A_1 receptors

Compound	R^1	R^2	A_3EC_{50} (nM)	E_{MAX} (%)	A_3 IC_{50}[a] (nM)	I_{MAX} (%)	A_1 EC_{50} (nM)	A_1 IC_{50} (nM)
1	3-Iodobenzyl	CONHMe	2.0	100[b]	ND		ND	ND
10	H	CH_2OH	$>10^5$		$>10^5$		ND	ND
11	H	CONHMe	$>10^5$		$>10^5$		ND	ND
12	3-Iodobenzyl	CH_2OH	$>10^5$		150	100	ND	ND
13	3-Iodobenzyl	CONHMe	$>10^5$		$>10^5$		ND	ND
14	H	CH_2OH	$>10^5$		$>10^5$		ND	ND
15	H	CONHMe	$>10^5$		170	100	ND	ND
16	3-Iodobenzyl	CH_2OH	$>10^5$		8.2	100	>3000	>3000
17	3-Iodobenzyl	CONHMe	$>10^5$		12	100	>3000	>3000
18	H	CH_2OH	$>10^5$		15	100	>3000	>3000
19	H	CONHMe	453	100	ND		ND	ND
20	3-Iodobenzyl	CH_2OH	$>10^5$		1.7	100	>3000	>3000
21	3-Iodobenzyl	CONHMe	1.9	100	ND		>3000	>3000

ND, not determined.

[a] IC_{50} was not determined for compounds showing agonist activity.

[b] Reference level.

considered as highly selective A_3 ligands, at least over the A_1 receptor.

In conclusion, we have prepared a series of LNA and 2′-amino-LNA analogs of IB-MECA. Human adenosine A_3 receptor activation data showed that these derivatives are not agonists, but fairly potent antagonists. Comparisons with the corresponding 2′-*O*-methyl adenosine analogs indicated that this lack of agonist activity is caused by conformational restriction of the furanose ring, not by alkylation of the 2′-oxygen. Highly potent antagonists were obtained when the 2′-oxygen in the LNA adenosines was replaced with an NH-group.

3. Experimental

3.1. General procedures

Anhydrous solvents were dried over 4 Å molecular sieves (4–8 mesh). Reagents were used as supplied. 2′-*O*-methyl adenosine was purchased from ChemGenes (Wilmington, MA), 2,6-dichloropurine was purchased from ChangChem Ltd (Nantong, China), and all other reagents were purchased through Sigma–Aldrich Denmark. Dry column vacuum chromatography (DCVC) was performed according to the published procedure.[25] Reverse phase preparative HPLC (RP-HPLC) was performed on a Biotage C18HS 12+S column. A gradient of 20–100% acetonitrile in water was used as eluent. ^{1}H NMR spectra were recorded on a Bruker AC-300 (300 MHz) spectrometer. Electrospray ionization mass spectra were recorded on an Agilent 1100 series LC–MS. High resolution mass spectra (Fast Atom Bombardment, FAB or Chemical Ionization, CI) were recorded by Kent Mass Spectrometry (Kent, UK). Human Adenosine A_3 receptor assay was run at Eurosreen, Belgium.

3.1.1. (1*R*,3*R*,4*R*,7*S*)-(7-Benzyloxy-3-(adenin-9-yl)-2,5-dioxabicyclo[2:2:1]hept-1-yl)-*N*-methylcarboxamide (23). To a solution of **22**[20] (5.0 g, 13.5 mmol) in acetonitrile (25 mL) and H_2O (25 mL) were added (diacetoxyiodo)-benzene (9.6 g, 29.7 mmol) and TEMPO (0.64 g, 4.1 mmol), and the mixture was stirred at 40 °C for 2 h. The solvents were removed under reduced pressure and the residue was triturated with diethyl ether. The white precipitate was filtered off, washed with ether, and dried under vacuum (4.9 g, 94%). The intermediate (4.4 g, 11.5 mmol) was dissolved in 99.9% ethanol (60 mL) and cooled to 0 °C. $SOCl_2$ was added dropwise over 10 min and stirring was continued at room temperature for 3 h. The mixture was concentrated in vacuo and the residual solvent was coevaporated with methanol. The residual oil was redissolved in a 2 M solution of methylamine in methanol (40 mL, 80 mmol) and the mixture was stirred at room temperature for 16 h. The solvent and reagent were removed under reduced pressure and the residue was coevaporated twice with methanol to give the target product **23** as a brown solid foam (5.1 g, 99%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.35 (1H, q, J = 4.7 Hz, N*H*Me), 8.31 (1H, s, H-2), 8.15 (1H, s, H-8), 7.37 (2H, s, NH_2), 7.32–7.29 (5H, m, Ph), 6.07 (1H, s, H-1′), 4.89 (1H, s, H-2′), 4.71 (1H, s, H-3′), 4.68 (1H, d, J = 11.9 Hz, PhCH_2), 4.63 (1H, d, J = 11.9 Hz, PhCH_2), 4.39 (1H, d, J = 8.2 Hz, H_a-1″), 3.98 (1H, d, J = 8.2 Hz, H_b-1″), 2.70 (3H, d, J = 4.7 Hz, CH_3). ESI-MS m/z 397.2 (MH^+).

3.1.2. (1*R*,3*R*,4*R*,7*S*)-(7-Hydroxy-3-(adenin-9-yl)-2,5-dioxabicyclo[2:2:1]hept-1-yl)-*N*-methylcarboxamide (11). Compound **23** (2.5 g, 6.3 mmol) was dissolved in methanol (50 mL) and added HCO_2NH_4 (4.0 g, 63 mmol) and $Pd(OH)_2$ 20% on carbon (0.5 g, 0.58 mmol). The mixture was stirred at reflux for 16 h and then filtered through a pad of Celite. Evaporation of the solvent under reduced pressure gave **11** as a brown solid (1.7 g, 90%). The crude product was used without further purification in the next step. A 140 mg sample was purified by RP-HPLC to give 75 mg of pure material. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.36 (1H, s, H-2), 8.30 (1H, q, J = 4.7 Hz, N*H*Me), 8.18 (1H, s, H-8), 7.27 (2H, s, NH_2), 6.01 (1H, br s, OH), 6.00 (1H, s, H-1′), 4.60 (1H, s, H-2′), 4.57 (1H, s, H-3′), 4.35 (1H, d, J = 8.0 Hz, H_a-1″), 3.91 (1H, d, J = 8.0 Hz, H_b-1″), 2.68 (3H, d, J = 4.7 Hz, CH_3). CI HR-MS: theoretical mass ($C_{12}H_{14}N_6O_4$): 307.11547 $[M+H]^+$, measured mass: 307.115598.

3.1.3. (1*S*,3*R*,4*R*,7*S*)-7-Hydroxy-1-hydroxymethyl-3-(*N*^{6}-(3-iodobenzyl)-adenin-9-yl)-2,5-dioxabicyclo[2:2:1]heptane (12). To a solution of **10**[20] (1.0 g, 3.6 mmol) in anhydrous DMF (50 mL) was added 3-iodobenzyl bromide (2.13 g, 7.2 mmol) and the mixture was stirred at 70 °C for 16 h. Additional 3-iodobenzyl bromide (1.1 g, 3.6 mmol) was added and stirring continued for 16 h. The solvent was removed under reduced pressure and the residual oil was triturated with a mixture of ether and acetone (4:1). The resulting brown gum was redissolved in methanol (15 mL) and NH_4OH (32%, aq) (10 mL), and stirred for 16 h. The solvents were removed under reduced pressure and the residue was purified by DCVC (d = 6 cm, 0–20% MeOH in EtOAc, 100 mL fractions, 0.5% increase pr fraction) to give **12** as a white solid (0.45 g, 25%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.48 (1H, br s, CH_2N*H*), 8.27 (1H, s, H-2), 8.22 (1H, s, H-8), 7.72 (1H, br s, 3-I-Ph), 7.59 (1H, d, J = 7.9 Hz, 3-I-Ph), 7.37 (1H, d, J = 7.7 Hz, 3-I-Ph), 7.10 (1H, t, J = 7.8 Hz, 3-I-Ph), 5.92 (1H, s, H-1′), 5.70 (1H, br s, OH), 5.06 (1H, br s, OH), 4.67 (2H, br s, 3-I-PhCH_2), 4.43 (1H, s, H-2′ or H-3′), 4.26 (1H, s, H-2′ or H-3′), 3.92 (1H, d, J = 8.2 Hz, H_a-1″), 3.79–3.76 (3H, m, H-5′, H_b-1″). FAB HR-MS: theoretical mass ($C_{18}H_{18}IN_5O_4$): 496.04817 $[M+H]^+$, measured mass: 496.047325.

3.1.4. (1*R*,3*R*,4*R*,7*S*)-(7-Hydroxy-3-(*N*^{6}-(3-iodobenzyl)-adenin-9-yl)-2,5-dioxabicyclo[2:2:1]hept-1-yl)-*N*-methylcarboxamide (13). Same procedure as for the synthesis of **12** using crude **11** (90 mg, 0.29 mmol) as starting material. The crude product was purified by DCVC (d = 3 cm, 0–8% MeOH in DCM, 25 mL fractions, 0.5% increase pr fraction) to give **13** as a white solid (40 mg, 26%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.40 (1H, s, CH_2N*H*), 7.84 (1H, s, H-2 or H-8), 7.72 (1H, s, H-2 or H-8), 7.62 (1H, d, J = 7.9 Hz, 3-I-Ph), 7.39

(1H, d, $J = 7.3$ Hz, 3-I-Ph), 7.26 (1H, s, 3-I-Ph), 7.06 (1H, t, $J = 7.8$ Hz, 3-I-Ph), 6.89 (1H, br s, N*H*Me), 6.27 (1H, br s, OH), 6.10 (1H, s, H-1′), 4.83 (2H, br s, 3-I-PhCH_2), 4.80 (1H, s, H-2′ or H-3′), 4.75 (1H, s, H-2′ or H-3′), 4.54 (1H, d, $J = 8.4$ Hz, H_a-1″), 4.10 (1H, d, $J = 8.4$ Hz, H_b-1″), 2.90 (3H, d, $J = 5.0$ Hz, CH_3). FAB HR-MS: theoretical mass ($C_{19}H_{19}IN_6O_4$): 523.05907 $[M+H]^+$, measured mass: 523.05852.

3.1.5. (1*R*,3*R*,4*R*,7*S*)-7-Benzyloxy-5-*tert*-butoxycarbonyl-1-hydroxymethyl-3-(adenine-9-yl)-2-oxa-5-aza-bicyclo [2:2:1]heptane (25). To a solution of **24**[22] (500 mg, 0.91 mmol) in DCM (20 mL) and triethylamine (3 mL) was added di-*tert*-butyl dicarbonate (418 μL, 1.82 mmol) and the solution was stirred at room temperature for 2 h. The solution was washed with saturated aq $NaHCO_3$ (20 mL), dried over Na_2SO_4, filtered, and evaporated to a yellow oil (ESI-MS *m*/*z* 651.2 $[M+H]^+$). The oil was redissolved in DMSO (10 mL) and added NaOBz (525 mg, 3.6 mmol) and the mixture was stirred at 90 °C for 2 h. H_2O (20 mL) was added and the mixture was extracted with DCM (3× 50 mL). The combined organic phase was washed with brine (2× 50 mL), dried over Na_2SO_4, filtered, and evaporated to a yellow solid foam (ESI-MS *m*/*z* 677.1 $[M+H]^+$). The solid was dissolved in saturated NH_3 in methanol (30 mL) and stirred at room temperature for 16 h. The solvent was removed in vacuo and the residue was purified by DCVC ($d = 2$ cm, 0–5% MeOH in DCM, 25 mL fractions, 0.5% increase pr fraction) to give **32** as a white solid (0.22 g, 50%). ^{1}H NMR (DMSO-d_6, 300 MHz, 2 rotamers 1:1) δ 8.22, 8.22 (1H, s, H-2), 8.17, 8.14 (1H, s, H-8), 7.34 (2H, br s, NH_2), 7.29–7.24 (5H, m, Ph), 5.98, 5.95 (1H, s, H-1′), 5.22 (1H, m, OH), 4.97, 4.73 (1H, s, H-2′), 4.63-4.49 (2H, m, CH_2Ph), 4.31, 4.30 (1H, s, H-3′), 3.81–3.73 (2H, m, CH_2OH), 3.45 (1H, d, $J = 10.1$ Hz, H_a-1″), 3.26 (1H, d, $J = 10.1$ Hz, H_b-1″), 1.43 (9H, s, $C(CH_3)_3$); ESI-MS *m*/*z* 469.1 $[M+H]^+$.

3.1.6. (1*R*,3*R*,4*R*,7*S*)-(7-Hydroxy-3-(adenin-9-yl)-2-oxa-5-azabicyclo[2:2:1]hept-1-yl)-*N*-methylcarboxamide (15). Compound **25** (300 mg, 0.64 mmol) was converted to **15** using the same procedures as for the synthesis of **23** and **11**. The crude product was purified by RP-HPLC to give 39 mg (21%) of pure material. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.34 (1H, s, H-2), 8.17 (1H, s, H-8), 8.16 (1H, br s, NHMe), 7.32 (2H, s, NH_2), 5.88 (1H, s, H-1′), 4.32 (1H, s, H-3′), 3.69 (1H, s, H-2′), 3.50 (1H, d, $J = 10.3$ Hz, H_a-1″), 2.85 (1H, d, $J = 10.3$ Hz, H_b-1″), 2.68 (3H, d, $J = 4.6$ Hz, CH_3). CI HR-MS: theoretical mass ($C_{12}H_{15}N_7O_3$): 306.13146 $[M+H]^+$, measured mass: 306.131584.

3.1.7. (1*S*,3*R*,4*R*,7*S*)-7-Hydroxy-1-hydroxymethyl-3-(N^6-(3-iodobenzyl)-adenin-9-yl)-2-oxa-5-azabicyclo[2:2:1]heptane (16). Compound **25** (150 mg, 0.32 mmol) was dissolved in methanol (4 mL) and added HCO_2NH_4 (101 mg, 1.60 mmol) and $Pd(OH)_2$ 20% on carbon (0.05 g, 0.05 mmol). The mixture was stirred at reflux for 5 h and then filtered through a pad of Celite. The solvent was removed under reduced pressure to give a white solid. The solid was redissolved in anhydrous DMF (5 mL), added 3-iodobenzyl bromide (285 mg, 0.96 mmol), and the mixture was stirred at 70 °C for 16 h. Additional 3-iodobenzyl bromide (142 mg, 0.48 mmol) was added and stirring continued for 16 h. The solvent was removed under reduced pressure and the residual oil was triturated with a mixture of ether and acetone (4:1). The resulting brown gum was redissolved in methanol (2 mL), and NH_4OH (32%, aq) (2 mL) and stirred for 16 h. The solvents were removed under reduced pressure and the residue was redissolved in TFA (4 mL). The mixture was stirred at room temperature for 1 h and then evaporated to a yellow oil. The crude product was purified by RP-HPLC to give **16** (16 mg, 10%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.43 (1H, br s, CH_2NH), 8.26 (1H, s, H-2), 8.21 (1H, s, H-8), 7.73 (1H, br s, 3-I-Ph), 7.57 (1H, d, $J = 7.7$ Hz, 3-I-Ph), 7.35 (1H, d, $J = 7.6$, Hz, 3-I-Ph), 7.11 (1H, t, $J = 7.7$ Hz, 3-I-Ph), 5.83 (1H, s, H-1′), 5.35 (1H, br s, OH), 4.97 (1H, br s, OH), 4.67 (2H, br s, 3-I-PhCH_2), 4.10 (1H, s, H-3′), 3.74 (2H, br s, H-5′), 3.54 (1H, s, H-2′), 2.99 (1H, d, $J = 10.0$ Hz, H_a-1″), 2.74 (1H, d, $J = 10.0$ Hz, H_b-1″). FAB HR-MS: theoretical mass ($C_{18}H_{19}IN_6O_3$): 495.06416 $[M+H]^+$, measured mass: 495.063836.

3.1.8. (1*R*,3*R*,4*R*,7*S*)-7-Benzyloxy-5-*tert*-butoxycarbonyl-1-hydroxymethyl-3-(N^6-(3-iodobenzyl)-adenine-9-yl)-2-oxa-5-aza-bicyclo[2:2:1]heptane (26). Compound **25** (300 mg, 0.64 mmol) was converted to **26** using the same procedures as for the synthesis of **12**. The crude product was purified by DCVC ($d = 2$ cm, 0–8% MeOH in DCM, 25 mL fractions, 0.5% increase pr fraction) to give **26** as a white solid (130 mg, 30%). ^{1}H NMR ($CDCl_3$, 300 MHz, 2 rotamers 2:3) δ 8.36, 8.35 (1H, s, CH_2NH), 7.84, 7.80 (1H, s, H-8), 7.74, 7.73 (1H, s, H-2), 7.61 (1H, d, $J = 7.9$ Hz, 3-I-Ph), 7.32–7.27 (7H, m, Ph, 3-I-Ph), 7.06 (1H, t, $J = 7.8$ Hz, 3-I-Ph), 6.03, 5.95 (1H, s, H-1′), 4.92, 4.69 (1H, s, H-2′), 4.80 (2H, br s, 3-I-PhCH_2) 4.62–4.55 (3H, m, H-3′, CH_2Ph,), 4.03, 4.01 (1H, d, $J = 12.5$ Hz, CH_2OH), 4.03, 3.90 (1H, d, $J = 12.5$ Hz, CH_2OH), 3.64, 3.60 (1H, d, $J = 10.1$ Hz, H_a-1″), 3.41, 3.35 (1H, d, $J = 10.1$ Hz, H_b-1″), 1.49, 1.46 (9H, s, $C(CH_3)_3$); ESI-MS *m*/*z* 685.3 $[M+H]^+$.

3.1.9. (1*R*,3*R*,4*R*,7*S*)-(7-Hydroxy-3-(N^6-(3-iodobenzyl)-adenin-9-yl)-2-oxa-5-azabicyclo[2:2:1]hept-1-yl)-*N*-methylcarboxamide (17). To a solution of **26** (110 mg 0.16 mmol) in acetonitrile (2 mL) and H_2O (2 mL) were added (diacetoxyiodo)benzene (320 mg, 1.0 mmol), and TEMPO (15 mg, 0.1 mmol) and the mixture was stirred at 40 °C for 2 h. The solvents were removed under reduced pressure and the residue was triturated with diethyl ether. The white precipitate was filtered off, washed with ether, and dried under vacuum. The intermediate was dissolved in 99.9% ethanol (5 mL) and cooled to 0 °C. $SOCl_2$ (0.26 mL, 3.5 mmol) was added and stirring was continued at room temperature for 3 h. The mixture was concentrated in vacuo and the residual solvent was coevaporated with methanol. The residual oil was redissolved in a 2 M solution of methylamine in methanol (5 mL, 10 mmol) and the mixture was stirred at room temperature for 16 h. The solvent and reagent were removed under reduced pressure to give the intermediate as a brown foam. The crude

intermediate was redissolved in anhydrous DCM (4 mL) and added methanesulfonic acid (2 mL mmol), and the mixture was stirred at room temperature for 3 h. The reaction mixture was diluted with anhydrous DCM (10 mL), cooled to 0 °C, and neutralized by the careful addition of triethylamine (4.5 mL). The mixture was washed with saturated aq $NaHCO_3$ (10 mL), and brine (10 mL) and then dried over Na_2SO_4. The solvent was removed under reduced pressure to give a yellow foam. The crude final product was purified by RP-HPLC to give **17** (14 mg).[1] H NMR (DMSO-d_6, 300 MHz) δ 8.48 (1H, br s, CH_2NH), 8.38 (1H, s, H-2), 8.24 (1H, s, H-8), 8.10 (1H, br s, N*H*Me), 7.73 (1H, s, 3-I-Ph), 7.60 (1H, d, J = 7.9 Hz, 3-I-Ph), 7.38 (1H, d, J = 7.6 Hz, 3-I-Ph), 7.11 (1H, t, J = 7.7 Hz, 3-I-Ph), 5.92 (1H, s, H-1′), 5.67 (1H, br s, OH), 4.68 (2H, br s, 3-I-PhCH_2), 4.36 (1H, s, H-3′), 3.77 (1H, s, H-2′), 3.52 (1H, d, J = 10.4 Hz, H_a-1″), 2.89 (1H, d, J = 10.4 Hz, H_b-1″), 2.68 (3H, d, J = 4.6 Hz, CH_3). FAB HR-MS: theoretical mass ($C_{19}H_{20}IN_7O_3$): 522.07506 $[M+H]^+$, measured mass: 522.073557.

3.1.10. 2′-*O*-Methyladenosine-*N*-methyl-5′-uronamide (19). To a solution of **18** (3.0 g, 10.7 mmol) in acetonitrile (25 mL) and H_2O (25 mL) were added (diacetoxyiodo)-benzene (10.3 g, 32.0 mmol) and TEMPO (0.50 g, 3.2 mmol) and the mixture was stirred at 40 °C for 4 h. The white precipitate was filtered off, washed with acetonitrile, and dried under vacuum (1.50 g). The reaction mixture was evaporated and the residue was triturated with diethyl ether. The white precipitate was filtered off, washed with ether, and dried under vacuum (1.45 g). The two portions were combined to give a total yield of the uronic acid of 2.95 g, 94%. The intermediate (1.37 g, 4.6 mmol) was dissolved in 99.9% ethanol (30 mL) and cooled to 0 °C. $SOCl_2$ (3.4 mL, 46 mmol) was added dropwise over 10 min and stirring was continued at room temperature for 3 h after which a white precipitate was formed. The mixture was concentrated in vacuo and the residual solvent was coevaporated with methanol to give a white solid material. The material was redissolved in a 2 M solution of methylamine in methanol (40 mL, 80 mmol) and the mixture was stirred at room temperature for 16 h. The white precipitate was filtered off, washed with diethyl ether, and dried under vacuum to give the target product **19** as a white solid (1.34 g, 94%) ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.85 (1H, m, N*H*CH_3), 8.47 (1H, s, H-2), 8.24 (1H, s, H-8), 7.43 (2H, br s, NH_2), 6.11 (1H, d, J = 7.1 Hz, OH), 5.79 (1H, d, J = 3.9 Hz, H-1′), 4.45-4.36 (3H, m, H-2′, H-3′ and H-4′), 3.24 (3H, s, OCH_3), 2.72 (3H, d, J = 4.3 Hz, NHCH_3). CI HR-MS: theoretical mass ($C_{12}H_{16}N_6O_4$): 309.13112 $[M+H]^+$, measured mass: 309.131270.

3.1.11. N^6-(3-Iodobenzyl)-2′-*O*-methyladenosine (20). From **18** (200 mg, 0.71 mmol) using the same procedure as for **12**. The crude product was purified by RP-HPLC to give **20** (60 mg, 17%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.36 (1H, s, H-2), 7.72 (1H, s, H-8 or 3-I-Ph), 7.71 (1H, s, H-8 or 3-I-Ph), 7.61 (1H, d, J = 7.9 Hz, 3-I-Ph), 7.28 (1H, d, J = 7.7 Hz, 3-I-Ph), 7.05 (1H, t, J = 7.8 Hz, 3-I-Ph), 6.82 (1H, br d, J = 6.8 Hz, 3′-OH), 6.69 (1H, br t, J = 6.7 Hz, 5′-OH), 5.81 (1H, d, J = 7.5 Hz, H-1′), 4.81 (2H, br s, 3-I-PhCH_2), 4.73 (1H, dd, J = 7.5 Hz and 4.6 Hz, H-2′), 4.60 (1H, d, J = 4.6 Hz, H-3′), 4.33 (1H, s, H-4′), 3.93 (1H, d, J = 12.9 Hz, H_a-5′), 3.74 (1H, t, J = 12.1 Hz, H_b-5′), 3.33 (3H, s, OCH_3). FAB HR-MS: theoretical mass ($C_{18}H_{20}IN_5O_4$): 498.06382 $[M+H]^+$, measured mass: 498.064358.

3.1.12. N^6-(3-Iodobenzyl)-2′-*O*-methyladenosine-*N*-methyl-5′-uronamide (21). From **19** (200 mg, 0.65 mmol) using the same procedure as for **13**. The crude product was purified by DCVC (d = 2 cm, 0–6% MeOH in DCM, 25 mL fractions, 0.5% increase pr fraction) to give **21** as a white solid (164 mg, 48%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.34 (1H, s, H-2), 7.82 (1H, s, H-8 or 3-I-Ph), 7.73 (1H, s, H-8 or 3-I-Ph), 7.57 (1H, d, J = 8.1 Hz, 3-I-Ph), 7.32 (1H, d, J = 7.3 Hz, 3-I-Ph), 7.04 (1H, t, J = 7.8 Hz, 3-I-Ph), 5.99 (1H, d, J = 8.0 Hz, H-1′), 4.79 (2H, br s, 3-I-PhCH_2), 4.62 (1H, s, H-4′), 4.56 (1H, d, J = 4.4 Hz, H-3′), 4.47 (1H, dd, J = 7.4 and 4.4 Hz, H-2′), 3.31 (3H, s, OCH_3), 2.91 (3H, s, NHCH_3). FAB HR-MS: theoretical mass ($C_{19}H_{21}IN_6O_4$): 525.07472 $[M+H]^+$, measured mass: 525.074340.

3.2. Human adenosine A_3 receptor assay

AequoScreen™ (Euroscreen, Belgium) cell lines expressing the A_3 human recombinant receptor and the promiscuous G protein $G_{\alpha 16}$ were used throughout the study. AequoScreen™ cells were cultured following recommended conditions for at least one week prior to the test. The day before the test, cells were harvested with PBS-EDTA, washed, and re-suspended in BSA-DMEM–F12 (Dulbecco's modified Eagle's medium—Ham's F12 with 0.1% BSA). Suspended cells were then incubated at room temperature with Coelenterazine H overnight. For agonist and antagonist testing, 50 μl of the cell suspension was injected onto 50 μl of the test compound or control in 96-well plates, and the resulting emission of light measured for the determination of cell activation. After the first reading, and following an incubation time of 15–30 min, 100 μl of the reference agonist (IB-MECA) at a concentration equal to the EC_{80} of the day of the experiment was injected onto the cell suspension containing the test compounds. The resulting emission of light was measured for the determination of antagonistic effects. Light emission for agonist and antagonist tests was recorded using a Hamamatsu FDSS-6000 reader. For agonist data, percentages of activation were calculated on the basis of the activation (luminescence data) induced by the reference agonist at a saturating concentration (EC_{100}). For antagonist data, percentages of inhibition were calculated on the basis of the activation (luminescence data) induced by the reference agonist at a concentration equal to the EC_{80}. The test compounds were tested as duplicate determinations at eight concentrations of 100, 10, 1, 0.1 μM and 10, 1, 0.1, and 0.01 nM for agonist activity and 50, 5, 0.5, 0.05 μM and 5, 0.5, 0.05, and 0.005 nM for antagonist activity. Dose–response data, EC_{50}/IC_{50}, from test compound were analyzed with XLfit (IDBS) software.

3.3. Human adenosine A_1 receptor assay

GPCR-expressing clones were transfected to coexpress human adenosine A_3 receptor (GenBank Accession No. L22607), mitochondrial apoaequorin, and Ga16. Cells were collected from plates with PBS containing 5 mM EDTA, pelleted, and re-suspended at 107 cells/ml in DMEM-F12 medium. Cells were then incubated with 5 μM Coelenterazine H (Molecular Probes) for 4 h at room temperature, washed in DMEM-F12 culture medium, and re-suspended at a concentration of 2×106 cells/ml. Cells were then mixed with test molecules and light emission by the aequorin is recorded with a Hamamatsu FDSS6000 luminometer for 30 s. The test compounds were tested as duplicate determinations at eight concentrations of 3000, 1000, 300, 100, 30, 10, 3, and 1 nM. Data were analyzed with the PRISM software (GraphPad Prism Software, San Diego, CA) using non-linear regression applied to a sigmoidal dose–response model. Results are expressed as percent of the agonist response. Controls include assays using cells not expressing GPCR (mock transfected), in order to exclude possible non-specific effects of the candidate compound.

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