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A novel class of fluorinated A_{2A} adenosine receptor agonist with application to last step enzymatic [^{18}F]fluorination for PET imaging

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Abstract: The A_{2A} adenosine receptor belongs to a family of G-coupled protein receptors that have been subjected to extensive investigation over the last few decades. Due to their prominent role in the biological functions of the heart, lungs, CNS and brain, they have become a target for the treatment of illnesses ranging from cancer immunotherapy to Parkinson's disease. The imaging of such receptors using positron emission tomography (PET) has also been of interest, potentially providing a valuable tool to analyse and diagnose various myocardial and neurodegenerative disorders, as well as offering support to drug-discovery trials. Reported herein is the design, synthesis and evaluation of two novel 5'-fluorodeoxy-adenosine (FDA) based receptor agonists, each substituted at the C-2 position with a terminally functionalised ethynyl unit. The structures enable a synthesis of ^{18}F-labelled analogues via direct, last-step, radiosynthesis from chlorinated precursors using the fluorinase enzyme (5'-fluoro-5'-deoxyadenosine synthase) which catalyses a transhalogenation reaction. This delivers a new class of A_{2A} adenosine receptor agonist which can be directly radiolabelled for exploration in PET studies.

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Introduction

Adenosine is a purine nucleoside that presents ubiquitously throughout the body and is involved in numerous vital biological functions.[1] Along with its involvement in important physiological processes as a substructure of endogenous molecules such as ATP[2] or RNA, adenosine acts at an extracellular level, where it is the native ligand for adenosine receptors. These adenosine receptors are divided into four subtypes: A_1, A_{2A}, A_{2B}, and A_3, each belonging to the broader family of G-protein-coupled receptors (GPCRs).[3] Adenosine signalling is widespread throughout mammalian organisms and each class of adenosine receptor possess distinct distributions and control over a broad spectrum of physiological and pathophysiological functions.[4]

Several studies have been conducted into the various subclasses of adenosine receptors, mostly relating to the inherent wider biological implications of their stimulation.[4-5] The A_{2A} adenosine receptors are responsible for the modulation of secondary messenger pathways, in particular the regulation of intracellular cyclic adenosine monophosphate (cAMP) biosynthesis.[6] Upon binding of adenosine to the adenosine A_{2A} receptor, and following its subsequent activation, levels of adenylyl cyclase are enhanced, thus stimulating cAMP production. A_{2A} adenosine receptors are found in numerous regions throughout the body, but they are most notably expressed in the immune cells of the spleen, leukocytes, blood platelets, and in the heart where adenosine is responsible for regulating the vasodilation of the coronary arteries, thus mediating coronary blood flow. Adenosine receptors are also significant in particular regions of the brain including the thymus, striatopallidal GABAergic neurons and the olfactory bulb, where they play an important role in the regulation of glutamate and dopamine release.[1, 7] A_{2A} adenosine receptors have been the subject of increased focus in recent years due to their association with numerous pathogenic, myocardial and neurological diseases. As such, they have become a therapeutic target for treatment of disorders ranging from inflammation, ischemia reperfusion injury, insomnia, infectious diseases, depression to CNS disorders and Parkinson's disease.[1, 7-8]

Considerable effort has been made to establish structure-activity relationships of adenosine-based ligands with respect to their stimulation of adenosine receptors, and from these studies a general pharmacophore has been established.[9] Predominant approaches to develop A_{2A} adenosine receptor agonists originate through modification of adenosine itself, and from these attempts it has been acknowledged that the adenosine scaffold must be largely maintained as the structural basis for agonist activity (see Figure 1). Furthermore, modifications to distinct regions of this scaffold can have discrete and significant effects on the binding and selectivity of adenosine analogues. Concerning the A_{2A} adenosine receptor specifically, it has been shown that the 2'- and 3'-hydroxyl groups on the ribose sugar are essential for full agonist activity, although modifications at the C-2 and N-6 positions of the adenine base (which often bring rise to increased metabolic stability), as well as modifications to the 5' position of the ribose ring are tolerated. However, N-6 substitution generally decreases A_{2A} adenosine receptor potency, and in many instances substitution at this position enhances the affinity and selectivity of A_1 and A_3 receptor binding.[7, 9a]

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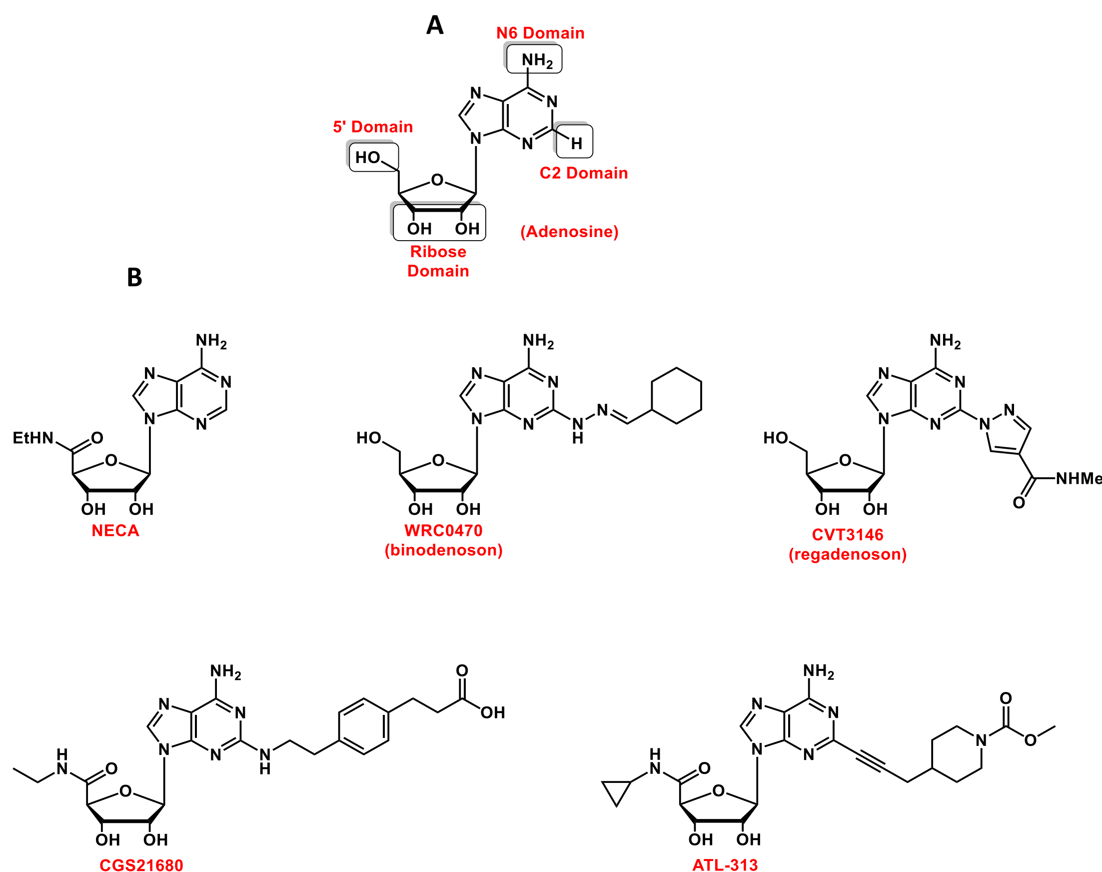

Figure 1. (A) General adenosine receptor agonist pharmacophore. (B) Examples of A_{2A} adenosine receptor agonists.

Herein we describe the synthesis of novel C-2 ethynyl adenosines **1** and **2** modified at C-5' of the ribose ring with a fluorodeoxy moiety (see Figure 2), along with assessment of their affinities for the human A_{2A} adenosine receptor and their agonist behaviour.

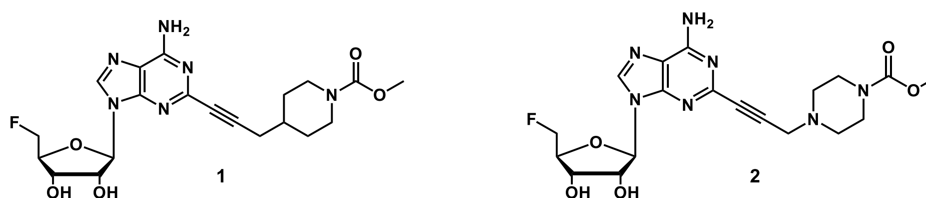

Figure 2. Structures of novel fluorinated A_{2A} adenosine receptor agonists **1** and **2**.

The fluorine in these agonists presents an immediate opportunity to develop radiotracers for PET imaging using the fluorine-18 isotope, as the 5'-fluorodeoxyadenosine motif is compatible with the enzymatic introduction of [^{18}F]fluorine from [^{18}F]fluoride. The wild type fluorinase enzyme[10] (5'-fluoro-5'-deoxyadenosine synthase) catalyses the reaction between S-adenosyl-L-methionine (SAM) and fluoride ion, generating 5'-fluoro-5'-deoxyadenosine (FDA) and L-(S)-methionine (L-Met). However, the enzyme has been shown to catalyse a transhalogenation reaction using C-2 acetylene substituted 5'-chlorodeoxyadenosine substrates to prepare ^{18}F-labelled C-2 acetylene substituted 5'-fluorodeoxyadenosines under experimentally benign conditions (buffers at pH 7.8) as illustrated in Scheme 1.[11] This has led to the strategy of tethering PEGylated peptide cargo (R group in Scheme 1) to permit last step [^{18}F]radiolabelling of cancer targeting peptides.[11-12]

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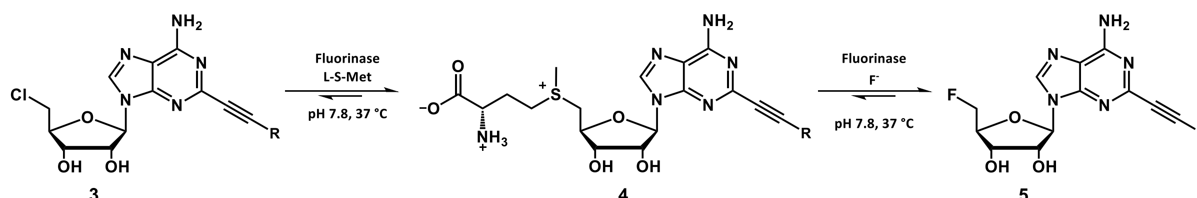

Scheme 1. Fluorinase-catalysed transhalogenation reactions with C-2 modified 5'-chloro-5'-deoxyadenosine substrates.

This study encompasses a strategy for both the chemical and enzymatic syntheses of **1** and **2**, which are derivatives of the adenosine receptor agonists in Figure 1.[13] As the fluoromethyl group at C-5' of this structural class of agonist had the potential to render them amenable to enzymatic fluorination from their chlorinated precursors using [^{18}F]fluoride, we demonstrated this in the radiochemical synthesis of **[^{18}F]1**, exemplifying the potential application of this class of A_{2a} adenosine receptor agonist as tracers for clinical imaging.[9e, 14]

Results and Discussion

Synthesis

The synthesis of **1** and **2**, along with their chlorinated precursors **19** and **24**, required an approach involving a Sonogashira coupling reaction between the C-2 iodinated adenosine scaffold and the appropriate functionalised alkyne coupling partner. For this study, 5′-fluoro-5′-deoxy-2-iodoadenosine **13** and 5′-chloro-5′-deoxy-2-iodoadenosine **11** were synthesised using previously reported strategies[12b] as illustrated in Scheme 2. Briefly, **8** was synthesised from guanosine using a two-step procedure first involving the per acetylation of the ribose moiety, followed by chlorination at C-6 of the guanine base using $POCl_3$. **8** was then iodinated at C-2 to afford **9**, which was subjected to simultaneous amination and deprotection to give 2-Iodoadenosine **10**. With **10** in hand, treatment with thionyl chloride followed by treatment with ammonia in aqueous methanol afforded the 5′-chloro-2-iodinated coupling partner **11**. Alternatively, acetonide protection of **11**, before fluorination with TsF and TBAF, followed by deprotection with TFA, afforded **13** in good yield.

The formation of alkyne **18** was achieved from commercially available *N*-Boc-4-piperidinemethanol **14**. Introduction of a tosyl group to *N*-Boc-4-piperidinemethanol **14** afforded **15** which, after flash chromatography, allowed for the insertion of a terminal alkyne using lithium acetylide/ethylenediamine complex in DMSO. Removal of the Boc group gave the free amine **17**, which followed by treatment with TEA and methyl chloroformate afforded **18** in good yield. Sonogashira cross-coupling was then performed using an excess of the alkyne **18** over **11** in the presence of a $Pd_2(dba)_3$ catalyst, CuI, and trimethylamine, in DMF. The product was subjected to C_{18} cartridge purification, followed by semi-prep HPLC to afford the coupled product **19** in good yield and high purity. In line with the proposed strategy of using the fluorinase to generate ^{18}F analogues of these A_{2A} adenosine receptor agonists, **1** was prepared enzymatically by transhalogenation of **19** in good yield as illustrated in Scheme 3 (See supporting Information for full experimental details for preparative transhalogenation).

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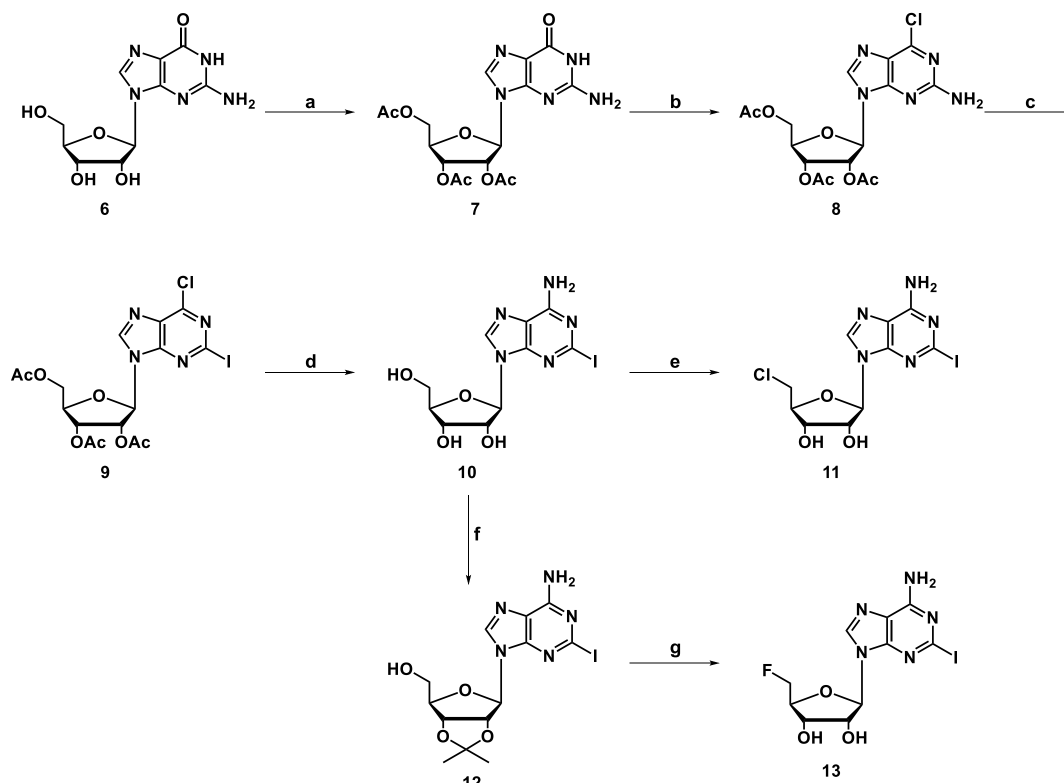
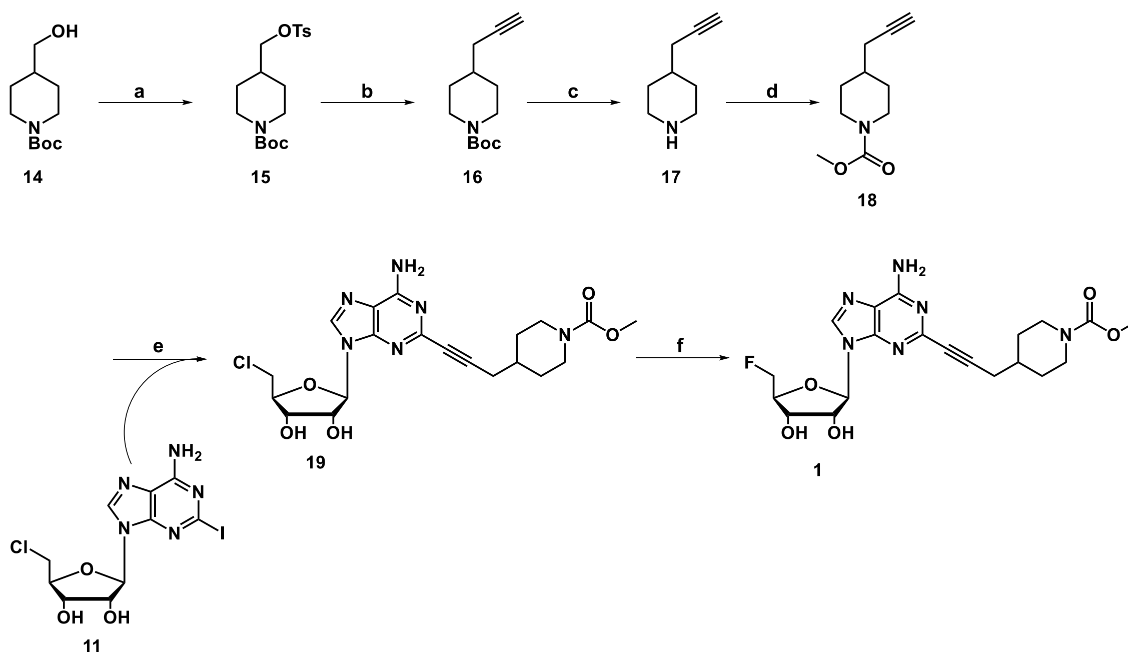


Scheme 2. Synthesis of 5′-chloro-5′-deoxy-2-iodoadenosine **11** and 5′-fluoro-5′-deoxy-2-iodoadenosine **13**. Reagents and conditions: a) Ac_2O, pyridine, DMF. b) $POCl_3$, Et_4NCl, dimethylaniline, CH_3CN. c) CuI, I_2, CH_2I_2, isoamyl nitrite, THF. d) NH_3, MeOH. e) $SOCl_2$, pyridine, CH_3CN, then NH_3, MeOH, H_2O. f) Acetone, 2,2-DMP, $HClO_4$. g) TBAF, TsF, THF, then TFA, MeOH.



Scheme 3. Synthesis of **19** and **1**. Reagents and conditions: a) Tosyl chloride, Et_3N, THF. b) Lithium acetylide, ethylenediamine complex, DMSO. c) TFA; d) Et_3N, Methyl chloroformate. e) $Pd_2(dba)_3$, Et_3N, CuI, DMF; f) Fluorinase, l-Se-Met, KF, phosphate buffer (pH 7.8).

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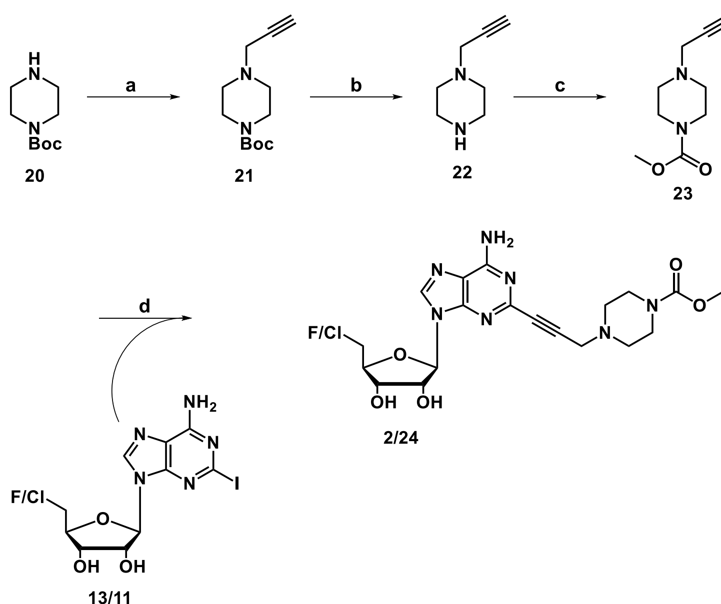
Product **2** was acquired from a similar a strategy commencing with 1-Boc-piperazine **20**. Introduction of the acetylene was accomplished using TEA and propargyl bromide, which after purification afforded **21** in very good yield. As before, the methyl carbamate moiety was introduced using successive TFA deprotection of the Boc protected amine, followed by treatment with methyl chloroformate in the presence of an excess of base to afford **23**. With **23** in hand Sonogashira cross-coupling was performed using the previously established protocol, with both the 5′-fluoro-5′-deoxy-2-iodoadenosine **13** and 5′-chloro-5′-deoxy-2-iodoadenosine **11** coupling partners as illustrated in Scheme 4. Purification by C_{18} cartridge followed by semi prep HPLC afforded **2** and **24** in good yields and in high purity



Scheme 4. Synthesis of **2** and **24**. Reagents and conditions: a) Propargyl bromide, Et_3N; b) TFA. c) Et_3N, Methyl chloroformate. d) $Pd_2(dba)_3$, Et_3N, CuI, DMF.

Radioligand displacement assays on Hek_{293} membranes expressing the human A_{2A} receptor

With **1** and **2** in hand their relative affinities to the A_{2A} adenosine receptor and any potential agonist activity was evaluated. Radioligand displacement experiments at the human A_{2A} adenosine receptor were performed with a range of concentrations of each potential ligand, in the presence of [^{3}H]-ZM241385. As a reference, CGS21680, a known agonist which possesses a high affinity to the A_{2A} adenosine receptor,[15] was also included in these experiments (Figure 3). Subsequent concentration-response curves were well behaved, and revealed the affinities of **1** and **2** for the human adenosine A_{2A} receptor to be both in the nanomolar range, with K_i values of 39 nM and 176 nM, respectively. Encouragingly, the affinity of the reference agonist CGS21680 under these conditions was also 39 nM, notably comparable to that of **1** (see also Table 1). It follows that the fluoromethyl group at the 5'-position is well tolerated by the A_{2A} adenosine receptor. A prerequisite for any viable PET tracer is a high affinity for the target receptor; with this in mind the [^{18}F] analogue of **1** was deemed the most promising of the two new agonists as a potential PET tracer, and as such was explored in hot radiolabelling studies.

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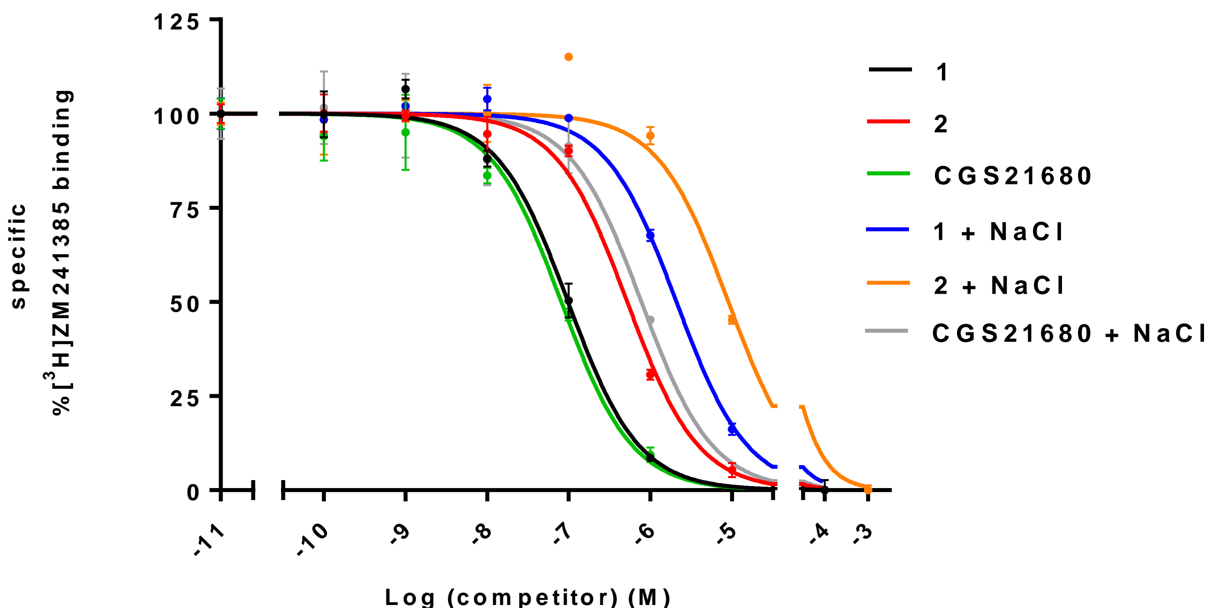

Figure 3. Concentration-response curves of **1**, **2** and the control CGS21680 under standard conditions and in the presence of 1M NaCl on Hek293 cell membranes stably expressing the hA_{2A} receptor.

To determine the agonist behavior of **1** and **2** each experiment was repeated in the presence of 1M NaCl. The binding of a sodium ion to the allosteric binding pocket of the human A_{2A} adenosine receptor stabilises the inactive conformation, and this results in an observed reduction in orthosteric binding of the agonists.[16] During these experiments the presence of sodium ion dramatically decreased the affinities of **1** and **2** to (sub)micromolar values (Table 1), consistent with both compounds acting as agonists of the human A_{2A} adenosine receptor.[17]

Table 1. Affinities of **1**, **2** and the control CGS21680 to the A_{2A} adenosine receptor in the absence and presence of sodium ion.

Compound	pKi ± SEM (control)	pKi ± SEM (+ 1 M NaCl)
1	**7.42 ± 0.07**	**6.19 ± 0.02**
2	**6.76 ± 0.01**	**5.52 ± 0.06**
CGS21680	**7.43 ± 0.09**	**6.56 ± 0.07**

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Evaluation of 1 and 2 as substrates for fluorinase mediated transhalogenation

In order to realise the application of last step enzyme catalysed ^{18}F-fluorination of **19** and **24** towards PET imaging of A_{2A} adenosine receptors, transhalogenation reactions with each of these chlorinated substrates were first explored with the fluorinase under 'cold' labelling conditions using [^{19}F]fluoride. In a typical experiment each 5'-chloro-5'-deoxy-adenosine substrate **19** and **24** was incubated with the fluorinase enzyme (0.7 mg mL^{-1}) in the presence of L-Se-Met (75 µM), potassium fluoride (50 mM) in phosphate buffer, at pH 7.8 (see the Experimental Section and Supporting Information for full experimental details and Figures). In order to monitor the progress of each assay, analytical time course experiments were conducted by removing aliquots from each reaction at specified time points. The enzyme was first removed by heat precipitation, followed by centrifugation, and conversions were determined by HPLC. Both **19** and **24** proved to be very good substrates for fluorinase mediated transhalogenation to generate **1** and **2** respectively (see Figures 4 and 5). The identities of both fluorinated products were confirmed by both mass spectrometry, and against fully characterised synthetic reference samples. The success of these reactions demonstrates that the methyl piperizine/piperidine-1-carboxylate groups of **19** and **24** do not negatively interfere with the action of the fluorinase enzyme, despite being on a short tether to the 5'-chloro-5'-deoxy-adenosine recognition/substrate motif. This opened up the prospect for direct 'last-step' [^{18}F]fluorine-labelling using fluorinase catalysis for PET.

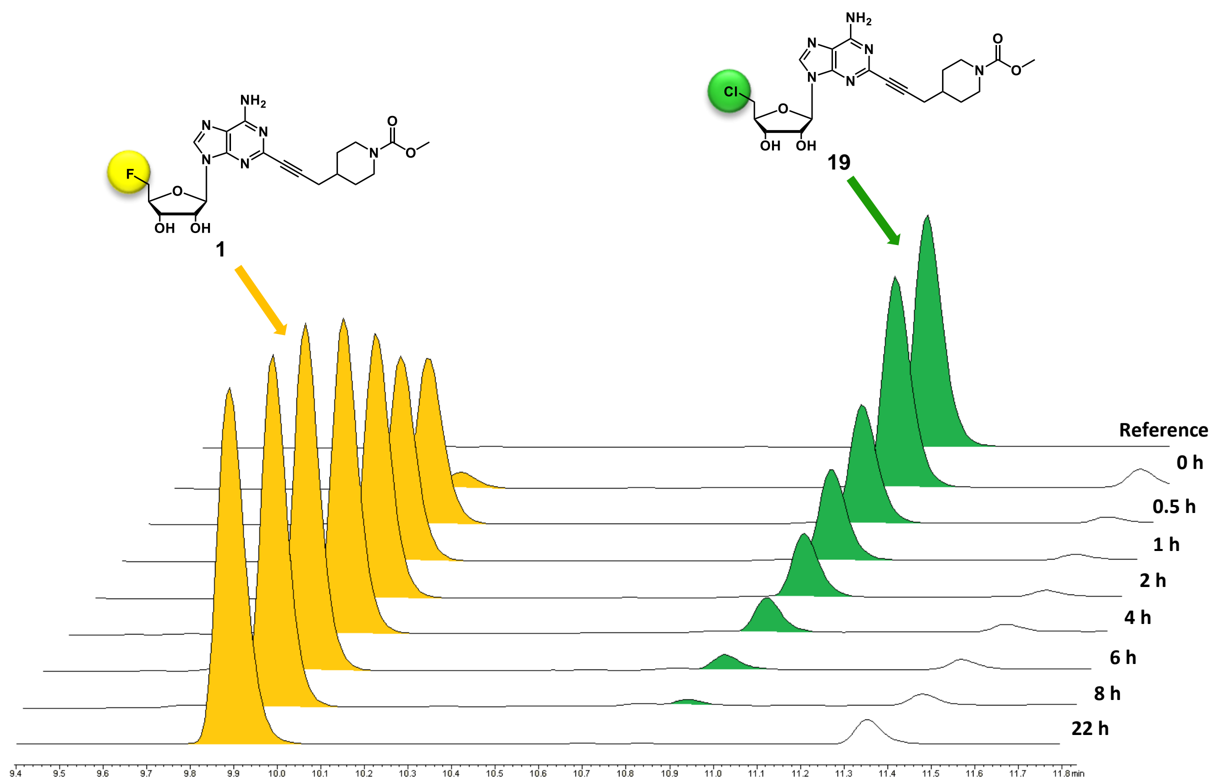

Figure 4. HPLC time course (UV, 254 nm) of the incubation of **19**, green (t_R = 10.9 min), with the fluorinase, l-Se-Met, KF, phosphate buffer (pH 7.8) at 37 °C. Traces show the formation of **1**, yellow (t_R = 9.9 min), and the consumption of **19**. For full conditions see the Experimental Section and Supporting Information.

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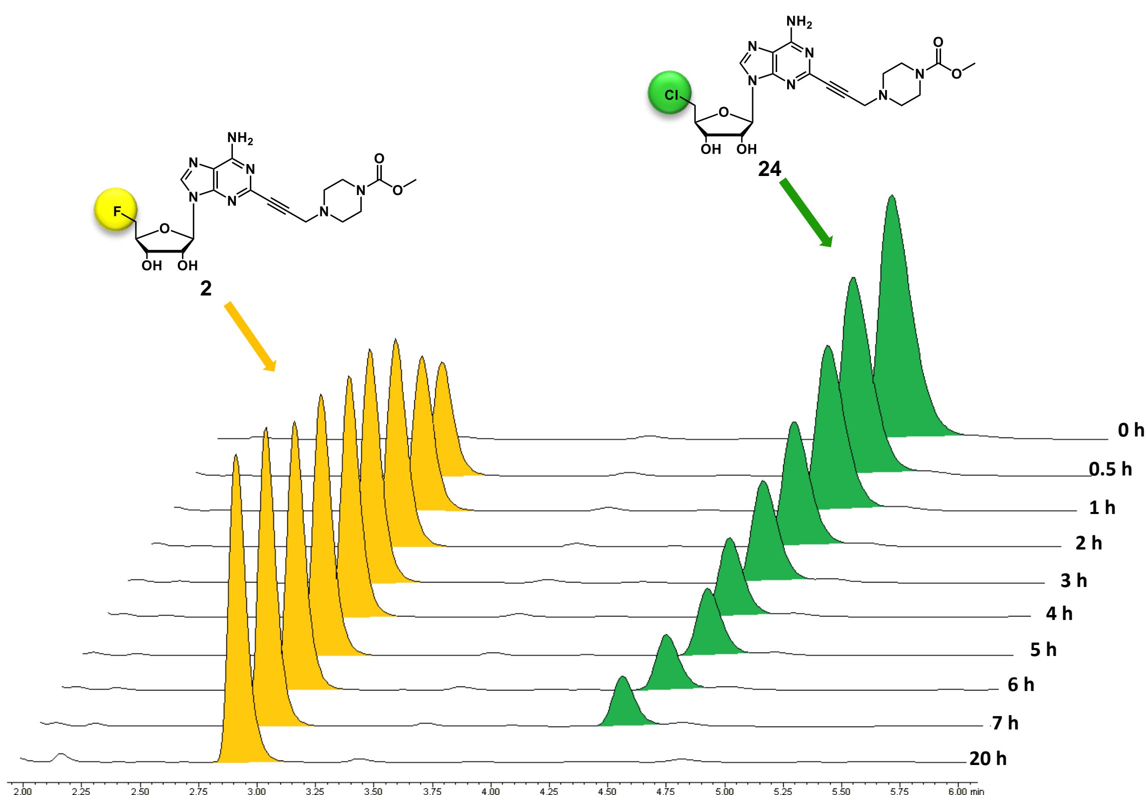

Figure 5. HPLC time course (UV, 254 nm) of the incubation of **24**, green (t_R = 4.5 min), with the fluorinase, l-Se-Met, KF, phosphate buffer (pH 7.8) at 37 °C. Traces show the formation of **2**, yellow (t_R = 2.8 min), and the consumption of **24**. For full conditions see the Experimental Section and Supporting Information.

There have been numerous studies probing the substrate specificity of the fluorinase enzyme. To date, these have revealed only a limited tolerance at N-6 and C-2 of the adenine base,[18] or more elaborate additions to the acetylene terminus of elongated PEG chains projecting from C-2.[11] **19** and **24** represent a new class of fluorinase substrate, whereby a more bulker cyclic substituent is incorporated just one methylene unit removed from the C-2 alkyne of the ClDEA motif, thus demonstrating a development in fluorinase substrate specificity.

Last step enzymatic ^{18}F labelling of 19 to [^{18}F]1

The ability to generate ^{18}F-labelled analogues of human A_{2A} adenosine receptor agonists provides an opportunity to study a variety of physiological disorders *in vivo,* as well as offering support to drug discovery trials targeting this receptor. As a proof of principle study, a transhalogenation reaction was performed with **19** in the presence of [^{18}F]fluoride, to observe its conversion to **[^{18}F]1**. Radiolabelling trials of this type are typically conducted using similar conditions to that described with [^{19}F]fluoride, but in this instance an aqueous [^{18}F]fluoride solution obtained directly from the cyclotron is used. Another significant distinction is that under hot labelling conditions the aqueous solution of [^{18}F]fluoride is generated in GBq as a dilute solution in [^{18}O]water. This solution is then utilised directly at MBq levels. Thus the final [^{18}F]fluoride ion concentrations are necessarily very low, in the pico-molar range, and as such these fluorinase-catalyzed radiochemical reactions are no longer catalytic as the enzyme (micromolar) is in considerable excess over the [^{18}F]fluoride (picomolar). In the event **19**, [^{18}F]fluoride ion, L-Se-Met and fluorinase enzyme were incubated at 37 °C in phosphate buffer (pH 7.8)

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(see Supporting Information for full experimental details and Figures). Product **[^{18}F]1** was generated in approximately 10% radiochemical conversion after a 30 min incubation. A semi-prep HPLC trace (radiochemistry detector) of the reaction mixture at 30 min is shown in Figure 6.

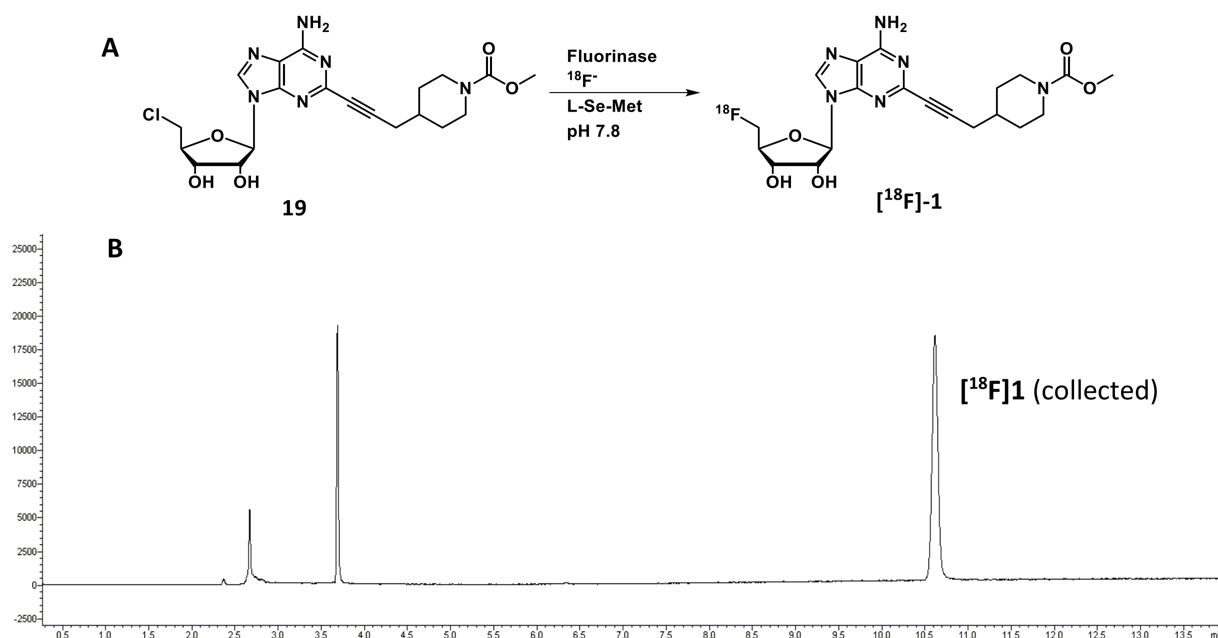

Figure 6. (A) Reaction scheme of the fluorinase catalysed transhalogenation of **19** to **[^{18}F]1**. (B) HPLC radioactivity trace of a fluorinase catalysed conversion of **19** to **[^{18}F]1** in the presence of ^{18}F$^-$, l-Se-Met in phosphate buffer (pH 7.8), at 37 °C after 30 min incubation. **[^{18}F]1** was observed at t_R = 10.6 min.

After a 30 min reaction the enzyme was heat denatured and cleanly removed by centrifugation. The clarified supernatant was then purified using semi-preparative HPLC to obtained **[^{18}F]1** (see Figure 6). Once collected, **[^{18}F]1** was then diluted with water and loaded onto a C_{18} reverse phase cartridge, washed with water and eluted with ethanol to reveal pure **[^{18}F]1** as illustrated in Figure 7. A typical procedure from [^{18}F]fluoride (205 MBq) to EtOH elution of **[^{18}F]1** (7 MBq) took 1 h, 50 min, and with a radiochemical yield of 3.5% (decay uncorrected) and afforded a radiochemical purity of >99%.

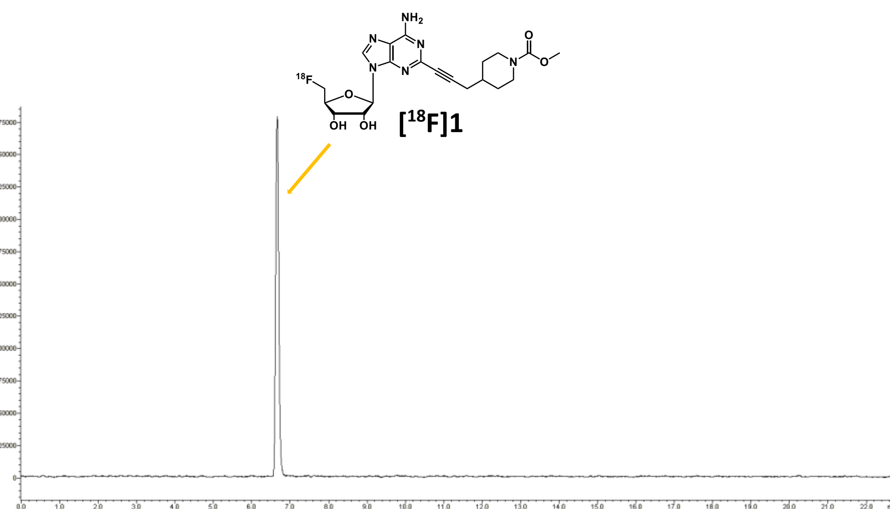

Figure 7. Analytical radiochemical HPLC trace for **[^{18}F]1** (after semi-prep HPLC purification).

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This fluorinase mediated ^{18}F-labelling methodology provides a novel route for generating a new class of human A_{2A} adenosine receptor targeting PET tracer candidates, while offering the principle advantages of an enzyme mediated synthesis (aqueous and ambient conditions, neutral pH). A common drawback with the development of small molecular weight PET tracers from known agonists is that it often necessitates the addition of a structurally bulky radiolabelled prosthetic or chelating group. The addition of such a group can often perturb the binding and pharmacokinetics of the ligand, and in the case of the human A_{2A} adenosine receptor SAR studies have shown a relatively strict structural scaffold must be maintained when developing adenosine based agonists. This fluorinase mediated strategy allows for smoother transition from the discovery of any new 5'-fluorodeoxy-adenosine (FDA) based receptor agonists of this class to the development of its corresponding ^{18}F-PET tracer analogue.

Conclusions

This study demonstrates a new class of A_{2A} adenosine receptor agonist which is designed around a 5'-fluorodeoxy-adenosine (FDA) scaffold. From this class, two novel agonists were synthesized and their affinity and agonist behavior against the human A_{2A} adenosine receptor was determined. The substrate scope of the fluorinase enzyme was further explored and expanded in the fluorinase mediated synthesis of **[^{18}F]1**, which demonstrated an efficient radiolabeling strategy that avoids the need to sacrifice structural integrity in the development of PET tracers from known agonists. Such a strategy can be applied to future A_{2A} adenosine receptor agonists of this structural class.

Experimental Section

Cold Transhalogenation assay of 19 to 1, and 24 to 2

In a total reaction volume of 1000 µL (in 50 mM phosphate buffer, at pH 7.8), recombinant fluorinase (0.7 mg/mL^{-1}) was incubated with **19** (0.1 mM) or **24** (0.2 mM), L-SeMet (0.075 mM) and KF (50 mM) at 37 °C. Samples (50 µL) were periodically removed, the protein precipitated by heating at 95 °C for 5 min, before being clarified by centrifugation (13 000 rpm, 10 min). Samples of the supernatant (40 µL) were removed for analysis by HPLC. HPLC analysis was performed on a Shimadzu Prominence system using a Kinetix 5µm XB-C18 100A (150 mm × 4.6 mm) column and a guard cartridge. Mobile phase: 0.05% TFA in water (solvent A) and 0.05% TFA in MeCN (solvent B); Linear Gradient: 15% solvent B to 95% solvent B over 25 min, 95% for 5 min, and back to 15% B for 10 min to re-equilibrate the column. Flow rate: 1 mL/min^{-1}; Detection: 254 nm; Injection volume: 40 µL.

[^{18}F] Labelling of 19 to [^{18}F]1

A typical $^{18}F^{-}$ labelling experiment of **24** was performed as follows: L-selenomethionine (40 µL of a 2mM solution in water) and compound **24** (0.6 mg in 100 µL of water) were added successively to an Eppendorf tube containing a solution of fluorinase (5 mg in 50 mM phosphate buffer, 60 µL). The contents were mixed well with a pipette and to this mixture was added [$^{18}F^{-}$]fluoride in [^{18}O]water (205 MBq, 50 µL), making a total volume of 250 µL. The contents were again well mixed and incubated

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at 37 °C for 30 min. After this time the reaction was stopped and the mixture denatured by heating at 95 °C for 5 min, before being clarified by centrifugation (13 000 rpm, corresponding to 16060 g, 5 min). The supernatant was injected into a Shimadzu Prominence HPLC system equipped with a quaternary pump, a degasser, a diode array detector and a radioactivity detector using a Phenomenex Kingsorb C18 (250 × 10.00 mm, 5µ) column and a guard cartridge. Mobile phase: 0.05% TFA in water (solvent A) and 0.05% TFA in MeCN (solvent B); Linear Gradient: 15% solvent B to 38% solvent B over 16 min, 95% for 5 min, and back to 15% B for 10 min to re-equilibrate the column. Flow rate: 5 mL/min^{-1}. The radioactive fraction corresponding to the reference of [^{18}F]**1** was collected, diluted with water (50 mL) and loaded onto a preactivated Waters Oasis HLB® Cartridge (conditioned with 2 mL EtOH, 5 mL water). The cartridge was washed with 20 mL of water and the desired product was collected by eluting with 0.5 mL of ethanol, to give about 7 MBq (3.5 %, decay uncorrected) of >99% pure product of **[^{18}F]1**.

Radioligand displacement assays on Hek_{293} membranes expressing the human A_{2A} receptor

Radioligand displacement experiments were performed using 6 concentrations of competing ligand in the presence of 1.7 nM [^{3}H]ZM241385. At this concentration total radioligand binding did not exceed 10% of that added to prevent ligand depletion. The incubations were performed under standard conditions and in the presence of 1M NaCl to test the effect of NaCl on agonist affinity. Nonspecific binding was determined in the presence of 100 µM NECA (5'-(N-Ethylcarboxamido)adenosine). Membrane aliquots containing 30 µg of protein were incubated in a total volume of 100 µL of assay buffer (25 mM Tris-HCl, pH 7.4) at 25 °C for 2 h to ensure equilibrium was reached.

Incubations were terminated by rapid vacuum filtration to separate the bound and free radioligand through prewetted 96-well GF/B filter plates using a PerkinElmer Filtermate-harvester (Perkin Elmer, Groningen, the Netherlands) after the indicated incubation time. Filters were subsequently washed 12 times with ice-cold wash buffer (25 mM Tris-HCl, pH 7.4). The plates were dried at 55 °C and Microscint™-20 cocktail (Perkin Elmer, Groningen, The Netherlands) was added. After 3 h the filter-bound radioactivity was determined by scintillation spectrometry using a 2450 MicroBeta Microplate Counter (Perkin Elmer, Groningen, The Netherlands).

See Supporting Information for experimental detail on: Compound synthesis, characterisation, large scale enzymatic synthesis of **1**, fluorinase overexpression and purification, and $HEK_{293}hA_{2A}R$ cell culture, membrane preparation and data analysis

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Keywords: adenosine receptors · biocatalysis · fluorinase · ^{18}F labelling · positron emission tomography

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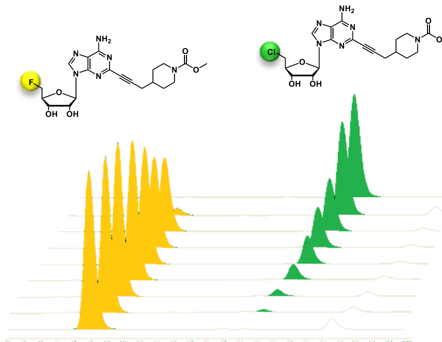
FULL PAPER

The synthesis and evaluation of a new class of A_{2a} adenosine receptor agonist, designed around a 5'-fluorodeoxy-adenosine scaffold, is reported. The fluoromethyl group at the C-5' position of this class allows for the radiosynthesis of ^{18}F-PET tracer analogues via enzymatic fluorination of chlorinated precursors with [^{18}F]fluoride.



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A novel class of fluorinated A_{2A} adenosine receptor agonist with application to last step enzymatic [^{18}F]fluorination for PET imaging

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