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Synthesis, F-18 radiolabeling, and microPET evaluation of 3-(2,4-dichlorophenyl)-*N*-alkyl-*N*-fluoroalkyl-2,5-dimethylpyrazolo[1,5-*a*]pyrimidin-7-amines as ligands of the corticotropin-releasing factor type-1 (CRF_1) receptor

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ABSTRACT

A series of 3-(2,4-dichlorophenyl)-*N*-alkyl-*N*-fluoroalkyl-2,5-dimethylpyrazolo[1,5-*a*]pyrimidin-7-amines were synthesized and evaluated as potential positron emission tomography (PET) tracers for the corticotropin-releasing factor type-1 (CRF_1) receptor. Compounds **27**, **28**, **29**, and **30** all displayed high binding affinity ($\leqslant$1.2 nM) to the CRF_1 receptor when assessed by in vitro competition binding assays at 23 °C, whereas a decrease in affinity ($\geqslant$10-fold) was observed with compound **26**. The log $P_{7.4}$ values of [^{18}F]**26**–[^{18}F]**29** were in the range of ~2.2–2.8 and microPET evaluation of [^{18}F]**26**–[^{18}F]**29** in an anesthetized male cynomolgus monkey demonstrated brain penetrance, but specific binding was not sufficient enough to differentiate regions of high CRF_1 receptor density from regions of low CRF_1 receptor density. Radioactivity uptake in the skull, and sphenoid bone and/or sphenoid sinus during studies with [^{18}F]**28**, [^{18}F]**28-d_8**, and [^{18}F]**29** was attributed to a combination of [^{18}F]fluoride generated by metabolic defluorination of the radiotracer and binding of intact radiotracer to CRF_1 receptors expressed on mast cells in the bone marrow. Uptake of [^{18}F]**26** and [^{18}F]**27** in the skull and sphenoid region was rapid but then steadily washed out which suggests that this behavior was the result of binding to CRF_1 receptors expressed on mast cells in the bone marrow with no contribution from [^{18}F]fluoride.

1. Introduction

Corticotropin-releasing factor (CRF) is a 41-residue peptide[5] that is the main regulator of the hypothalamic-pituitary-adrenal axis through its actions on CRF type-1 (CRF_1) receptors[1,6,7] expressed in the pituitary[8] which, in turn, signal for the release of adrenocorticotropic hormone.[9–13] Central nervous system (CNS) CRF has been linked to a variety of disorders including depression, stress, anxiety, post-traumatic stress disorder, and addiction.[14–24] CRF has been shown to be involved in the stress-induced phosphorylation of tau which implies a potential link between stress and Alzheimer's disease pathology.[25,26] It has also been suggested that CRF can play a neuroprotective role.[25,27–31] CRF is also found in the periphery where it is involved in inflammation[32–38] and cancer,[39–51] and it has been suggested that CRF may be one of the links between stress and cancer.[48,52–59]

Pharmacological manipulation of CNS CRF_1 receptors has been pursued as a means to treat stress, anxiety, and depression,[60–65] and this has resulted in the synthesis of numerous small-molecule CRF_1 receptor antagonists as potential therapeutics.[66–71] The development of therapeutic CRF_1 receptor antagonists can be aided by the availability of radiolabeled CRF_1 receptor antagonists that allow for imaging with positron emission tomography (PET) or single-photon emission computed tomography (SPECT). PET or SPECT imaging enables the measurement of receptor density thereby allowing for a determination of the levels of receptor expression

Abbreviations: CRF, corticotropin-releasing factor; CRF_1, CRF type-1 receptor[1]; PET, positron emission tomography; SPECT, single-photon emission computed tomography; BBB, blood–brain barrier; DMA, dimethylacetamide; PEG, polyethylene glycol; SUV, standard uptake value[2–4]; TACs, time–activity curves; ROIs, regions of interest; CPCU, chemical processing control unit; rcy, radiochemical yield; EOB, end-of-bombardment.

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in normal and altered psychiatric states, and how this changes during pharmacologic treatment.[72–74] Furthermore, utilization of PET or SPECT imaging in conjunction with a therapeutic allows for a determination of receptor occupancy, dose finding, and mechanism of action studies of the therapeutic.[75–79] Thus, there has been great interest in the development of radiolabeled CRF_1 receptor antagonists, and several examples (**1**–**12**)[80–89] are shown in Figure 1. Compounds [^{18}F]**1** and [^{123}I]**2** had limited aqueous solubility (20% EtOH in saline was required to dissolve the compounds),[80] and biological evaluation in rats showed very low brain uptake indicating that the compounds did not diffuse through the blood–brain barrier (BBB).[90–93] In vivo biodistribution studies with [^{76}Br]**3** in rats demonstrated brain penetrance, and in vitro autoradiography studies demonstrated specific binding.[82] Compounds [^{11}C]**6**, [^{11}C]**7**, and [^{11}C]**8** all penetrated the BBB of a baboon but did not display specific binding and were rapidly metabolized.[84,85] Compounds [^{18}F]**9**–[^{18}F]**11** and [^{11}C]**12** have recently been reported, and these compounds were able to penetrate the BBB of a monkey but they did not display specific binding.[87,88] Thus, attempts to develop a viable CRF_1 receptor radiotracer have been hampered by low brain entry, little or no in vivo specific binding when brain entry is achieved, and rapid metabolism. A successful CRF_1 receptor radiotracer will, therefore, require improvements in binding affinity that will enable specific binding to be detected in vivo, a reduction in lipophilicity to increase brain penetrance and reduce non-specific binding, and increased metabolic stability. As part of an effort to develop a viable CRF_1 receptor PET tracer we have been investigating the effects of various *N*-fluoroalkyl groups (Scheme 1) on the binding affinity, lipophilicity, and PET imaging properties of 3-(2,4-dichlorophenyl)-*N*-alkyl-*N*-fluoroalkyl-2,5-dimethylpyrazolo[1,5-*a*]pyrimidin-7-amines.[94] This structural class[94,95] was chosen because the 2,4-dichloro-substituents would be resistant to metabolic transformation (unlike methyl-, alkyl ether-, and alkyl amino-substituents) and would be less lipophilic than bromo- or iodo-substituents. Furthermore, as shown in Scheme 1, the core molecule can be assembled and then a library of compounds can be prepared through a divergent synthetic approach.

2. Results and discussion

2.1. Chemistry

Amino-pyrazole **13**[89,95,96] (Scheme 1) was refluxed in ethyl acetoacetate (an alternative to using ethyl acetoacetate in toluene with acid catalyst[94] or in refluxing AcOH[96]) to give **14** which was then reacted with $POCl_3$ to give **15**. Compound **15** was reacted with primary alkylammonium chlorides or primary amines to give compounds **16**–**20**, or with dialkylamines to give compounds **21**–**25**. Alcohols **24** and **25** were deprotonated with NaH in DMA and then reacted with 1-tosyloxy-2-fluoroethane to give compounds **26** and **27**. Compounds **16**–**18** were deprotonated with NaH in DMA and then reacted with 1-bromo-4-fluorobutane to give compounds **28**–**30**, or reacted with alkyl-ditosylates to give the radiolabeling precursors **31**–**34**. Attempts to *N*-alkylate **19** with 1-tosyloxy-3-fluoropropane, and **20** with 1-tosyloxy-2-fluoroethane or 1,2-ditosyloxyethane did not give the desired products, presumably due to the formation of a cyclic-quaternary ammonium salt.[97] 1,4-Ditosyloxybutane (**35**) and 1,4-ditosyloxybutane-d_8 (**35-d_8**) were prepared by refluxing 1,4-dibromobutane or 1,4-dibromobutane-d_8, respectively, with AgOTs in CH_3CN (Scheme 2).[98,99]

2.2. In vitro competition binding assays

The binding affinities of compounds **21**–**30** at the CRF_1 receptor were determined using in vitro competition binding assays at 23 °C with transfected human CRF_1 receptors in HEK293T cells (Table 1). Compounds **21** and **22** have equal affinities indicating that the CRF_1 receptor does not discriminate between symmetrical and unsymmetrical *N*,*N*-dialkyl groups with an equal number of CH_2 groups (in this limited series of compounds). The binding affinity that we determined for **22** (K_i = 0.46 ± 0.06 nM) was similar to the previously reported value of K_i = 0.9 ± 0.1 nM.[96] Addition of a terminal fluorine atom to the butyl group of **22** to give **29** did not change the binding affinity, and replacement of the *N*-ethyl group of **29** with *N*-fluoroethyl to give **30** had a negligible effect on the binding affinity. Replacement of the ethyl group of **29** with a methyl group to give **28**, in an attempt to reduce lipophilicity, resulted in a ~2.3-fold loss of affinity. The ethers **26** and **27** were also prepared in an effort to reduce lipophilicity. Insertion of an oxygen atom into the butyl group of **29** to give **27** resulted in a ~2.5-fold loss of affinity whereas a nearly 12-fold loss of affinity occurred upon changing **28** to **26**.

2.3. Radiochemistry

Radiolabeling of [^{18}F]**28** was initially performed by a two-step method (Scheme 3) where **16** was deprotonated with NaH in anhydrous DMA, and then reacted with [^{18}F]fluorobutyltosylate which was prepared from **35** in a Siemens/CTI chemical processing control unit (CPCU). Subsequently, a one-step method was developed

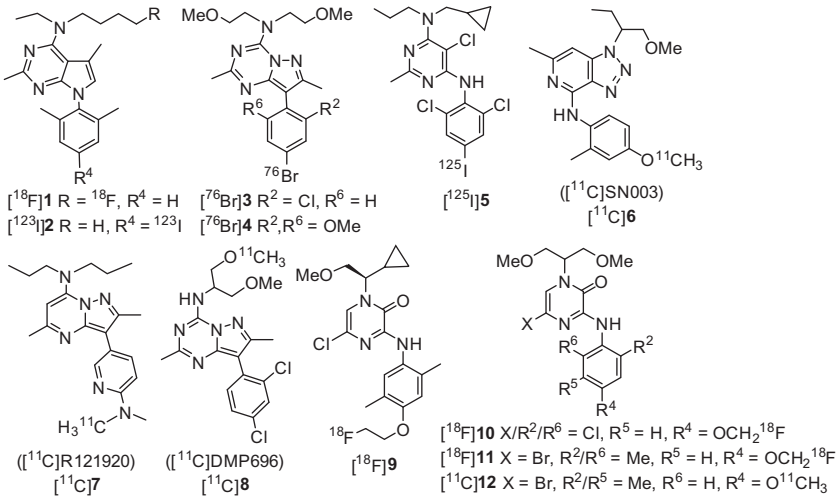
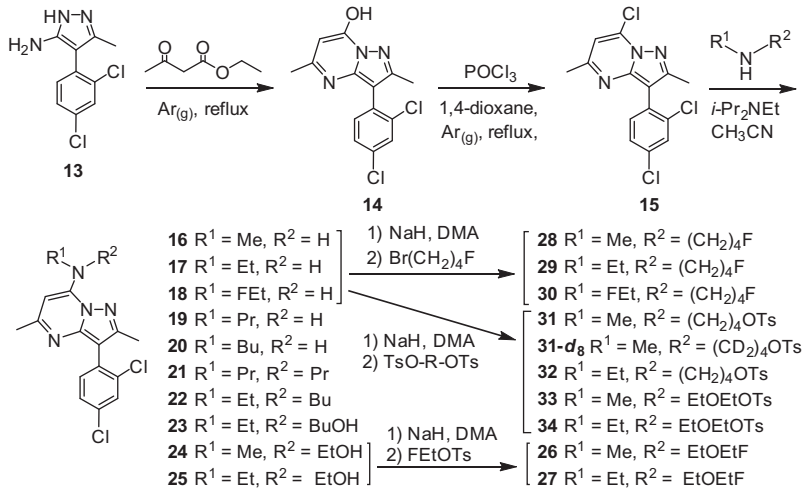
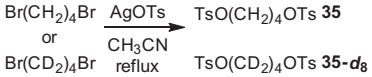


Figure 1. Examples of previously reported CRF_1 receptor radiotracers.

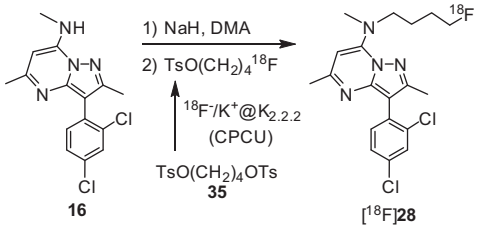


Scheme 1.

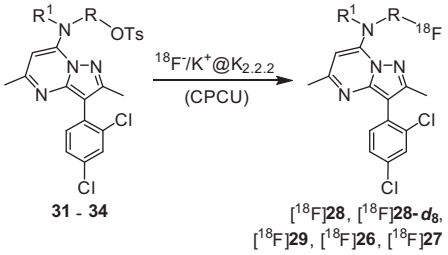


Scheme 2.

which utilized the alkyltosylate precursors **31**–**34** (Scheme 4) in the CPCU. This shortened the radiosynthesis time by ~35 min and simplified the radiolabeling procedure because it moved the water-sensitive NaH deprotonation to the precursor synthesis step (Scheme 1). This also increased the radiochemical yield for [^{18}F]**28** (11% for the two-step method versus 44% for the one-step method, see Experimental Sections 4.29 and 4.30). The crude radiotracers were purified by semi-preparative HPLC (we initially used MeOH in the solvent mixture but later replaced it with EtOH which successfully reduced radiolysis of the radiotracer[101–103] during purification and isolation), collected on a Waters tC$_{18}$ Sep-Pak, eluted from the Sep-Pak with EtOH, and collected in a sealed sterile dose vial as a 10%EtOH/saline solution. Radiochemical yields (decay-corrected) were then calculated at this point (see Experimental Sections 4.27–4.32). The solutions were then passed through successive 1 mm and 0.2 mm Acrodisc PTFE filters (pre-rinsed with EtOH) and collected in a sealed sterile dose vial as the formulated dose. The octanol/aqueous buffer partition coefficients (log$P_{7.4}$) of [^{18}F]**26**–[^{18}F]**29** (Table 2) were measured by the shake-flask method as previously reported by Wilson and Houle,[104,105] and were found to be between log$P_{7.4}$ = ~2.2–2.8 which is in the appropriate range for brain entry.[90,91,93]



Scheme 3.



Scheme 4.

2.4. MicroPET imaging

Compound [^{18}F]**28** (prepared by the two-step radiolabeling method) was evaluated by microPET for brain uptake in an anesthetized male Sprague–Dawley rat. As shown in Figure S1 (Supplementary material), brain penetrance was achieved but high uptake was also observed in the liver and Harderian glands.[106] The time–activity curves (TACs) in Figure 2 show that rapid brain uptake occurred (SUV$^{2-4}$ = 2 at 5 min) followed by a slow washout. Liver uptake was very high and rapid (SUV = 5.4 at 3 min) followed by a steady washout, whereas uptake in the Harderian glands continued throughout the course of the study. The lack of bone

Table 1
Results of in vitro competition binding assays at 23 °C using transfected human CRF$_1$ receptors in HEK293T cells

Compd	R^1	R^2	K_i (nM) ± SEM[a]	*n*=
21	Pr	Pr	0.47 ± 0.08	3
22	Et	Bu	0.46 ± 0.06	3
26	Me	EtOEtF	12.4 ± 1.06	3
27	Et	EtOEtF	1.17 ± 0.07	3
28	Me	BuF	1.07 ± 0.21	4
29	Et	BuF	0.47 ± 0.10	5
30	FEt	BuF	0.59 ± 0.04	3

[a] Versus [^{125}I]-Tyr^0-sauvagine.[100]

Table 2
Log$P_{7.4}$ values

Compd	Log$P_{7.4}$ ± SD	*n*=
[^{18}F]**26**	2.75 ± 0.02[a]	4
[^{18}F]**27**	2.80 ± 0.01[a]	4
[^{18}F]**28**	2.20 ± 0.02[b]	4
[^{18}F]**28**	2.44 ± 0.09[a]	3
[^{18}F]**28-d_8**	2.49 ± 0.01[a]	4
[^{18}F]**29**	2.81 ± 0.01[a]	4

[a] 1-Step radiolabeling method.
[b] 2-Step radiolabeling method.

visualization suggested that [^{18}F]**28** was stable to defluorination in the rat. Based on this preliminary data, compound [^{18}F]**28** (prepared by the one-step radiolabeling method) was evaluated by microPET for brain uptake and specific binding in an anesthetized male cynomolgus monkey. The microPET images in Figure S2 (Supplementary material) show that brain penetrance was achieved but the TACs in Figure 3 show that uptake of [^{18}F]**28** was equal across all brain regions including regions with high CRF_1 receptor density (cerebellum and cortex)[85,88,107,108] and regions of low CRF_1 receptor density (striatum and thalamus), thus suggesting little or no specific binding. This uniform brain distribution is similar to what was reported for [^{11}C]**6**, [^{11}C]**7**, [^{11}C]**8**, [^{18}F]**10**, [^{18}F]**11**, and [^{11}C]**12**.[84,85,87] Furthermore, high radioactivity uptake in the skull, and sphenoid bone and/or sphenoid sinus, suggests defluorination. The deuterated version, [^{18}F]**28-*d*$_8$**, was therefore prepared in an effort to reduce defluorination.[109,110] As shown by the TACs in Figure 4, uptake of [^{18}F]**28-*d*$_8$** in the skull and sphenoid region was reduced somewhat relative to [^{18}F]**28** but defluorination was not completely prevented. Skull uptake of [^{18}F]**28** and [^{18}F]**28-*d*$_8$** was rapid but then leveled off for [^{18}F]**28-*d*$_8$** and only slightly increased with time for [^{18}F]**28**. Radioactivity uptake in the sphenoid region was also rapid for both [^{18}F]**28** and [^{18}F]**28-*d*$_8$**, but then there was a brief washout followed by a slow increase. This seems to suggest that radioactivity uptake in the sphenoid region is not solely the result of metabolic defluorination to generate [^{18}F]fluoride and, in fact, compounds [^{11}C]**6**, [^{11}C]**7**, and [^{11}C]**8**, which cannot generate [^{18}F]fluoride, also show uptake in the same region.[85] Thus, some of the observed sphenoid uptake of [^{18}F]**28** and [^{18}F]**28-*d*$_8$** may be the result of binding in bone marrow. Mast cells, which are derived from bone marrow[111,112] and are also located intracranially,[113–115] have been shown to express CRF_1 receptors,[43,112,116–124] and this would help to explain the uptake of [^{18}F]**28** and [^{18}F]**28-*d*$_8$** (and [^{11}C]**6**, [^{11}C]**7**, and [^{11}C]**8**) in the sphenoid region. Mast cells present in the skin also express CRF_1 receptors[125–127] which explains the radioactivity uptake observed in the skin in the coronal and sagittal images in Figures S2 and S3 (Supplementary material). The pituitary, which is located in the hypophyseal fossa of the sphenoid bone, also contains CRF_1 receptors[8] and the pituitary can be observed in the sagittal image in Figure S2 (Supplementary material) but radioactivity spillover from the sphenoid region prevented an accurate generation of

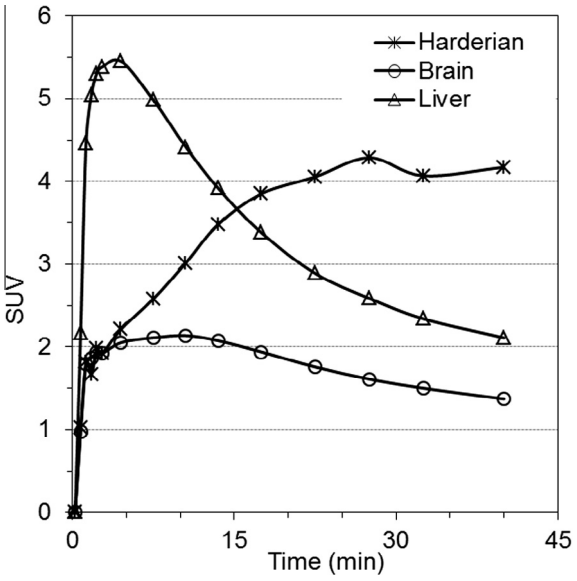


Figure 2. MicroPET TACs of [^{18}F]**28** in the brain, liver, and Harderian glands of an anesthetized male Sprague–Dawley rat.

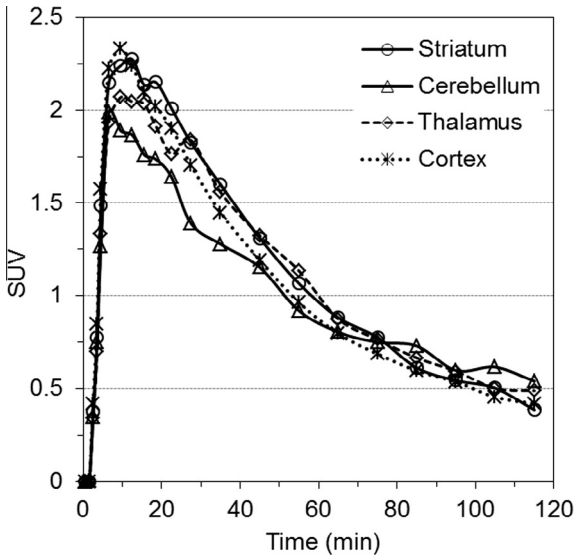


Figure 3. MicroPET TACs of [^{18}F]**28** in the brain of an anesthetized male cynomolgus monkey.

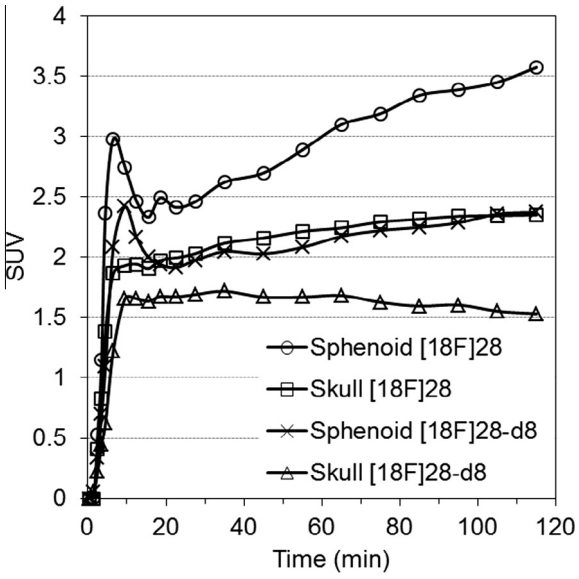


Figure 4. MicroPET TACs comparing the uptake of [^{18}F]**28** and [^{18}F]**28-*d*$_8$** in the skull, and sphenoid bone and/or sphenoid sinus of an anesthetized male cynomolgus monkey.

TACs for the pituitary. The brain uptake and washout of [^{18}F]**28-*d*$_8$** (Fig. 5) was very similar to that of [^{18}F]**28** (Fig. 3) which indicates that the deuterated *N*-fluorobutyl group did not alter the in vivo behavior of the radiotracer other than what is shown in Figure 4.

The distribution of [^{18}F]**29** in the brain (Fig. S4, Supplementary material) was similar to that of [^{18}F]**28** and [^{18}F]**28-*d*$_8$**, but the overall amount of uptake was less (Fig. 6) while the retention was slightly longer and the washout was slightly slower which may be the result of the greater lipophilicity of [^{18}F]**29** relative to [^{18}F]**28**, or the ~2-fold stronger binding affinity of [^{18}F]**29** relative to [^{18}F]**28**, or a combination of both factors. The skull uptake of [^{18}F]**29** (Fig. 7) was less than that of [^{18}F]**28** and [^{18}F]**28-*d*$_8$** (Fig. 4) which suggests that an *N*-ethyl group may be able to reduce metabolic defluorination through increased sterics relative to an *N*-methyl group. The uptake of [^{18}F]**29** in the sphenoid region

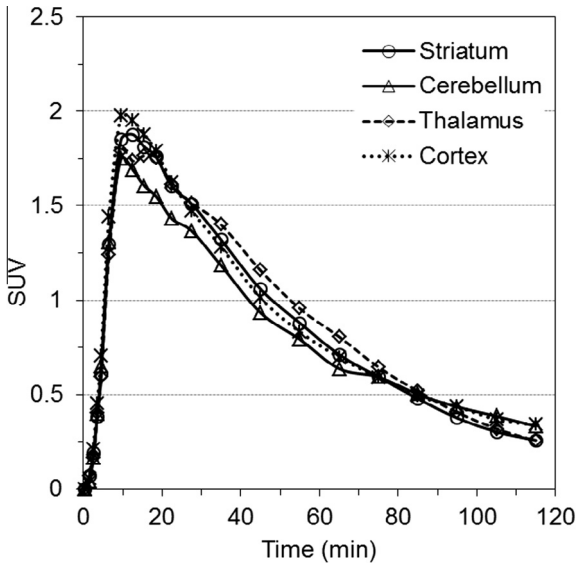


Figure 5. MicroPET TACs of [^{18}F]**28-d_8** in the brain of an anesthetized male cynomolgus monkey.

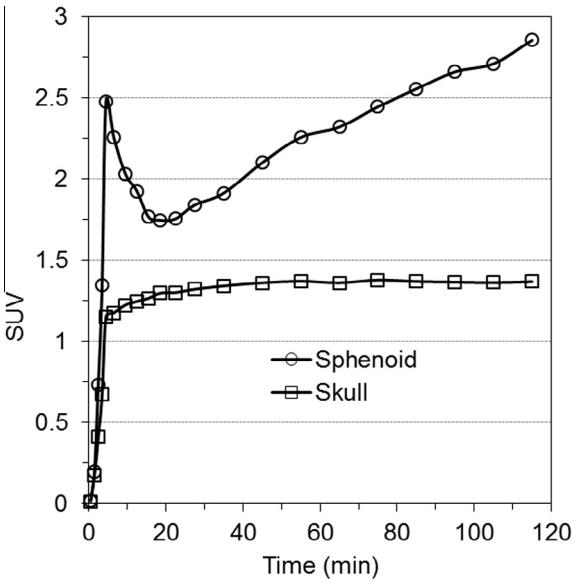


Figure 7. MicroPET TACs of [^{18}F]**29** in the skull, and sphenoid bone and/or sphenoid sinus of an anesthetized male cynomolgus monkey.

(Fig. 7) behaved similarly to [^{18}F]**28** and [^{18}F]**28-d_8** (Fig. 4) with an initial rapid uptake followed by a quick partial washout and then a steady increase. As described above, the radioactivity uptake in the sphenoid region may initially be rapid uptake of [^{18}F]**29** by mast cells in the bone marrow followed by a brief washout and then a steady uptake of [^{18}F]fluoride generated by metabolic defluorination. But, the continuous metabolic generation of [^{18}F]fluoride would also be expected to be reflected by a continuous increase of radioactivity in bone[109] although that is not what is observed in the skull in Figure 7 with [^{18}F]**29** or in Figure 4 with [^{18}F]**28-d_8**. Furthermore, as described above, uptake of [^{18}F]fluoride cannot account for the observed uptake of [^{11}C]**6**, [^{11}C]**7**, and [^{11}C]**8** in the sphenoid region.[85] The increase of radioactivity in the sphenoid region, after the initial brief washout, (Figs. 4 and 7) corresponds to the beginning of radiotracer washout from the brain (Figs. 3, 5 and 6) which suggests that the continuous radioactivity uptake (from ~20 to 115 min) in the sphenoid region is at least partly the result of intact radiotracer exiting the brain and is not solely the result of metabolically generated [^{18}F]fluoride.

The distribution of [^{18}F]**26** and [^{18}F]**27** in the brain (Figs. S5 and S6, respectively, Supplementary material) was similar to that of [^{18}F]**28**, [^{18}F]**28-d_8**, and [^{18}F]**29**. The TACs of [^{18}F]**27** in the brain (Fig. 8) were also similar to those of [^{18}F]**28**, [^{18}F]**28-d_8**, and [^{18}F]**29** with peak brain uptake achieved between ~5 and 15 min followed by a steady washout and with no discernable difference between regions with high CRF_1 receptor density (cerebellum and cortex)[85,88,107,108] and regions of low CRF_1 receptor density (striatum and thalamus) which suggests little or no specific binding. The TACs of [^{18}F]**26** in the brain (Fig. 9) show that it entered the brain more rapidly and washed out more rapidly than [^{18}F]**27**, [^{18}F]**28**, [^{18}F]**28-d_8**, and [^{18}F]**29**. This is further demonstrated by Figure S7 (Supplementary material) which compares the whole-brain TACs (a single ROI was generated for the whole

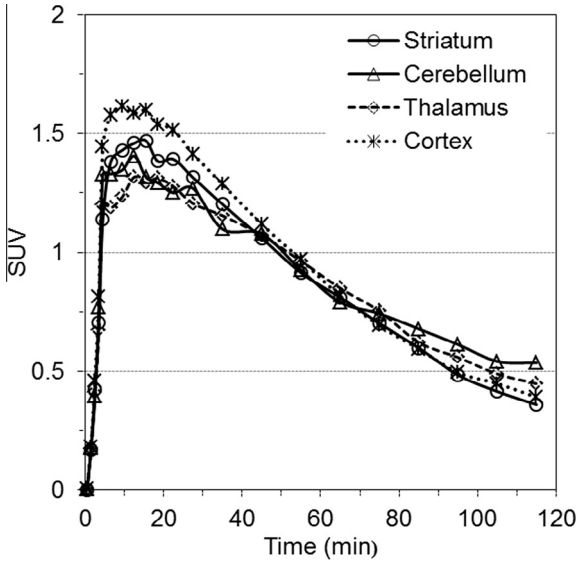


Figure 6. MicroPET TACs of [^{18}F]**29** in the brain of an anesthetized male cynomolgus monkey.

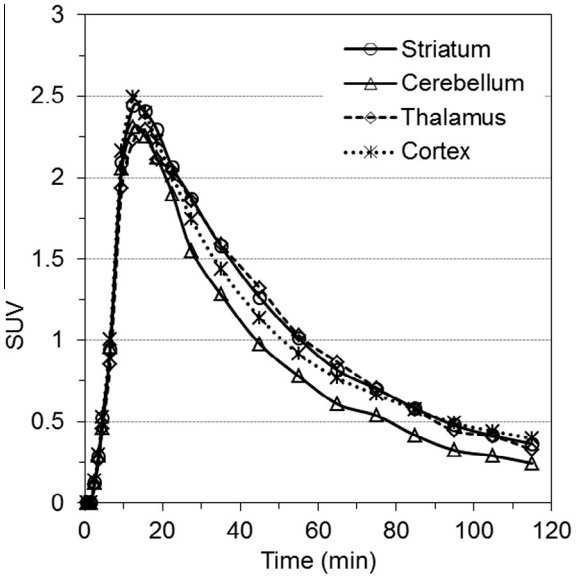


Figure 8. MicroPET TACs of [^{18}F]**27** in the brain of an anesthetized male cynomolgus monkey.

brain) for each of the radiotracers and which confirms the more rapid kinetics of [^{18}F]**26**. Compound **26** has ~10–12-fold lower binding affinity than **27** or **28** and ~26-fold lower binding affinity than **29** (Table 1). Thus, the rapid washout of [^{18}F]**26** from the brain suggests that the higher binding affinity compounds, [^{18}F]**27**, [^{18}F]**28**, [^{18}F]**28-*d*$_8$**, and [^{18}F]**29**, may actually be displaying a small amount of specific binding to the CRF_1 receptor which slows the washout from the brain. The rate of washout from the brain cannot be attributed to lipophilicity-induced non-specific binding because [^{18}F]**27** and [^{18}F]**29** have the same log$P_{7.4}$ value (Table 2) and are only slightly more lipophilic than [^{18}F]**26**, yet each compound has a different shaped whole-brain TAC in Figure S7. Therefore, it may be possible that [^{18}F]**27**, [^{18}F]**28**, [^{18}F]**28-*d*$_8$**, and [^{18}F]**29** are binding to the CRF_1 receptor but that dissociation from the receptor is too rapid[128,129] for a difference between regions with high CRF_1 receptor density (cerebellum and cortex) and regions of low CRF_1 receptor density (striatum and thalamus) to be detected.

Figures 10 and 11 show the TACs for [^{18}F]**26** and [^{18}F]**27**, respectively, in the skull and sphenoid region. Both [^{18}F]**26** and [^{18}F]**27** show rapid uptake and then a steady washout from these regions but do not show the steady continuous uptake that was observed with [^{18}F]**28**, [^{18}F]**28-*d*$_8$**, and [^{18}F]**29** which supports the notion that some of the uptake of [^{18}F]**28**, [^{18}F]**28-*d*$_8$**, and [^{18}F]**29** in the sphenoid region, and possibly the skull, is due to binding to CRF_1 receptors expressed on mast cells in the bone marrow[43,112,116] and not solely the result of metabolically generated [^{18}F]fluoride. Furthermore, the lower binding affinity compound, [^{18}F]**26**, washes out faster from the skull and sphenoid region than the higher binding affinity compound, [^{18}F]**27**, which is in agreement with the idea that binding to the CRF_1 receptor expressed on bone marrow mast cells is being observed. Also, the rate of washout of [^{18}F]**27** from the sphenoid region changes significantly at ~20 min which corresponds to the beginning of washout of [^{18}F]**27** from the brain, while the change in washout of [^{18}F]**26** from the sphenoid region is less pronounced at ~20 min, which is also in agreement with the higher binding affinity compound, [^{18}F]**27**, binding to the CRF_1 receptor expressed on mast cells in bone marrow.

The behavior of [^{18}F]**27**, [^{18}F]**28**, [^{18}F]**28-*d*$_8$**, and [^{18}F]**29** in the brain of a cynomolgus monkey is similar to what was reported for [^{11}C]**7** and [^{11}C]**8** in the brain of a baboon,[85] and [^{18}F]**10** and [^{11}C]**12** in the brain of a rhesus monkey[88] with regards to the shapes of the TACs, relatively even distribution throughout the brain, and an apparent lack of specific binding. All of these compounds displayed high binding affinities to the CRF_1 receptor when evaluated in in vitro binding assays and compounds [^{18}F]**10** and [^{18}F]**11** displayed specific binding in autoradiography studies with rhesus brain slices.[88] Furthermore, **8** blocked the binding of [^{125}I]sauvagine, [^{125}I]oCRF, and [^{3}H]**6** in rat autoradiography studies,[130,131] and numerous other studies have used autoradiography to confirm the presence of the CRF_1 receptor and to quantify its density.[85,88,107,108,132–134] Thus, it is not clear why specific binding is not being observed during PET studies. It is possible that endogenous CRF is competing with the PET tracers for binding at the CRF_1 receptor during the PET studies or that there is interference from the isoflurane anesthesia which has already been proven to alter the results of some PET studies.[135–139] A speculative mechanism of how isoflurane, mediated through CNS mast cells, increases

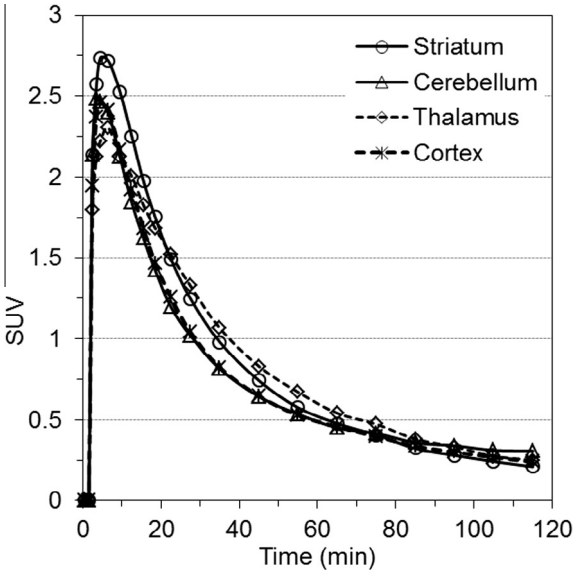


Figure 9. MicroPET TACs of [^{18}F]**26** in the brain of an anesthetized male cynomolgus monkey.

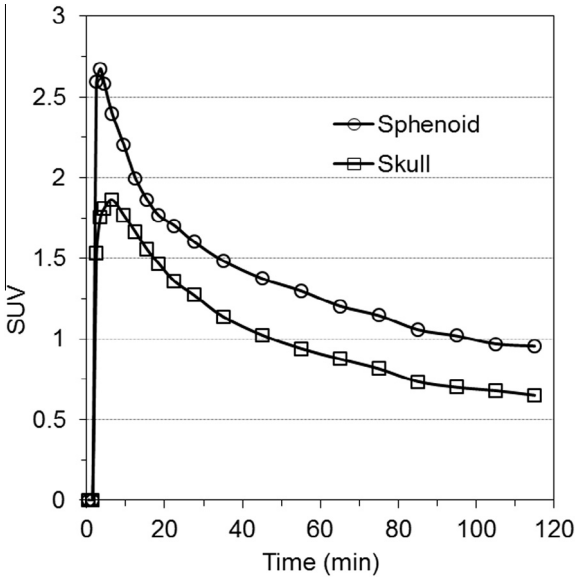


Figure 10. MicroPET TACs of [^{18}F]**26** in the skull, and sphenoid bone and/or sphenoid sinus of an anesthetized male cynomolgus monkey.

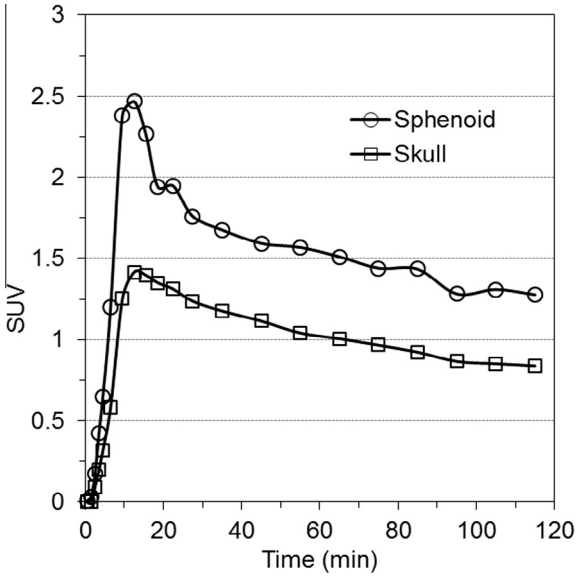


Figure 11. MicroPET TACs of [^{18}F]**27** in the skull, and sphenoid bone and/or sphenoid sinus of an anesthetized male cynomolgus monkey.

the concentration of endogenous CRF in the brain and which then competes with the PET tracer, is as follows: Mast cells[111] are located peripherally but also intracranially in the dura[113] as well as in the brain, with high levels in the thalamus, hypothalamus, and grey matter, including the cortex;[124,140] they can also move from the blood to the brain,[115] and they interact with CRF to regulate the permeability of the BBB.[114,141] They are involved in the allergic response, including the allergic response to anesthesia,[142] and they can also be activated by non-allergic triggers,[143] including CRF.[113,118,121,122,125,126] Mast cells express CRF_1 receptors[43,112,116,118,119] and also synthesize and secrete CRF,[117] and thus the two are closely linked. Activation of mast cells results in the release of histamine and other chemical messengers,[111] and histamine has been shown to increase levels of CRF in rats.[144,145] This effect could be further enhanced by histamine receptor agonists or blocked by histamine receptor antagonists or anti-CRF.[144,146,147] In dog brain, mast cell degranulation results in an increase in histamine release and a subsequent increase in plasma cortisol and ACTH.[120,148] This effect can be blocked by intracerebroventricular administration of anti-CRF or a histamine receptor antagonist, thus confirming the link between histamine and CRF release in the brain. In rats, isoflurane anesthesia has been shown to increase the concentration of histamine in the hypothalamus, and this was attributed to inhibition of histamine metabolism.[149] Furthermore, ketamine, an anesthetic commonly used to initially anesthetize non-human primates prior to using isoflurane during PET imaging, has been shown to stimulate the release of histamine from lung mast cells.[142] Thus, we speculate that anesthesia could be increasing the histamine concentration in the brain through mast cell activation, and then the histamine causes an increase in CRF concentration. The increased CRF concentration would then compete directly with the PET tracer for binding at the CRF_1 receptor, or cause a downregulation or internalization of the receptor, effectively reducing the number of available CRF_1 receptors for PET tracer binding. Because high concentrations of CNS mast cells correspond to the same locations as high concentrations of CRF_1 receptor, a reduction in the number of available CRF_1 receptors caused by the anesthesia, and mediated through mast cells, would make PET tracer binding appear similar to brain regions with low mast cell and CRF_1 receptor density, and would explain the discrepancies between the PET data and the autoradiography data. This speculative mechanism could be tested by using an awake non-human primate, but restraint stress in rats has been shown to increase histamine levels and blood corticosterone.[147] Thus, histamine receptor blockade would still be necessary,[144,146,147] but this histamine receptor blockade could potentially be employed in the presence of anesthesia,[150] thus eliminating the need for an awake non-human primate.

3. Summary

This work focused on the synthesis, binding affinity determination, F-18 radiolabeling, and microPET evaluation of a series of *N*-fluoroalkyl-pyrazolo[1,5-*a*]pyrimidin-7-amines as ligands of the CRF_1 receptor. Compounds **27**, **28**, **29**, and **30** all displayed high binding affinity ($\leqslant$1.2 nM) to the CRF_1 receptor when assessed by in vitro competition binding assays at 23 °C, whereas a decrease in affinity ($\geqslant$10-fold) was observed with compound **26**. A one-step F-18 radiolabeling method was developed which took less time and afforded higher radiochemical yields than the initial two-step method that was employed for [^{18}F]**28**. The log$P_{7.4}$ values of [^{18}F]**26**–[^{18}F]**29** were in the range of ~2.2–2.8 which is appropriate for passive diffusion across the BBB. MicroPET evaluation of [^{18}F]**26**–[^{18}F]**29** in an anesthetized male cynomolgus monkey demonstrated that these compounds all penetrated the brain but did not display specific binding sufficient enough to differentiate regions of high CRF_1 receptor density from regions of low CRF_1 receptor density. This is similar to what has been reported for [^{11}C]**6**, [^{11}C]**7**, [^{11}C]**8**, [^{18}F]**10**, [^{18}F]**11**, and [^{11}C]**12**,[84,85,87] and may be due to interference from anesthesia.[135–139] Radioactivity uptake in the skull and sphenoid region during studies with [^{18}F]**28**, [^{18}F]**28-d_8**, and [^{18}F]**29** was attributed to a combination of [^{18}F]fluoride generated by metabolic defluorination of the radiotracer and binding of intact radiotracer to CRF_1 receptors expressed on mast cells in the bone marrow. Uptake of [^{18}F]**26** and [^{18}F]**27** in the skull and sphenoid region was rapid but then steadily washed out which suggests that this behavior was the result of binding to CRF_1 receptors expressed on mast cells in the bone marrow with no contribution from [^{18}F]fluoride. This indicates that the [^{18}F]fluoroethoxy ethyl group is more stable to defluorination than the [^{18}F]fluorobutyl group and is a suitable substitute for the [^{18}F]fluorobutyl group.

4. Experimental section

4.1. General

NMR spectra were obtained on a Varian Mercury spectrometer at the specified frequencies. ^{1}H chemical shifts are referenced to internal TMS or residual $CHCl_3$ (7.26 ppm), and ^{13}C chemical shifts are referenced to $CDCl_3$ (77.23 ppm). For ^{1}H NMR spectroscopy ~4–6 mg of sample was dissolved in ~1 mL $CDCl_3$, whereas for ^{13}C NMR spectroscopy ~30–40 mg of sample was dissolved in ~1 mL $CDCl_3$. Solvents were from EMD and were used as received. 4-Ethylamino-1-butanol was purchased from TCI. Ethyl ammonium chloride was purchased from Alfa Aesar. 1,4-Dibromobutane-d_8 was purchased from CDN Isotopes, Inc. Diethyleneglycol *p*-toluenesulfonate, 1,4-dibromobutane, 1-bromo-4-fluorobutane, primary amines, dialkylamines, *N*,*N*-diisopropylethylamine, 2-fluoroethanol, 3-fluoropropanol, tosyl chloride, NaH (60% dispersion in mineral oil), and anhydrous DMA (100-mL septum-capped bottle) were purchased from Aldrich. 2-Fluoroethyl tosylate and 3-fluoropropyl tosylate were prepared from the corresponding alcohols. NaH was not rinsed with hexane prior to use. Dry silica gel purifications were performed by placing silica gel (Whatman Purasil 60 Å, 230–400 mesh) in a medium-fritted filter tube (~31 cm total height, 24/40 ground glass joints, 16 cm h (frit to joint bottom) × 4 cm i.d.) or in a 60-mL medium-fritted filter funnel (43 mm h × 43 mm i.d.) and eluting under vacuum using a vacuum-takeoff adapter (24/40) and 125-mL flat-bottomed boiling flasks (24/40) for fraction collection. The same method was employed for vacuum flash chromatography except that the silica was pretreated with hexane (100 mL). Radial chromatography was performed with a Harrison Research Chromatotron using Analtech rotors (Silica Gel GF). Preparative-TLC was performed on Analtech Uniplate Silica Gel GF 20 × 20 cm, 2000 micron plates (catalog #02015). HRMS was performed by the Emory University Mass Spectrometry Center. Purity of target compounds was determined by elemental analysis (www.atlanticmicrolab.com). HPLC was performed with Waters XTerra (Prep-RP_{18}, 5 mm, 19 × 100 mm) and Waters NovaPak (C_{18}, 4 mm, 3.9 × 150 mm) columns. Radiochemistry development, log*P* determination, and rodent microPET imaging were performed at the Emory University Center for Systems Imaging (CSI) which houses a Siemens RDS 111 cyclotron and a Siemens Inveon MicroPET/CT. Non-human primate microPET imaging was performed at Yerkes National Primate Research Center which houses a PETNET facility (the source of $H^{18}F_{(aq)}$) and a Siemens MicroPET Focus 220. One-step ^{18}F-radiolabelings were performed in a Siemens/CTI Chemical Processing Control Unit (CPCU).

4.2. 3-(2,4-Dichlorophenyl)-2,5-dimethylpyrazolo[1,5-*a*]pyrimidin-7-ol (14)

Compound **13** (3.90 g, 16.11 mmol) and ethyl acetoacetate (100 mL) were stirred at reflux under $Ar_{(g)}$ for 20 h, then cooled to 0 °C, filtered, rinsed with cold EtOAc (25 mL × 2), hexane (25 mL), and dried under vacuum to afford **14** (3.51 g, 71%) as an off-white powder (insoluble in $CHCl_3$, CH_3OH, DMSO, acetone, H_2O).

4.3. 7-Chloro-3-(2,4-dichlorophenyl)-2,5-dimethylpyrazolo[1,5-*a*]pyrimidine (15)

Compound **14** (1.35 g, 4.38 mmol) was suspended in 1,4-dioxane (30 mL) followed by addition of $POCl_3$ (2.1 mL, 22.5 mmol, 5.1 equiv). The reaction mixture was stirred at reflux under $Ar_{(g)}$ for 3 h, then cooled, and the solvent was removed azeotropically with heptane to give an orange/red syrup that was dissolved in CH_2Cl_2 (50 mL) and poured into ice-water (100 mL). The aqueous layer was basified to pH 8–9 with conc. $NH_4OH_{(aq)}$, the layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (20 mL × 2). The combined CH_2Cl_2 layers were dried over $MgSO_4$ and the solvent was removed to give an orange/red syrup that was redissolved in CH_2Cl_2 (~10 mL), poured onto dry silica (14 cm h × 4 cm i.d.), and eluted under vacuum: hexane (100 mL), %CH_2Cl_2/hexane—25% (100 mL), 50% (100 mL), 75% (100 mL), CH_2Cl_2 (400 mL) to afford **15** (1.22 g, 85%) as a white solid: ^{1}H NMR (300 MHz, $CDCl_3$) d 7.55 (s, 1H), 7.34 (apparent s—overlapping resonances, 2H), 6.83 (s, 1H), 2.56 (s, 3H), 2.45 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) d 159.15, 154.54, 147.56, 137.77, 135.82, 134.47, 133.96, 129.93, 129.42, 127.36, 108.81, 107.66, 24.93, 13.93.

4.4. 3-(2,4-Dichlorophenyl)-*N*,2,5-trimethylpyrazolo[1,5-*a*]pyrimidin-7-amine (16)

Compound **15** (293 mg, 8.97×10^{-4} mol), $MeNH_2$·HCl (253 mg, 3.75 mmol, 4.2 equiv), *i*Pr_2NEt (2 mL, 11.5 mmol, 12.8 equiv), and CH_3CN (20 mL) were stirred at reflux under $Ar_{(g)}$ for 150 min. The solvent was removed to give an oil and then hexane was added and removed to give an off-white solid that was dried under vacuum. The solid was dissolved in CH_2Cl_2, poured onto dry silica (15 cm h × 4 cm i.d.), and eluted under vacuum: hexanes/EtOAc/NEt_3 v/v/v 95:4:1 (100 mL), 90:8:2 (100 mL), 75:20:5 (400 mL), 50:45:5 (100 mL), 20:75:5 (100 mL) to afford a white foam (295 mg). Purification by radial chromatography (4 mm silica): hexanes/EtOAc/NEt_3 v/v/v 98:1:1 (100 mL), 95:4:1 (100 mL), 90:8:2 (200 mL), 75:20:5 (125 mL) afforded **16** (285 mg, 99%) as a white foam: TLC R_f = 0.23 (silica, 75:20:5 v/v/v hexanes/EtOAc/NEt_3); ^{1}H NMR (300 MHz, $CDCl_3$) d 7.51 (d, 1H, J = 2.1 Hz), 7.37 (d, 1H, J = 8.1 Hz), 7.30 (dd, 1H, J = 2.1 Hz, J = 8.1 Hz), 6.19 (br d (unresolved q), 1H, J = 5.1 Hz), 5.81 (s, 1H), 3.10 (d, 3H, J = 5.1 Hz), 2.48 (s, 3H), 2.35 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) d 160.22, 152.27, 146.95, 146.83, 135.77, 134.26, 133.62, 130.57, 129.74, 127.21, 104.96, 85.47, 28.60, 25.55, 13.86; HRMS (APCI) $[MH]^+$ Calcd for $C_{15}H_{15}Cl_2N_4$: 321.0668, found: 321.0670; HPLC: Waters XTerra t_R = 8.9 min (70:30:0.1 v/v/v MeOH/H_2O/NEt_3, 9 mL/min), t_R = 21.4 min (60:40:0.1 v/v/v MeOH/H_2O/NEt_3, 9 mL/min).

4.5. 3-(2,4-Dichlorophenyl)-*N*-ethyl-2,5-dimethylpyrazolo[1,5-*a*]pyrimidin-7-amine (17)

Compound **15** (415 mg, 1.27 mmol), $EtNH_3Cl$ (430 mg, 5.27 mmol, 4.1 equiv), *i*Pr_2NEt (3 mL, 17.2 mmol, 13.5 equiv) and CH_3CN (25 mL) were stirred at reflux under $Ar_{(g)}$ for 140 min, cooled, and the solvent was removed to give a faint brown syrup that was dried under vacuum to give a tan solid. The solid was dissolved in CH_2Cl_2, poured onto dry silica (14 cm h × 4 cm i.d.), and eluted under vacuum: hexane/EtOAc/NEt_3 v/v/v 95:4:1 (100 mL), 90:8:2 (100 mL), 75:20:5 (400 mL) to give an off-white foam (451 mg). Purification by radial chromatography (4 mm silica): hexane/EtOAc/NEt_3 v/v/v 98:1:1 (100 mL), 95:4:1 (200 mL), 90:8:2 (200 mL), 75:20:5 (125 mL) afforded a white foam (407 mg) that was further purified by radial chromatography (4 mm silica): $CHCl_3$ (200 mL) to give **17** (396 mg, 93%) as a white foam: ^{1}H NMR (300 MHz, $CDCl_3$) d 7.51 (d, 1H, J = 2.1 Hz), 7.37 (d, 1H, J = 8.1 Hz), 7.30 (dd, 1H, J = 2.1 Hz, J = 8.1 Hz), 6.13 (t, 1H, J = 5.7 Hz), 5.82 (s, 1H), 3.44 (dq, 2H, J = 5.7 Hz, J = 7.2 Hz), 2.47 (s, 3H), 2.35 (s, 3H), 1.42 (t, 3H, J = 7.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) d 160.14, 152.22, 147.01, 145.86, 135.77, 134.26, 133.60, 130.59, 129.74, 127.21, 104.96, 85.63, 37.02, 25.56, 14.60, 13.85; HRMS (APCI) $[MH]^+$ Calcd for $C_{16}H_{17}Cl_2N_4$: 335.0825, found: 335.0826; HPLC: Waters Xterra t_R = 11.1 min (70:30:0.1 v/v/v MeOH/H_2O/NEt_3, 9 mL/min).

4.6. 3-(2,4-Dichlorophenyl)-*N*-(2-fluoroethyl)-2,5-dimethylpyrazolo[1,5-*a*]pyrimidin-7-amine (18)

Compound **15** (271 mg, 8.30×10^{-4} mol), 2-fluoroethylamine·HCl (357 mg, 3.59 mmol, 4.3 equiv), *i*-Pr_2NEt (1.3 mL, 7.5 mmol, 9.0 equiv), and CH_3CN (25 mL) were stirred at reflux under $Ar_{(g)}$ for 7 h, and then the solvent was removed to give a light yellow oil. Hexane was added and removed to give a syrup that was dried under vacuum to give a light yellow solid. The solid was dissolved in CH_2Cl_2, poured onto dry silica (15 cm h × 4 cm i.d.), and eluted under vacuum: hexane (50 mL), %CH_2Cl_2/hexane—25% (100 mL), 50% (100 mL), 75% (100 mL), CH_2Cl_2 (800 mL), hexane/EtOAc/NEt_3 v/v/v 75:20:5 (200 mL), 50:45:5 (200 mL), 20:75:5 (100 mL) to give **15** (24 mg, 9% recovery) and **18** as an off-white solid (284 mg). Purification by radial chromatography (2 mm silica): hexane/EtOAc/NEt_3 v/v/v 98:1:1 (100 mL), 95:4:1 (100 mL), 90:8:2 (200 mL), 75:20:5 (100 mL), 50:45:5 (50 mL) afforded **18** (257 mg, 88%) as a white foam: ^{1}H NMR (300 MHz, $CDCl_3$) d 7.51 (d, 1H, J = 2.1 Hz), 7.36 (d, 1H, J = 8.1 Hz), 7.31 (dd, 1H, J = 8.1 Hz, J = 2.1 Hz), 6.47 (t, 1H, J = 6.0 Hz), 5.85 (s, 1H), 4.72 (dt, 2H, $^2J_{HF}$ = 46.8 Hz, J = 5.1 Hz), 3.73 (ddt, 2H, $^3J_{HF}$ = 25.7 Hz, J = 6.0 Hz, J = 5.1 Hz), 2.47 (s, 3H), 2.36 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) d 160.21, 152.52, 146.96, 145.84, 135.80, 134.25, 133.73, 130.45, 129.79, 127.26, 105.26, 85.70, 81.83 (d, $^1J_{CF}$ = 169.9 Hz), 42.55 (d, $^2J_{CF}$ = 21.0 Hz), 25.62, 13.87; HRMS (APCI) $[MH]^+$ Calcd for $C_{16}H_{16}Cl_2FN_4$: 353.0731, found: 353.0732.

4.7. 3-(2,4-Dichlorophenyl)-2,5-dimethyl-*N*-propylpyrazolo[1,5-*a*]pyrimidin-7-amine (19)

Compound **15** (207 mg, 6.34×10^{-4} mol), $PrNH_3Cl$ (255 mg, 2.67 mmol, 4.2 equiv), *i*Pr_2NEt (1.8 mL, 10.3 mmol, 16.2 equiv), and CH_3CN (10 mL) were stirred at reflux under $Ar_{(g)}$ for 135 min, then cooled, and the solvent was removed to give an off-white solid. The solid was dissolved in CH_2Cl_2, poured onto dry silica (8 cm h × 4 cm i.d.), and eluted under vacuum: hexane (50 mL), hexane/EtOAc/NEt_3 v/v/v 95:4:1 (50 mL), 90:8:2 (100 mL), 75:20:5 (300 mL) to afford a light yellow solid (223 mg). Purification by radial chromatography (2 mm silica): hexane/EtOAc/NEt_3 v/v/v 98:1:1 (100 mL), 95:4:1 (150 mL), 90:8:2 (100 mL) afforded **19** (213 mg, 96%) as a white solid: ^{1}H NMR (300 MHz, $CDCl_3$) d 7.51 (d, 1H, J = 2.1 Hz), 7.37 (d, 1H, J = 8.1 Hz), 7.30 (dd, 1H, J = 2.1 Hz, J = 8.1 Hz), 6.20 (t, 1H, J = 5.7 Hz), 5.82 (s, 1H), 3.36 (partially resolved dq, 2H, J = 7.2 Hz), 2.46 (s, 3H), 2.35 (s, 3H), 1.80 (sextet, 2H, J = 7.2 Hz), 1.08 (t, 3H,

J = 7.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) d 160.13, 152.23, 147.03, 146.05, 135.79, 134.27, 133.61, 130.62, 129.76, 127.23, 104.98, 85.64, 44.01, 25.58, 22.59, 13.86, 11.69; HRMS (APCI) $[MH]^+$ Calcd for $C_{17}H_{19}N_4Cl_2$: 349.0981, found: 349.0983.

4.8. *N*-Butyl-3-(2,4-dichlorophenyl)-2,5-dimethylpyrazolo[1,5-*a*]pyrimidin-7-amine (20)

Compound **15** (244 mg, 7.47×10^{-4} mol), $BuNH_2$ (0.30 mL, 3.0 mmol, 4.0 equiv), iPr$_2$NEt (0.30 mL, 1.7 mmol, 2.3 equiv), and CH_3CN (20 mL) were stirred at reflux under $Ar_{(g)}$ for 1 h, cooled, and the solvent was removed to give a yellow residue that was dried under vacuum. The solid was dissolved in CH_2Cl_2, poured onto dry silica (43 mm h × 43 mm i.d.), and eluted under vacuum: CH_2Cl_2 (650 mL) to afford **20** (247 mg, 91%) as a white foam: ^{1}H NMR (300 MHz, $CDCl_3$) d 7.51 (d, 1H, J = 2.1 Hz), 7.36 (d, 1H, J = 8.1 Hz), 7.30 (dd, 1H, J = 2.1 Hz, J = 8.1 Hz), 6.17 (t, 1H, J = 5.4 Hz), 5.81 (s, 1H), 3.39 (dt, 2H, J = 5.4 Hz, J = 7.2 Hz), 2.46 (s, 3H), 2.35 (s, 3H), 1.76 (m, 2H), 1.50 (m, 2H), 1.01 (t, 3H, J = 7.5 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) d 160.11, 152.18, 147.00, 146.02, 135.76, 134.26, 133.58, 130.60, 129.72, 127.20, 104.94, 85.61, 41.96, 31.25, 25.55, 20.27, 13.90, 13.85; HRMS (APCI) $[MH]^+$ Calcd for $C_{18}H_{21}N_4Cl_2$: 363.1138, found: 363.1140.

4.9. 3-(2,4-Dichlorophenyl)-2,5-dimethyl-*N*,*N*-dipropylpyrazolo[1,5-*a*]pyrimidin-7-amine (21)

Compound **15** (155 mg, 4.75×10^{-4} mol), Pr_2NH (0.30 mL, 2.2 mmol, 4.6 equiv), iPr$_2$NEt (0.23 mL, 1.3 mmol, 2.7 equiv), and CH_3CN (8 mL) were stirred at reflux under $Ar_{(g)}$ for 3.5 h, then cooled, and the solvent was removed to give a residue that was dried under vacuum briefly. The residue was dissolved in CH_2Cl_2, poured onto dry silica (43 mm h × 43 mm i.d.), and eluted under vacuum: hexane (50 mL), hexane/EtOAc/NEt_3 v/v/v 95:4:1 (100 mL), 90:8:2 (100 mL), 75:20:5 (200 mL) to give a yellow oil (170 mg). Purification by radial chromatography (2 mm silica): hexane/EtOAc/NEt_3 v/v/v 98:1:1 (250 mL) gave a yellow oil (149 mg) that was repurified by radial chromatography (1 mm silica): hexane/EtOAc/NEt_3 v/v/v 98:1:1 (150 mL) to afford **21** (129 mg, 69%) as a white solid: ^{1}H NMR (300 MHz, $CDCl_3$) d 7.50 (d, 1H, J = 2.1 Hz), 7.36 (d, 1H, J = 8.1 Hz), 7.29 (dd, 1H, J = 2.1 Hz, J = 8.1 Hz), 5.80 (s, 1H), 3.71 (t, 4H, J = 7.5 Hz), 2.42 (s, 3H), 2.34 (s, 3H), 1.74 (sextet, 4H, J = 7.5 Hz), 0.96 (t, 6 H, J = 7.5 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) d 159.12, 151.57, 149.61, 148.78, 135.72, 134.32, 133.38, 131.06, 129.73, 127.17, 140.00, 92.05, 53.20, 25.29, 21.31, 14.13, 11.53; HRMS (APCI) $[MH]^+$ Calcd for $C_{20}H_{25}Cl_2N_4$: 391.1451, found: 391.1456; Anal. Calcd for $C_{20}H_{24}Cl_2N_4$: C, 61.38; H, 6.18; N, 14.32; found: C, 61.65; H, 6.31; N, 14.16.

4.10. *N*-Butyl-3-(2,4-dichlorophenyl)-*N*-ethyl-2,5-dimethylpyrazolo[1,5-*a*]pyrimidin-7-amine (22)

Compound **15** (105 mg, 3.21×10^{-4} mol), BuNHEt (0.20 mL, 1.5 mmol, 4.7 equiv), iPr$_2$NEt (0.15 mL, 8.6×10^{-4} mol, 2.7 equiv), and CH_3CN (5 mL) were stirred at reflux under $Ar_{(g)}$ for 2.5 h, cooled, and the solvent was removed to give a residue that was dried under vacuum. The residue was dissolved in CH_2Cl_2, poured onto dry silica (43 mm h × 43 mm i.d.), and eluted under vacuum: hexane (50 mL), hexane/EtOAc/NEt_3 v/v/v 95:4:1 (50 mL), 90:8:2 (100 mL), 75:20:5 (200 mL) to give an off-white solid (96 mg). Purification by radial chromatography (1 mm silica): hexane/EtOAc/NEt_3 v/v/v 98:1:1 (150 mL) afforded **22** (90 mg, 72%) as a colorless syrup that became an off-white solid when stored in a freezer: ^{1}H NMR (300 MHz, $CDCl_3$) d 7.50 (d, 1H, J = 2.1 Hz), 7.36 (d, 1H, J = 8.1 Hz), 7.29 (dd, 1H, J = 2.1 Hz, J = 8.1 Hz), 5.83 (s, 1H), 3.81 (q, 2H, J = 6.9 Hz), 3.74 (t, 2H, J = 7.8 Hz), 2.43 (s, 3H), 2.34 (s, 3H), 1.70 (pentet, 2H, J = 7.8 Hz), 1.39 (sextet, 2H, J = 7.5 Hz), 1.29 (t, 3H, J = 6.9 Hz), 0.97 (t, 3H, J = 7.5 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) d 159.16, 151.65, 149.53, 148.73, 135.75, 134.32, 133.42, 131.02, 129.74, 127.17, 104.11, 92.32, 50.43, 45.74, 30.03, 25.28, 20.40, 14.08, 13.10; HRMS (APCI) $[MH]^+$ Calcd for $C_{20}H_{25}Cl_2N_4$: 391.1451, found: 391.1459; Anal. Calcd for $C_{20}H_{24}Cl_2N_4$: C, 61.38; H, 6.18; N, 14.32; found: C, 61.63; H, 6.19; N, 14.28; HPLC: Waters Xterra t_R = 33.8 min (70:30:0.1 v/v/v MeOH/H_2O/NEt_3, 9 mL/min), t_R = 16.0 min (75:25:0.1 v/v/v MeOH/H_2O/NEt_3, 9 mL/min), Waters NovaPak t_R = 23.5 min (75:25:0.1 v/v/v MeOH/H_2O/NEt_3, 1.1 mL/min).

4.11. 4-((3-(2,4-Dichlorophenyl)-2,5-dimethylpyrazolo[1,5-*a*]-pyrimidin-7-yl)(ethyl)amino)butan-1-ol (23)

Compound **15** (106 mg, 3.25×10^{-4} mol), EtNHBuOH (0.15 mL, 1.16 mmol, 3.6 equiv), iPr$_2$NEt (0.15 mL, 8.6×10^{-4} mol, 2.6 equiv), and CH_3CN (10 mL) were stirred at reflux under $Ar_{(g)}$ for 2.5 h, cooled, and the solvent was removed to give a faint yellow syrup. The syrup was dissolved in CH_2Cl_2, poured onto dry silica (43 mm h × 43 mm i.d.), and eluted under vacuum: hexane (50 mL), hexane/EtOAc/NEt_3 v/v/v 90:8:2 (50 mL), 75:20:5 (100 mL), 50:45:5 (100 mL), 20:75:5 (150 mL) to give a sticky, yellow residue (134 mg). Purification by radial chromatography (2 mm silica): hexane/EtOAc/NEt_3 v/v/v 90:8:2 (100 mL), 75:20:5 (100 mL), 50:45:5 (100 mL), 20:75:5 (50 mL) gave an off-white foam (123 mg) that was further purified by radial chromatography (1 mm silica): 1% MeOH/$CHCl_3$ (100 mL) to afford **23** (118 mg, 89%) as a white foam: ^{1}H NMR (300 MHz, $CDCl_3$) d 7.50 (d, 1H, J = 2.1 Hz), 7.35 (d, 1H, J = 8.4 Hz), 7.29 (dd, 1H, J = 2.1, J = 8.4 Hz), 5.84 (s, 1H), 3.87 (br s, 2H), 3.73 (q, 2H, J = 6.0 Hz), 3.72 (q, 2H, J = 7.2 Hz), 2.43 (s, 3H), 2.35 (s, 3H), 2.01 (t, 1H, J = 6.0 Hz), 1.88 (pentet, 2H, J = 7.2 Hz), 1.65 (pentet, 2H, J = 7.2 Hz), 1.30 (t, 3H, J = 7.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) d 159.36, 151.90, 149.60, 148.55, 135.76, 134.30, 133.50, 130.82, 129.70, 127.18, 104.24, 92.13, 62.15, 50.05, 45.73, 29.62, 25.17, 24.68, 13.94, 12.72; HRMS (APCI) $[MH]^+$ Calcd for $C_{20}H_{25}Cl_2N_4O$: 407.1400, found: 407.1404.

4.12. 2-((3-(2,4-Dichlorophenyl)-2,5-dimethylpyrazolo[1,5-*a*]-pyrimidin-7-yl)(methyl)amino)ethanol (24)

Compound **15** (144 mg, 4.41×10^{-4} mol), CH_3CN (15 mL), iPr$_2$NEt (0.20 mL, 1.1 mmol, 2.5 equiv), and MeNHEtOH (0.14 mL, 1.8 mmol, 4.1 equiv) were stirred at reflux under $Ar_{(g)}$ for 3 h, then cooled, and the solvent was removed to give a yellow syrup. Hexane was added and removed to give an off-white solid that was dried under vacuum. The solid was dissolved in CH_2Cl_2, poured onto dry silica (14 cm h × 4 cm i.d.), and eluted under vacuum: hexane/EtOAc/NEt_3 v/v/v 90:8:2 (100 mL), 75:20:5 (100 mL), 50:45:5 (100 mL), 20:75:5 (400 mL) to afford **24** (152 mg, 94%) as an off-white solid: ^{1}H NMR (300 MHz, $CDCl_3$) d 7.52 (d, 1H, J = 1.8 Hz), 7.34 (d, 1H, J = 8.1 Hz), 7.31 (dd, 1H, J = 1.8 Hz, J = 8.1 Hz), 6.23 (t, 1H, J = 3.6 Hz), 5.99 (s, 1H), 4.02 (partially resolved dt, 2H, J = 4.5 Hz), 3.95 (br d, 2H), 3.10 (s, 3H), 2.47 (s, 3H), 2.35 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) d 160.49, 151.69, 149.83, 148.85, 135.73, 134.20, 133.87, 130.11, 129.81, 127.26, 104.92, 93.00, 59.76, 53.46, 37.89, 25.28, 13.74; HRMS (APCI) $[MH]^+$ Calcd for $C_{17}H_{19}Cl_2N_4O$: 365.0930, found: 365.0931.

4.13. 2-((3-(2,4-Dichlorophenyl)-2,5-dimethylpyrazolo[1,5-*a*]-pyrimidin-7-yl)(ethyl)amino)ethanol (25)

Compound **15** (143 mg, 4.38×10^{-4} mol), CH_3CN (15 mL), iPr$_2$NEt (0.20 mL, 1.1 mmol, 2.5 equiv), and EtNHEtOH (0.15 mL,

1.5 mmol, 3.4 equiv) were stirred at reflux under $Ar_{(g)}$ for 3.5 h, then cooled, and the solvent was removed to give a faint yellow liquid. Hexane was added and removed to give a faint yellow syrup that was dried under vacuum to give an off-white solid. The solid was dissolved in CH_2Cl_2, poured onto dry silica (14 cm h × 4 cm i.d.), and eluted under vacuum: hexane/EtOAc/NEt_3 v/v/v 90:8:2 (50 mL), 75:20:5 (50 mL), 50:45:5 (100 mL), 20:75:5 (200 mL) to afford **25** (162 mg, 98%) as a white foam: ^{1}H NMR (300 MHz, $CDCl_3$) d 7.51 (d, 1H, J = 1.5 Hz), 7.34 (d, 1H, J = 8.1 Hz), 7.30 (dd, 1H, J = 1.5 Hz, J = 8.1 Hz), 6.15 (t, 1H, J = 3.9 Hz), 5.99 (s, 1H), 4.08 (br d, 2H), 3.98 (partially resolved dt, 2H, J = 4.8 Hz), 3.56 (q, 2H, J = 7.2 Hz), 2.46 (s, 3H), 2.34 (s, 3H), 1.36 (t, 3H, J = 7.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) d 160.18, 151.53, 149.36, 148.55, 135.71, 134.22, 133.79, 130.23, 129.78, 127.25, 104.68, 92.74, 59.72, 50.92, 44.58, 25.29, 13.75, 11.84; HRMS (APCI) $[MH]^+$ Calcd for $C_{18}H_{21}Cl_2N_4O$: 379.1087, found: 379.1089.

4.14. 3-(2,4-Dichlorophenyl)-*N*-(2-(2-fluoroethoxy)ethyl)-*N*,2,5-trimethylpyrazolo[1,5-*a*]pyrimidin-7-amine (26)

Compound **24** (126 mg, 3.45×10^{-4} mol) was dissolved in DMA (2 mL) under $Ar_{(g)}$ and cooled to 0 °C. NaH (60%, 40 mg, 1.00 mmol, 2.9 equiv) was added, the mixture was stirred at 0 °C under $Ar_{(g)}$ for 30 min, and then a DMA (0.5 mL) solution of 2-fluoroethyl tosylate (207 mg, 9.48×10^{-4} mol, 2.7 equiv) was added. The reaction mixture was warmed to ambient temperature, stirred for 16 h, then diluted with CH_2Cl_2 (1 mL) and hexane (3 mL), poured onto dry silica (43 mm h × 43 mm i.d.), and eluted under vacuum: hexane (25 mL), %CH_2Cl_2/hexane—25% (50 mL), 50% (50 mL), 75% (50 mL), CH_2Cl_2 (150 mL). The desired fractions were combined, concentrated, dissolved in EtOAc (25 mL), washed with H_2O (10 mL × 3), sat. $NaCl_{(aq)}$ (10 mL), and dried over $MgSO_4$. The solvent was removed to give a yellow syrup that was dissolved in CH_2Cl_2, poured onto dry silica (43 mm h × 43 mm i.d.), and eluted under vacuum: %CH_2Cl_2/hexane—50% (50 mL), 75% (50 mL), CH_2Cl_2 (100 mL), hexane/EtOAc/NEt_3 v/v/v 75:20:5 (100 mL), 50:45:5 (100 mL), 20:75:5 (100 mL) to give a yellow syrup (131 mg). Purification by radial chromatography (2 mm silica): hexane/EtOAc/NEt_3 v/v/v 98:1:1 (100 mL), 95:4:1 (100 mL), 90:8:2 (100 mL), 75:20:5 (175 mL) gave a colorless syrup (110 mg) that was further purified by radial chromatography (2 mm silica): %CH_2Cl_2/hexane—5% (100 mL), 10% (100 mL), 15% (100 mL), 25% (100 mL), 35% (100 mL), 50% (100 mL), 75% (100 mL), 85% (100 mL), CH_2Cl_2 (300 mL), %MeOH/CH_2Cl_2—1% (100 mL), 2% (50 mL) to afford **26** (64 mg, 45%) as a white solid: ^{1}H NMR (300 MHz, $CDCl_3$) d 7.51 (d, 1H, J = 2.1 Hz), 7.36 (d, 1H, J = 8.4 Hz), 7.30 (dd, 1H, J = 8.4 Hz, J = 2.1 Hz), 5.90 (s, 1H), 4.46 (apparent dt, 2H, $^2J_{HF}$ = 47.7 Hz, $^3J_{HH}$ = 4.2 Hz), 4.29 (partially resolved t, 2H), 3.85 (t, 2H, J = 5.1 Hz), 3.66 (apparent dt, 2H, $^3J_{HF}$ = 30.0 Hz, $^3J_{HH}$ = 4.2 Hz), 2.45 (s, 3H), 2.34 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) d 159.63, 151.82, 149.67, 149.13, 135.73, 134.27, 133.54, 130.78, 129.74, 127.19, 104.38, 92.60, 83.18 (d, $^1J_{CF}$ = 168.2 Hz), 70.74, 70.26 (d, $^2J_{CF}$ = 19.7 Hz), 52.08, 40.33, 25.26, 14.04; HRMS (NSI) $[MH]^+$ Calcd for $C_{19}H_{22}Cl_2FN_4O$: 411.1149, found: 411.1156; Anal. Calcd for $C_{19}H_{21}Cl_2FN_4O$: C, 55.48; H, 5.15; N, 13.62; found: C, 55.51; H, 5.21; N, 13.70; HPLC: Waters XTerra t_R = 14.75 min (50:50:0.1 v/v/v EtOH/H_2O/NEt_3, 9 mL/min), t_R = 19.1 min (50:50:0.1 v/v/v EtOH/H_2O/NEt_3, 7 mL/min).

4.15. 3-(2,4-Dichlorophenyl)-*N*-ethyl-*N*-(2-(2-fluoroethoxy)-ethyl)-2,5-dimethylpyrazolo[1,5-*a*]pyrimidin-7-amine (27)

Compound **25** (158 mg, 4.17×10^{-4} mol) was flushed with $Ar_{(g)}$, then dissolved in DMA (1.5 mL) and cooled to 0 °C. NaH (60%, 42 mg, 1.05 mmol, 2.5 equiv) was added, the mixture was stirred at 0 °C under $Ar_{(g)}$ for 30 min, and then a DMA (0.5 mL) solution of 2-fluoroethyl tosylate (248 mg, 1.14 mmol, 2.7 equiv) was added. The reaction mixture was warmed to ambient temperature, stirred for 16 h, diluted with CH_2Cl_2 (1 mL) and hexane (3 mL), poured onto dry silica (43 mm h × 43 mm i.d.), and eluted under vacuum: hexane (25 mL), %CH_2Cl_2/hexane—25% (50 mL), 50% (50 mL), 75% (50 mL), CH_2Cl_2 (200 mL). The desired fractions were combined, concentrated, dissolved in EtOAc (25 mL), washed with H_2O (10 mL × 3), sat. $NaCl_{(aq)}$ (10 mL), and dried over $MgSO_4$. The solvent was removed to give a light yellow syrup that was dissolved in CH_2Cl_2, poured onto dry silica (43 mm h × 43 mm i.d.), and eluted under vacuum: hexane (50 mL), %CH_2Cl_2/hexane—25% (50 mL), 50% (50 mL), 75% (100 mL), CH_2Cl_2 (125 mL), hexane/EtOAc/NEt_3 v/v/v 75:20:5 (300 mL), 50:45:5 (50 mL) to give an opaque syrup (164 mg). Purification by radial chromatography (2 mm silica): hexane/EtOAc/NEt_3 v/v/v 98:1:1 (200 mL), 95:4:1 (100 mL), 90:8:2 (100 mL), 75:20:5 (100 mL) gave a colorless syrup (144 mg) that was further purified by radial chromatography (2 mm silica): %CH_2Cl_2/hexane—5% (100 mL), 10% (100 mL), 15% (100 mL), 25% (100 mL), 35% (100 mL), 50% (100 mL), 75% (200 mL), 85% (100 mL), CH_2Cl_2 (300 mL), %MeOH/CH_2Cl_2—1% (100 mL), 2% (50 mL) to afford **27** (89 mg, 50%) as a crystalline, white solid: ^{1}H NMR (300 MHz, $CDCl_3$) d 7.50 (d, 1H, J = 2.1 Hz), 7.35 (d, 1H, J = 8.4 Hz), 7.29 (dd, 1H, J = 8.4 Hz, J = 2.1 Hz), 5.91 (s, 1H) 4.51 (dddd, 2H, $^2J_{HF}$ = 47.4 Hz, $^3J_{HH}$ = 4.2 Hz, J = 1.2 Hz), 4.16 (t, 2H, $^3J_{HH}$ = 5.7 Hz), 3.87 (t, 2H, $^3J_{HH}$ = 5.7 Hz), 3.74 (q, 2H, J = 7.2 Hz), 3.69 (apparent dt, 2H, $^3J_{HF}$ = 30.0 Hz, $^3J_{HH}$ = 4.2 Hz), 2.44 (s, 3H), 2.33 (s, 3H), 1.31 (t, 3H, J = 7.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) d 159.37, 151.68, 149.46, 148.67, 135.70, 134.27, 133.46, 130.87, 129.72, 127.17, 104.18, 92.34, 83.18 (d, $^1J_{CF}$ = 168.2 Hz), 70.89, 70.38 (d, $^2J_{CF}$ = 19.4 Hz), 49.99, 46.77, 25.28, 14.05, 12.57; HRMS (APCI) $[MH]^+$ Calcd for $C_{20}H_{24}Cl_2FN_4O$: 425.1306, found: 425.1309; Anal. Calcd for $C_{20}H_{23}Cl_2FN_4O$: C, 56.48; H, 5.45; N, 13.17; found: C, 56.36; H, 5.48; N, 13.11; HPLC: Waters XTerra t_R = 12.2 min (55:45:0.1 v/v/v EtOH/H_2O/NEt_3, 9 mL/min), t_R = 20.5 min (50:50:0.1 v/v/v EtOH/H_2O/NEt_3, 9 mL/min).

4.16. 3-(2,4-Dichlorophenyl)-*N*-(4-fluorobutyl)-*N*,2,5-trimethylpyrazolo[1,5-*a*]pyrimidin-7-amine (28)

Compound **16** (105 mg, 3.27×10^{-4} mol) was flushed with $Ar_{(g)}$, then dissolved in DMA (2 mL) and cooled to 0 °C. NaH (60%, 38 mg, 9.50×10^{-4} mol, 2.9 equiv) was added, the mixture was stirred at 0 °C under $Ar_{(g)}$ for 30 min, and then 1-bromo-4-fluorobutane (0.12 mL, 1.12 mmol, 3.4 equiv) was added. The reaction mixture was warmed to ambient temperature, stirred for 16 h, diluted with CH_2Cl_2 (1 mL) and hexane (2 mL), poured onto dry silica (43 mm h × 43 mm i.d.), and eluted under vacuum: hexane (50 mL), %CH_2Cl_2/hexane—25% (50 mL), 50% (50 mL), 75% (50 mL), CH_2Cl_2 (200 mL). The desired fractions were combined, concentrated to a light yellow liquid, dissolved in EtOAc (25 mL), washed with H_2O (10 mL × 3), sat. $NaCl_{(aq)}$ (10 mL), dried over $MgSO_4$, and then the solvent was removed to give a faint yellow syrup that was dried under vacuum (121 mg). Purification by radial chromatography (2 mm silica): hexane/EtOAc/NEt_3 v/v/v 98:1:1 (400 mL), 95:4:1 (100 mL), 90:8:2 (150 mL) gave a colorless syrup (104 mg) that was further purified by radial chromatography (2 mm silica): $CHCl_3$ (150 mL) to give a colorless syrup (100 mg). Purification by flash column chromatography (10 g silica, 90:8:2 v/v/v hexane/EtOAc/NEt_3) gave a colorless syrup (94 mg) that was further purified by radial chromatography (2 mm silica): $CHCl_3$ (125 mL) to give a white solid (89 mg). A final purification by radial chromatography (2 mm silica): %*i*-PrOH/hexane—1% (100 mL), 2% (100 mL), 3% (100 mL) afforded **28** (87 mg, 67%) as a white solid: ^{1}H NMR (300 MHz, $CDCl_3$) d 7.51 (d, 1H, J = 2.1 Hz), 7.36 (d, 1H,

J = 8.4 Hz), 7.30 (dd, 1H, J = 8.4 Hz, J = 2.1 Hz), 5.84 (s, 1H), 4.51 (dt, 2H, $^2J_{HF}$ = 47.1 Hz, J = 6.0 Hz), 4.02 (br unresolved t, 2H), 3.17 (s, 3H), 2.45 (s, 3H), 2.35 (s, 3H), 1.85 (m, 2H), 1.79 (dm, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) d 159.53, 151.86, 149.53, 149.20, 135.74, 134.27, 133.53, 130.81, 129.74, 127.18, 104.41, 92.29, 83.90 (d, $^1J_{CF}$ = 164.0 Hz), 52.27, 38.91, 27.78 (d, $^2J_{CF}$ = 20.0 Hz), 25.25, 23.93, 14.04; HRMS (APCI) $[MH]^+$ Calcd for $C_{19}H_{22}Cl_2FN_4$: 395.1200, found: 395.1203; Anal. Calcd for $C_{19}H_{21}Cl_2FN_4$: C, 57.73; H, 5.35; N, 14.17; found: C, 57.72; H, 5.39; N, 14.13; HPLC: Waters XTerra t_R = 15.0 min (70:30:0.1 v/v/v MeOH/H_2O/NEt_3, 9 mL/min), t_R = 15.7 min (55:45:0.1 v/v/v EtOH/H_2O/NEt_3, 9 mL/min), Waters Nova-Pak t_R = 7.8 min (75:25:0.1 v/v/v MeOH/H_2O/NEt_3, 1.1 mL/min).

4.17. 3-(2,4-Dichlorophenyl)-*N*-ethyl-*N*-(4-fluorobutyl)-2,5-dimethylpyrazolo[1,5-*a*]pyrimidin-7-amine (29)

Compound **17** (113 mg, 3.37×10^{-4} mol) was flushed with $Ar_{(g)}$ for 45 min, then dissolved in DMA (2 mL), and cooled to 0 °C. NaH (60%, 18 mg, 7.50×10^{-4} mol, 2.2 equiv) was added, the mixture was stirred at 0 °C under $Ar_{(g)}$ for 1 h, and then 1-bromo-4-fluorobutane (0.15 mL, 1.4 mmol, 4.2 equiv) was added. The reaction mixture was warmed to ambient temperature, stirred for 20 h, diluted with CH_2Cl_2 (1 mL) and hexane (2 mL), poured onto dry silica (43 mm h × 43 mm i.d.), and eluted under vacuum: hexane (25 mL), %CH_2Cl_2/hexane—25% (50 mL), 50% (50 mL), 75% (100 mL), CH_2Cl_2 (150 mL) to afford a yellow syrup (126 mg). Purification by radial chromatography (2 mm silica): hexane/EtOAc/NEt_3 v/v/v 98:1:1 (200 mL), 95:4:1 (100 mL), 90:8:2 (100 mL), 75:20:5 (100 mL) afforded a colorless syrup (102 mg) that was again purified by radial chromatography (2 mm silica): %*i*-PrOH/hexane—1% (100 mL), 2% (100 mL), 3% (100 mL) to give a colorless syrup (86 mg). Further purification by radial chromatography (2 mm silica): $CHCl_3$ (100 mL) afforded **29** (82 mg, 59%) as a colorless syrup: ^{1}H NMR (300 MHz, $CDCl_3$) d 7.51 (d, 1H, J = 2.1 Hz), 7.36 (d, 1H, J = 8.4 Hz), 7.30 (dd, 1H, J = 2.1 Hz, J = 8.4 Hz), 5.84 (s, 1H), 4.51 (dt, 2H, J = 6.0 Hz, $^2J_{HF}$ = 47.1 Hz), 3.85 (br s, 2H), 3.75 (q, 2H, J = 7.2 Hz), 2.43 (s, 3H), 2.34 (s, 3H), 1.86 (m, 2H), 1.79 (dm, 2H, $^3J_{HF}$ = 32.4 Hz), 1.30 (t, 3H, J = 7.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) d 159.27, 151.74, 149.50, 148.56, 135.73, 134.29, 133.47, 130.92, 129.74, 127.19, 104.23, 92.31, 83.94 (d, $^1J_{CF}$ = 164.2 Hz), 50.04, 45.71, 27.96 (d, $^2J_{CF}$ = 20.0 Hz), 25.30, 24.27 (d, $^3J_{CF}$ = 15.9 Hz), 14.07, 12.84; HRMS (APCI) $[MH]^+$ Calcd for $C_{20}H_{24}Cl_2FN_4$: 409.1357, found: 409.1367; Anal. Calcd for $C_{20}H_{23}Cl_2FN_4$: C, 58.68; H, 5.66; N, 13.69; found: C, 58.38; H, 5.74; N, 13.41; HPLC: Waters Xterra t_R = 19.4 min (70:30:0.1 v/v/v MeOH/H_2O/NEt_3, 9 mL/min), t_R = 20.7 min (55:45:0.1 v/v/v EtOH/H_2O/NEt_3, 9 mL/min).

4.18. 3-(2,4-Dichlorophenyl)-*N*-(4-fluorobutyl)-*N*-(2-fluoroethyl)-2,5-dimethylpyrazolo[1,5-*a*]pyrimidin-7-amine (30)

Compound **18** (42 mg, 1.19×10^{-4} mol) was dissolved in DMA (1 mL) under $Ar_{(g)}$ and cooled to 0 °C. NaH (60%, 13 mg, 3.25×10^{-4} mol, 2.7 equiv) was added, the mixture was stirred at 0 °C under $Ar_{(g)}$ for 1 h, and then 1-bromo-4-fluorobutane (0.1 mL, 9.3×10^{-4} mol, 7.8 equiv) was added. The reaction mixture was warmed to ambient temperature, stirred for 21 h, and then diluted with CH_2Cl_2 (1 mL) and hexane (3 mL). The mixture was poured onto dry silica (33 mm h × 33 mm i.d.) and eluted under vacuum: hexane (25 mL), %CH_2Cl_2/hexane—25% (25 mL), 50% (25 mL), 75% (50 mL), CH_2Cl_2 (50 mL) to give a light yellow syrup (45 mg). Purification by radial chromatography (1 mm silica): hexane/EtOAc/NEt_3 v/v/v 98:1:1 (100 mL), 95:4:1 (100 mL), 90:8:2 (100 mL), 75:20:5 (100 mL) gave **18** (11 mg, 26% recovery) and **30** (29 mg) as a colorless syrup. Further purification by radial chromatography (1 mm silica): $CHCl_3$ (30 mL) afforded **30** (24 mg, 47%) as a colorless syrup: ^{1}H NMR (300 MHz, $CDCl_3$) d 7.51 (d, 1H, J = 1.8 Hz), 7.35 (d, 1H, J = 8.1 Hz), 7.30 (dd, 1H, J = 8.1 Hz, J = 1.8 Hz), 5.92 (s, 1H), 4.79 (dt, 2H, $^2J_{HF}$ = 48.0 Hz, J = 4.5 Hz), 4.51 (dt, 2H, $^2J_{HF}$ = 47.1 Hz, J = 5.7 Hz), 4.28 (d of br s, 2H, $^3J_{HF}$ = 25.2 Hz), 3.72 (t, 2H, J = 7.2 Hz), 2.45 (s, 3H), 2.32 (s, 3H), 1.89 (m, 2H), 1.83 (dm, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) d 159.53, 151.94, 149.40, 148.33, 135.73, 134.26, 133.64, 130.67, 129.78, 127.24, 104.52, 92.70, 84.32 (d, $^1J_{CF}$ = 167.6 Hz), 83.87 (d, $^1J_{CF}$ = 164.2 Hz), 52.12, 51.53 (d, $^2J_{CF}$ = 19.6 Hz), 27.89 (d, $^2J_{CF}$ = 19.9 Hz), 25.34, 23.31 (d, $^3J_{CF}$ = 4.0 Hz), 14.05; HRMS (NSI) $[MH]^+$ Calcd for $C_{20}H_{23}Cl_2F_2N_4$: 427.1262, found: 427.1262; Anal. Calcd for $C_{20}H_{22}Cl_2F_2N_4$: C, 56.21; H, 5.19; N, 13.11; found: C, 56.07; H, 5.29; N, 13.02; HPLC: Waters XTerra t_R = 16.7 min (55:45:0.1 v/v/v EtOH/H_2O/NEt_3, 9 ml/min).

4.19. 4-((3-(2,4-Dichlorophenyl)-2,5-dimethylpyrazolo[1,5-*a*]-pyrimidin-7-yl)(methyl)amino)butyl 4-methylbenzenesulfonate (31)

Compound **16** (28 mg, 8.72×10^{-5} mol) was flushed with $Ar_{(g)}$, then dissolved in DMA (0.5 mL), and cooled to 0 °C. NaH (60%, 13 mg, 3.25×10^{-4} mol, 3.7 equiv) was added, the mixture was stirred at 0 °C under $Ar_{(g)}$ for 25 min, and then a DMA (1 mL) solution of 1,4-ditosyloxybutane (**35**) (174 mg, 4.37×10^{-4} mol, 5.0 equiv) was added. The reaction mixture was warmed to ambient temperature, stirred for 19 h, then diluted with CH_2Cl_2 (1 mL) and hexane (1 mL), poured onto dry silica (33 mm h × 33 mm i.d.), and eluted under vacuum: hexane (10 mL), %CH_2Cl_2/hexane—25% (10 mL), 50% (50 mL), 75% (50 mL), CH_2Cl_2 (25 mL). The desired fractions were combined, concentrated, dissolved in EtOAc (25 mL), washed with H_2O (5 mL × 3), sat. $NaCl_{(aq)}$ (5 mL), and dried over $MgSO_4$. The solvent was removed to give a faint yellow oil that was dried under vacuum briefly to give an opaque residue that was purified by preparative-TLC (CH_2Cl_2) to give a faint yellow residue (27 mg). The residue was purified again by preparative-TLC, and then on Waters silica Sep-Pak Classics (2 in series): load—CH_2Cl_2 (1 mL), elution—hexane (1 mL), hexane/EtOAc/NEt_3 v/v/v 98:1:1 (3 mL), 95:4:1 (2 mL × 6), 90:8:2 (2 mL × 6), 75:20:5 (2 mL × 12) to afford **31** (13 mg, 27%) as a colorless residue: ^{1}H NMR (300 MHz, $CDCl_3$) d 7.78 (d, 2H, J = 8.1 Hz), 7.51 (d, 1H, J = 2.1 Hz), 7.35 (d, 1H, J = 8.4 Hz), 7.33 (d, 2H, J = 8.1 Hz), 7.30 (dd, 1H, J = 8.4 Hz, J = 2.1 Hz), 5.82 (s, 1H), 4.09 (t, 2H, J = 6.0 Hz), 3.95 (br s, 2H), 3.11 (s, 3H), 2.44 (s—overlapping resonances, 3H + 3H), 2.31 (s, 3H), 1.76 (m—overlapping resonances, 2H+2H).

4.20. 4-((3-(2,4-Dichlorophenyl)-2,5-dimethylpyrazolo[1,5-*a*]-pyrimidin-7-yl)(methyl)amino)butyl-1,1,2,2,3,3,4,4-d_8 4-methylbenzenesulfonate (31-d_8)

Compound **16** (50 mg, 1.56×10^{-4} mol) was flushed with $Ar_{(g)}$ for 1 h, then dissolved in DMA (1 mL) and cooled to 0 °C. NaH (60%, 25 mg, 6.25×10^{-4} mol, 4.0 equiv) was added, the mixture was stirred at 0 °C under $Ar_{(g)}$ for 30 min, and then 1,4-ditosyloxybutane-d_8 (**35-d_8**) (159 mg, 3.91×10^{-4} mol, 2.5 equiv) was added. The reaction mixture was warmed to ambient temperature, stirred for 3 h, diluted with CH_2Cl_2 (1 mL) and hexane (1 mL), poured onto dry silica (33 mm h × 33 mm i.d.), and eluted under vacuum: hexane (10 mL), hexane/EtOAc/NEt_3 v/v/v 95:4:1 (25 mL), 90:8:2 (25 mL), 75:20:5 (200 mL). The desired fractions were combined, concentrated to a faint yellow liquid, dissolved in EtOAc (25 mL), washed with H_2O (10 mL × 2), sat. $NaCl_{(aq)}$ (10 mL), and dried over $MgSO_4$. The solvent was removed to give a residue that was dried under vacuum (119 mg) and then purified by radial chromatography (2 mm silica): hexane/EtOAc/NEt_3 v/v/v

98:1:1 (100 mL), 95:4:1 (100 mL), 90:8:2 (100 mL), 75:20:5 (300 mL) afforded **16** (20 mg, 40% recovery) and **31-d_8** (21 mg, colorless residue) which was further purified by preparatory-TLC (75:20:5 v/v/v hexane/EtOAc/NEt_3) to afford **31-d_8** (15 mg, 17%) as an off-white foam: ^{1}H NMR (300 MHz, $CDCl_3$) d 7.78 (d, 2H, J = 8.4 Hz), 7.51 (d, 1H, J = 2.1 Hz), 7.35 (d, 1H, J = 8.4 Hz), 7.33 (d, 2H, J = 8.4 Hz), 7.30 (dd, 1H, J = 8.4 Hz, J = 2.1 Hz), 5.81 (s, 1H), 3.11 (s, 3H), 2.44 (s—overlapping resonances, 3H + 3H), 2.31 (s, 3H); HRMS (NSI) $[MH]^+$ Calcd for $C_{26}H_{21}D_8O_3N_4Cl_2S$: 555.1834, found: 555.1832.

4.21. 4-((3-(2,4-Dichlorophenyl)-2,5-dimethylpyrazolo[1,5-*a*]-pyrimidin-7-yl)(ethyl)amino)butyl 4-methylbenzenesulfonate (32)

Compound **17** (36 mg, 1.07×10^{-4} mol) was flushed with $Ar_{(g)}$, dissolved in DMA (0.5 mL), and cooled to 0 °C. NaH (60%, 14 mg, 3.50×10^{-4} mol, 3.3 equiv) was added, the mixture was stirred at 0 °C under $Ar_{(g)}$ for 25 min, and then a DMA (1 mL) solution of 1,4-ditosyloxybutane (**35**) (214 mg, 5.37×10^{-4} mol, 5.0 equiv) was added. The reaction mixture was warmed to ambient temperature, stirred for 19 h, diluted with CH_2Cl_2 (1 mL) and hexane (1 mL), poured onto dry silica (33 mm h × 33 mm i.d.), and eluted under vacuum: hexane (10 mL), %CH_2Cl_2/hexane—25% (10 mL), 50% (50 mL), 75% (50 mL), CH_2Cl_2 (50 mL). The desired fractions were combined, concentrated, and purified by preparative-TLC (CH_2Cl_2) to give a colorless residue (23 mg). The residue was purified again by preparative-TLC (75:20:5 v/v/v hexane/EtOAc/NEt_3), and then on Waters silica Sep-Pak Classics (2 in series): load—CH_2Cl_2 (1 mL), elution—hexane (2 mL), hexane/EtOAc/NEt_3 v/v/v 98:1:1 (3 mL), 95:4:1 (3 mL × 2), 90:8:2 (3 mL × 4), 75:20:5 (3 mL × 6) to afford **32** (15 mg, 25%) as a white foam that was used without further purification: ^{1}H NMR (300 MHz, $CDCl_3$) d 7.78 (d, 2H, J = 8.1 Hz), 7.50 (d, 1H, J = 2.1 Hz), 7.35 (d, 1H, J = 8.4 Hz), 7.33 (d, 2H, J = 8.1 Hz), 7.29 (dd, 1H, J = 8.4 Hz, J = 2.1 Hz), 5.82 (s, 1H), 4.09 (t, 2H, J = 6.0 Hz), 3.80 (br s, 2H), 3.68 (q, 2H, J = 7.2 Hz), 2.44 (s, 3H), 2.43 (s, 3H), 2.30 (s, 3H), 1.76 (m—overlapping resonances, 2H + 2H), 1.26 (t, 3H, J = 7.2 Hz); HRMS (NSI) $[MH]^+$ Calcd for $C_{27}H_{31}Cl_2N_4O_3S$: 561.1494, found: 561.1523; [MH-*p*TsOH]$^+$ Calcd for $C_{20}H_{23}Cl_2N_4$: 389.1300, found: 389.1297.

4.22. 2-(2-((3-(2,4-Dichlorophenyl)-2,5-dimethylpyrazolo[1,5-*a*]pyrimidin-7-yl)(methyl)amino)ethoxy)ethyl 4-methylbenzenesulfonate (33)

Compound **16** (47 mg, 1.46×10^{-4} mol) was flushed with $Ar_{(g)}$ for 30 min, then dissolved in DMA (0.5 mL) and cooled to 0 °C. NaH (60%, 14 mg, 3.50×10^{-4} mol, 2.4 equiv) was added, the mixture was stirred at 0 °C under $Ar_{(g)}$ for 30 min, and then a DMA (1 mL) solution of diethyleneglycol *p*-toluenesulfonate (212 mg, 5.11×10^{-4} mol, 3.5 equiv) was added. The reaction mixture was warmed to ambient temperature, stirred for 16 h, then diluted with CH_2Cl_2 (1 mL) and hexane (2 mL), poured onto dry silica (33 mm h × 33 mm i.d.), and eluted under vacuum: %CH_2Cl_2/hexane—25% (25 mL), 50% (25 mL), 75% (25 mL), CH_2Cl_2 (75 mL). The desired fractions were combined, concentrated to a faint yellow liquid, dissolved in EtOAc (25 mL), washed with H_2O (10 mL × 3), sat. $NaCl_{(aq)}$ (10 mL), and dried over $MgSO_4$. The solvent was removed to give a faint yellow residue (216 mg) that was purified by radial chromatography (2 mm silica): %CH_2Cl_2/hexane—25% (100 mL), 50% (100 mL), 75% (100 mL), CH_2Cl_2 (200 mL), %MeOH/CH_2Cl_2—1% (100 mL), 2% (100 mL) to give a white foam (45 mg). Further purification by radial chromatography (1 mm silica): $CHCl_3$ (100 mL) afforded **33** (42 mg, 51%) as a white foam: ^{1}H NMR (300 MHz, $CDCl_3$) d 7.77 (d, 2H, J = 8.4 Hz), 7.51 (d, 1H, J = 2.1 Hz), 7.36 (d, 1H, J = 8.4 Hz), 7.31 (d, 2H, J = 8.4 Hz), 7.30 (dd, 1H, J = 8.4 Hz, J = 2.1 Hz), 5.89 (s, 1H), 4.21 (partially resolved t, 2H), 4.08 (t, 2H, J = 4.8 Hz), 3.76 (t, 2H, J = 5.1 Hz), 3.61 (t, 2H, J = 4.8 Hz), 3.16 (s, 3H), 2.45 (s, 3H), 2.43 (s, 3H), 2.32 (s, 3H); HRMS (NSI) $[MH]^+$ Calcd for $C_{26}H_{29}Cl_2N_4O_4S$: 563.1281, found: 563.1277.

4.23. 2-(2-((3-(2,4-Dichlorophenyl)-2,5-dimethylpyrazolo[1,5-*a*]pyrimidin-7-yl)(ethyl)amino)ethoxy)ethyl 4-methylbenzenesulfonate (34)

Compound **17** (55 mg, 1.64×10^{-4} mol) was flushed with $Ar_{(g)}$ for 30 min, then dissolved in DMA (2 mL) and cooled to 0 °C. NaH (60%, 22 mg, 5.50×10^{-4} mol, 3.4 equiv) was added, the mixture was stirred at 0 °C under $Ar_{(g)}$ for 35 min, and then diethyleneglycol *p*-toluenesulfonate (242 mg, 5.84×10^{-4} mol, 3.6 equiv) was added. The reaction mixture was warmed to ambient temperature, stirred for 16 h, then diluted with CH_2Cl_2 (1 mL) and hexane (3 mL), poured onto dry silica (43 mm h × 43 mm i.d.), and eluted under vacuum: hexane (25 mL), %CH_2Cl_2/hexane—25% (50 mL), 50% (50 mL), 75% (50 mL), CH_2Cl_2 (100 mL). The desired fractions were combined, concentrated, dissolved in EtOAc (25 mL), washed with H_2O (10 mL × 3), sat. $NaCl_{(aq)}$ (10 mL), and dried over $MgSO_4$. The solvent was removed to give a colorless syrup that was dried under vacuum to give an off-white solid (284 mg). Purification by radial chromatography (2 mm silica): hexane/EtOAc/NEt_3 v/v/v 98:1:1 (100 mL), 95:4:1 (100 mL), 90:8:2 (100 mL), 75:20:5 (300 mL) gave **17** (30 mg, 55% recovery) and **34** (35 mg) as a sticky, faint yellow residue. Further purification by radial chromatography (1 mm silica): $CHCl_3$ (100 mL) afforded **34** (26 mg, 27%) as a sticky, colorless residue: ^{1}H NMR (300 MHz, $CDCl_3$) d 7.78 (d, 2H, J = 8.4 Hz), 7.50 (d, 1H, J = 2.1 Hz), 7.35 (d, 1H, J = 8.4 Hz), 7.32 (d, 2H, J = 8.4 Hz), 7.29 (dd, 1H, J = 8.4 Hz, J = 2.1 Hz), 5.89 (s, 1H), 4.11 (t, 2H, J = 4.5 Hz), 4.08 (t, 2H, J = 5.4 Hz), 3.77 (t, 2H, J = 5.4 Hz), 3.68 (q, 2H, J = 6.9 Hz), 3.64 (t, 2H, J = 4.5 Hz), 2.43 (s—overlapping resonances, 3H + 3H), 2.31 (s, 3H), 1.28 (t, 3H, J = 6.9 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) d 159.46, 151.70, 149.48, 148.61, 145.09,135.71, 134.29, 133.51, 133.15, 130.86, 130.04, 129.75, 128.11, 127.22, 104.21, 93.29, 71.00, 69.37, 68.70, 49.91, 46.83, 25.30, 21.85, 14.08, 12.54; HRMS (NSI) $[MH]^+$ Calcd for $C_{27}H_{31}Cl_2N_4O_4S$: 577.1438, found: 577.1439.

4.24. 1,4-Ditosyloxybutane (35)

AgOTs (2.51 g, 8.99 mmol, 2.4 equiv) was dissolved in CH_3CN (60 mL) under $Ar_{(g)}$ followed by addition of 1,4-dibromobutane (0.45 mL, 3.77 mmol). The reaction mixture was stirred at reflux under $Ar_{(g)}$ for 17 h, then cooled to 0 °C, and filtered. The precipitate was rinsed with CH_3CN and dried under vacuum to afford a green powder (AgBr, 1.37 g, 81%). The solvent was removed from the filtrate to give a white solid that was dried under vacuum, then suspended in CH_2Cl_2 (25 mL) and filtered. The precipitate was rinsed with CH_2Cl_2 and dried under vacuum to afford a white crystalline solid (AgOTs, 0.39 g, 16% recovery). The solvent was removed from the filtrate to give a colorless syrup that became an off-white solid under vacuum (1.54 g). The solid was dissolved in CH_2Cl_2, poured onto dry silica (8.5 cm h × 4 cm i.d.), and eluted under vacuum: hexane (25 mL), %CH_2Cl_2/hexane—25% (50 mL), 50% (100 mL), 75% (100 mL), CH_2Cl_2 (350 mL) to afford 1-bromo-4-tosyloxy-butane (**36**) (94 mg, 8%) as a colorless syrup (spectroscopic data reported in Section 4.25), and 1,4-ditosyloxy-butane (**35**) (1.37 g, 91%) as a white solid: TLC R_f = 0.58 (silica, CH_2 Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$) d 7.76 (d, 4H, J = 8.1 Hz), 7.35 (d, 4H, J = 8.1 Hz), 3.99 (m, 4 H), 2.46 (s, 6 H), 1.70 (m, 4 H); ^{13}C NMR (75 MHz, $CDCl_3$) d 145.14, 132.98, 130.11, 128.03, 69.58, 25.21, 21.85.

4.25. 1-Bromo-4-tosyloxy-butane (36)

(Obtained as a side product from Section 4.24): TLC R_f = 0.73 (silica, CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$) d 7.79 (d, 2H, J = 8.1 Hz), 7.36 (d, 2H, J = 8.1 Hz), 4.06 (t, 2H, J = 6.0 Hz), 3.37 (t, 2H, J = 6.0 Hz), 2.46 (s, 3H), 1.91 (m, 2H), 1.81 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) d 145.11, 133.07, 130.11, 128.07, 69.57, 32.83, 28.69, 27.61, 21.86.

4.26. 1,4-Ditosyloxybutane-d_8 (35-d_8, TsO-$CD_2CD_2CD_2CD_2$-OTs)

1,4-Dibromobutane-d_8 (0.44 g, 1.96 mmol) was dissolved in CH_3CN (35 mL) followed by addition of AgOTs (1.31 g, 4.69 mmol, 2.4 equiv). The reaction mixture was stirred at reflux under $Ar_{(g)}$ for 24 h, then cooled to 0 °C, and filtered. The precipitate was washed with CH_3CN and dried under vacuum to afford a green solid (AgBr, 0.61 g, 83%). The solvent was removed from the filtrate to give a white solid that was dried under vacuum, then suspended in CH_2Cl_2, filtered, rinsed with CH_2Cl_2, and dried under vacuum to afford a white crystalline solid (AgOTs, 0.20 g, 15% recovery). The filtrate was concentrated, poured onto dry silica (6 cm h × 4 cm i.d.), and eluted under vacuum: hexane (25 mL), %CH_2Cl_2/hexane—25% (50 mL), 50% (50 mL), 75% (50 mL), CH_2Cl_2 (400 mL) to afford **35-d_8** (0.72 g, 90%) as a white crystalline solid: ^{1}H NMR (300 MHz, $CDCl_3$) d 7.76 (d, 4H, J = 8.1 Hz), 7.35 (d, 4H, J = 8.1 Hz), 2.46 (s, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) d 145.11, 132.93, 130.08, 127.97, 68.83 (pentet, $^1J_{CD}$ = 22.7 Hz), 24.04 (pentet, $^1J_{CD}$ = 19.6 Hz), 21.81; HRMS (NSI) $[MH]^+$ Calcd for $C_{18}H_{15}D_8O_6S_2$: 407.1433, found: 407.1433.

4.27. 3-(2,4-Dichlorophenyl)-*N*-(2-(2-[^{18}F]fluoroethoxy)ethyl)-*N*,2,5-trimethylpyrazolo[1,5-*a*]pyrimidin-7-amine ([^{18}F]26)

$H^{18}F_{(aq)}$ (1786 mCi (66.1 GBq)) in $H_2{}^{18}O$ was obtained from PETNET and collected in a V-vial housed in a Capintec detector. The radioactivity sample was divided and 901 mCi (33.3 GBq) was delivered under $Ar_{(g)}$ pressure to a CPCU, collected on a trap/release cartridge, released with $K_2CO_{3(aq)}$ (0.9 mg in 0.6 mL H_2O), and added to a solution of Kryptofix 222 (5 mg in 1 mL CH_3CN) in the CPCU side-arm reaction vessel. The vessel was placed in a 110 °C oil bath and the solvent was evaporated with an $Ar_{(g)}$ flow. CH_3CN (3.5 mL) was added and evaporated in order to azeotropically dry the Kryptofix 222/$K^{18}F$. A solution of **33** (4.8 mg) in CH_3CN (1 mL) was added to the vessel containing the Kryptofix 222/$K^{18}F$, the mixture was heated at 110 °C for 15 min, and then cooled. HPLC solvent (3 mL) was added to the vessel and the solution was transferred under $Ar_{(g)}$ pressure to a hot cell and collected in a conical tube. The CPCU vessel was rinsed with HPLC solvent (3 mL) and this was added to the conical tube. The solution was drawn into a 10-mL HPLC injector loop and then purified by semi-preparative HPLC (Waters XTerra, 50:50:0.1 v/v/v EtOH/H_2O/NEt_3, 7 mL/min, t_R (range) = 14–17 min). The desired fractions were combined, diluted 1:1 v/v with H_2O, loaded onto a Waters tC_{18} Sep-Pak, and rinsed with 0.9% $NaCl_{(aq)}$ (30 mL). The radiotracer was eluted from the Sep-Pak with EtOH (1.5 mL) and collected in a sealed sterile vial containing 0.9% $NaCl_{(aq)}$ (13.5 mL) to give [^{18}F]**26** (204 mCi (7.5 GBq), 39.6% rcy decay-corrected). The solution was passed successively through a 1 mm filter and then a 0.2 mm filter (Acrodisc PTFE) under $Ar_{(g)}$ pressure and collected in a sealed sterile dose vial (164 mCi (6.1 GBq), 82.4% recovery, decay-corrected; pH = 5.5). Radioactivity remaining on filters: ~35 mCi (~1.3 GBq). The total synthesis time was ~93 min from transfer of the $H^{18}F_{(aq)}$ to the CPCU. The radiotracer was analyzed by analytical HPLC (Waters Nova-Pak, 3.9 × 150 mm, 75:25:0.1 v/v/v MeOH/H_2O/NEt_3, 1 mL/min, t_R = 5.4 min) to determine the radiochemical purity (99.4%) and the specific activity (1.2×10^4 Ci/mol).

4.28. 3-(2,4-Dichlorophenyl)-*N*-ethyl-*N*-(2-(2-[^{18}F]fluoroethoxy)-ethyl)-2,5-dimethylpyrazolo[1,5-*a*]pyrimidin-7-amine ([^{18}F]27)

$H^{18}F_{(aq)}$ (1929 mCi (71.4 GBq)) in $H_2{}^{18}O$ was obtained from PETNET and collected in a V-vial housed in a Capintec detector. Using $Ar_{(g)}$ pressure 1849 mCi (68.4 GBq) was delivered to a CPCU, collected on a trap/release cartridge, released with $K_2CO_{3(aq)}$ (0.9 mg in 0.6 mL H_2O), and added to a solution of Kryptofix 222 (5 mg in 1 mL CH_3CN) in the CPCU side-arm reaction vessel. The vessel was placed in a 110 °C oil bath and the solvent was evaporated with an $Ar_{(g)}$ flow. CH_3CN (3.5 mL) was added and evaporated in order to azeotropically dry the Kryptofix 222/$K^{18}F$. A solution of **34** (4.5 mg) in CH_3CN (1 mL) was added to the vessel containing the Kryptofix 222/$K^{18}F$, the mixture was heated at 110 °C for 15 min, and then cooled. HPLC solvent (3 mL) was added to the vessel and the solution was transferred under $Ar_{(g)}$ pressure to a hot cell and collected in a conical tube. The CPCU vessel was rinsed with HPLC solvent (3 mL) and this was added to the conical tube. The solution was drawn into a 10-mL HPLC injector loop and then purified by semi-preparative HPLC (Waters XTerra, 50:50:0.1 v/v/v EtOH/H_2O/NEt_3, 9 mL/min, t_R (range) = 16–18 min). The desired fractions were combined, diluted 1:1 v/v with H_2O, loaded onto a Waters tC_{18} Sep-Pak, and rinsed with 0.9% $NaCl_{(aq)}$ (30 mL). The radiotracer was eluted from the Sep-Pak with EtOH (1.5 mL) and collected in a sealed sterile vial containing 0.9% $NaCl_{(aq)}$ (13.5 mL) to give [^{18}F]**27** (107.4 mCi (4.0 GBq), 10.4% rcy decay-corrected). The solution was passed successively through a 1 mm filter and then a 0.2 mm filter (Acrodisc PTFE) under $Ar_{(g)}$ pressure and collected in a sealed sterile dose vial (56.3 mCi (2.1 GBq), 54% recovery, decay-corrected; pH = 5.5). Radioactivity remaining on filters: 47.5 mCi (1.8 GBq). The total synthesis time was ~98 min from transfer of the $H^{18}F_{(aq)}$ to the CPCU. The radiotracer was analyzed by analytical HPLC (Waters Nova-Pak, 75:25:0.1 v/v/v MeOH/H_2O/NEt_3, 1 mL/min, t_R = 7.25 min) to determine the radiochemical purity (99.5%) and the specific activity (>6.4×10^5 Ci/mol).

4.29. 3-(2,4-Dichlorophenyl)-*N*-(4-[^{18}F]fluorobutyl)-*N*,2,5-trimethylpyrazolo[1,5-*a*]pyrimidin-7-amine ([^{18}F]28)—two-step radiolabeling

$H^{18}F_{(aq)}$ (1186 mCi (43.9 GBq) at EOB) in $H_2{}^{18}O$ was delivered to a CPCU, collected on a trap/release cartridge, released with $K_2CO_{3(aq)}$ (0.9 mg in 0.6 mL H_2O), and added to a solution of Kryptofix 222 (5 mg in 1 mL CH_3CN) in the CPCU side-arm reaction vessel. The vessel was placed in a 110 °C oil bath and the solvent was evaporated with an $Ar_{(g)}$ flow. CH_3CN (3.5 mL) was added and evaporated in order to azeotropically dry the Kryptofix 222/$K^{18}F$. A solution of **35** (5.8 mg) in CH_3CN (1 mL) was added to the vessel containing the Kryptofix 222/$K^{18}F$, the mixture was heated at 110 °C for 10 min, and then cooled. EtOEt (~5 mL) was added, the solution was transferred under $Ar_{(g)}$ pressure out of the reaction vessel, through a Waters silica Sep-Pak Classic, and collected in a 15-mL conical tube that had been placed in an adjacent hot cell. The CPCU reaction vessel was rinsed with EtOEt (~5 mL) and this was transferred through the Sep-Pak and into the conical tube. The tube was placed in a warm water bath and the EtOEt was evaporated with an $Ar_{(g)}$ flow until ~2.5 mL remained (705 mCi (26.1 GBq), 82.5% rcy, decay-corrected from EOB, ~52 min). The tube was placed in a 110 °C oil bath and the remainder of the EtOEt was evaporated with an $Ar_{(g)}$ flow.

NaH (95%, 1.1 mg, 4.6×10^{-5} mol, 4.6 equiv) was placed in a 1-mL vial, capped with a septum, purged with $Ar_{(g)}$ for 90 min, and

then cooled to 0 °C. Compound **16** (4.0 mg, 1.0×10^{-5} mol) was placed in a 1-mL vial, capped with a septum, purged with $Ar_{(g)}$ for 90 min, dissolved in DMA (0.3 mL), added to the cold NaH, and transferred to the hot cell. This DMA solution then stood at ambient temperature in the hot cell for ~10–15 min during the transfer of [^{18}F]fluorobutyltosylate from the CPCU and evaporation of the EtOEt. The DMA solution was added to the conical tube containing [^{18}F]fluorobutyltosylate, the mixture was heated at 110 °C for 5 min, and then cooled at 0 °C for 1 min (630 mCi (23.3 GBq)). HPLC solvent (0.5 mL) was added, the solution was drawn into a 10-mL HPLC loop, the tube was rinsed with HPLC solvent (0.5 mL, loaded onto the loop), rinsed with MeOH (0.5 mL, loaded onto the loop), and then the mixture was purified by semi-preparative HPLC (Waters XTerra + guard pack 19 × 10 mm, 70:30:0.1 v/v/v MeOH/H_2O/NEt_3, 9 mL/min, t_R (range) = 15–18 min). The desired fractions were combined, diluted 1:1 v/v with H_2O, loaded onto a Waters tC_{18} Sep-Pak, and rinsed with 0.9% $NaCl_{(aq)}$ (15 mL). The radiotracer was eluted from the Sep-Pak with EtOH (1.5 mL) and collected in a sealed sterile vial containing 0.9% $NaCl_{(aq)}$ (13.5 mL) to give [^{18}F]**28** (65.8 mCi (2.4 GBq), 11.1% rcy, decay-corrected from EOB, ~110 min; 13.5% rcy, decay-corrected from measurement of [^{18}F]fluorobutyltosylate, ~58 min). The solution was passed successively through a 1 mm filter and then a 0.2 mm filter (Acrodisc PTFE) under $Ar_{(g)}$ pressure and collected in a sealed sterile dose vial (50.7 mCi (3.9 GBq), 81.6% recovery, decay-corrected; pH = 7.5). Radioactivity remaining on filters: 10.1 mCi (0.37 GBq). The total synthesis time was ~120 min from EOB. The radiotracer was analyzed by analytical HPLC (Waters Nova-Pak, 75:25:0.1 v/v/v MeOH/H_2O/NEt_3, 1 mL/min, t_R = 6.0 min) to determine the radiochemical purity (100%) and the specific activity (>9.9×10^5 Ci/mol).

4.30. 3-(2,4-Dichlorophenyl)-*N*-(4-[^{18}F]fluorobutyl)-*N*,2,5-trimethylpyrazolo[1,5-*a*]pyrimidin-7-amine ([^{18}F]28)—one-step radiolabeling

$H^{18}F_{(aq)}$ (1699 mCi (62.9 GBq)) in $H_2{}^{18}O$ was obtained from PETNET and collected in a V-vial housed in a Capintec detector. Using $Ar_{(g)}$ pressure 1614 mCi (59.7 GBq) was delivered to a CPCU, collected on a trap/release cartridge, released with $K_2CO_{3(aq)}$ (0.9 mg in 0.6 mL H_2O), and added to a solution of Kryptofix 222 (5 mg in 1 mL CH_3CN) in the CPCU side-arm reaction vessel. The vessel was placed in a 110 °C oil bath and the solvent was evaporated with an $Ar_{(g)}$ flow. CH_3CN (3.5 mL) was added and evaporated in order to azeotropically dry the Kryptofix 222/$K^{18}F$. A solution of **31** (4.7 mg) in CH_3CN (1 mL) was added to the vessel containing the Kryptofix 222/$K^{18}F$, the mixture was heated at 110 °C for 15 min, and then cooled. HPLC solvent (3 mL) was added to the vessel and the solution was transferred under $Ar_{(g)}$ pressure to a hot cell and collected in a conical tube. The CPCU vessel was rinsed with HPLC solvent (3 mL) and this was added to the conical tube. The solution was drawn into a 10-mL HPLC injector loop and then purified by semi-preparative HPLC (Waters XTerra, 70:30:0.1 v/v/v MeOH/H_2O/NEt_3, 9 mL/min, t_R (range) = 11.5–13.5 min). The desired fractions were combined, diluted 1:1 v/v with H_2O, loaded onto a Waters tC_{18} Sep-Pak, and rinsed with 0.9% $NaCl_{(aq)}$ (25 mL). The radiotracer was eluted from the Sep-Pak with EtOH (1.5 mL) and collected in a sealed sterile vial containing 0.9% $NaCl_{(aq)}$ (13.5 mL) to give [^{18}F]**28** (428 mCi (15.8 GBq), 43.9% rcy decay-corrected). The solution was passed successively through a 1 mm filter and then a 0.2 mm filter (Acrodisc PTFE) under $Ar_{(g)}$ pressure and collected in a sealed sterile dose vial (106 mCi (3.9 GBq), 25.8% recovery, decay-corrected; pH = 5.3). Radioactivity remaining on filters: 294 mCi (10.9 GBq). The total synthesis time was ~85 min from transfer of the $H^{18}F_{(aq)}$ to the CPCU. The radiotracer was analyzed by analytical HPLC (Waters Nova-Pak, 75:25:0.1 v/v/v MeOH/H_2O/NEt_3, 1 mL/min, t_R = 6.6 min) to determine the radiochemical purity (95.5%) and the specific activity (>1.1×10^6 Ci/mol).

4.31. 3-(2,4-Dichlorophenyl)-*N*-(4-[^{18}F]fluorobutyl-1,1,2,2,3,3,-4,4-d_8)-*N*,2,5-trimethylpyrazolo[1,5-*a*]pyrimidin-7-amine ([^{18}F]28-d_8)

$H^{18}F_{(aq)}$ (1886 mCi (69.8 GBq)) in $H_2{}^{18}O$ was obtained from PETNET and collected in a V-vial housed in a Capintec detector. The radioactivity sample was divided and 892 mCi (33.0 GBq) was delivered under $Ar_{(g)}$ pressure to a CPCU, collected on a trap/release cartridge, released with $K_2CO_{3(aq)}$ (0.9 mg in 0.6 mL H_2O), and added to a solution of Kryptofix 222 (5 mg in 1 mL CH_3CN) in the CPCU side-arm reaction vessel. The vessel was placed in a 110 °C oil bath and the solvent was evaporated with an $Ar_{(g)}$ flow. CH_3CN (3.5 mL) was added and evaporated in order to azeotropically dry the Kryptofix 222/$K^{18}F$. A solution of **31-d_8** (4.8 mg) in CH_3CN (1 mL) was added to the vessel containing the Kryptofix 222/$K^{18}F$, the mixture was heated at 110 °C for 15 min, and then cooled. HPLC solvent (3 mL) was added to the vessel and the solution was transferred under $Ar_{(g)}$ pressure to a hot cell and collected in a conical tube. The CPCU vessel was rinsed with HPLC solvent (3 mL) and this was added to the conical tube. The solution was drawn into a 10-mL HPLC injector loop and then purified by semi-preparative HPLC (Waters XTerra, 55:45:0.1 v/v/v EtOH/H_2O/NEt_3, 9 mL/min, t_R (range) = 11–14 min). The desired fractions were combined, diluted 1:1 v/v with H_2O, loaded onto a Waters tC_{18} Sep-Pak, and rinsed with 0.9% $NaCl_{(aq)}$ (25 mL). The radiotracer was eluted from the Sep-Pak with EtOH (1.5 mL) and collected in a sealed sterile vial containing 0.9% $NaCl_{(aq)}$ (13.5 mL) to give [^{18}F]**28-d_8** (208 mCi (7.7 GBq), 39.4% rcy decay-corrected). The solution was passed successively through a 1 mm filter and then a 0.2 mm filter (Acrodisc PTFE) under $Ar_{(g)}$ pressure and collected in a sealed sterile dose vial (36.2 mCi (1.3 GBq), 18.1% recovery, decay-corrected; pH = 5.0). Radioactivity remaining on filters: 160.7 mCi (5.9 GBq). The total synthesis time was ~90 min from transfer of the $H^{18}F_{(aq)}$ to the CPCU. The radiotracer was analyzed by analytical HPLC (Waters Nova-Pak, 75:25:0.1 v/v/v MeOH/H_2O/NEt_3, 1 mL/min, t_R = 8.8 min) to determine the radiochemical purity (98.4%) and the specific activity (>5.4×10^5 Ci/mol).

4.32. 3-(2,4-Dichlorophenyl)-*N*-ethyl-*N*-(4-[^{18}F]fluorobutyl)-2,5-dimethylpyrazolo[1,5-*a*]pyrimidin-7-amine ([^{18}F]29)

$H^{18}F_{(aq)}$ (2.01 Ci (74.4 GBq)) in $H_2{}^{18}O$ was obtained from PETNET and collected in a V-vial housed in a Capintec detector. The radioactivity sample was divided and 840 mCi (31.1 GBq) was delivered under $Ar_{(g)}$ pressure to a CPCU, collected on a trap/release cartridge, released with $K_2CO_{3(aq)}$ (0.9 mg in 0.6 mL H_2O), and added to a solution of Kryptofix 222 (5 mg in 1 mL CH_3CN) in the CPCU side-arm reaction vessel. The vessel was placed in a 110 °C oil bath and the solvent was evaporated with an $Ar_{(g)}$ flow. CH_3CN (3.5 mL) was added and evaporated in order to azeotropically dry the Kryptofix 222/$K^{18}F$. A solution of **32** (3.6 mg) in CH_3CN (1 mL) was added to the vessel containing the Kryptofix 222/$K^{18}F$, the mixture was heated at 110 °C for 15 min, and then cooled. HPLC solvent (3 mL) was added to the vessel and the solution was transferred under $Ar_{(g)}$ pressure to a hot cell and collected in a conical tube. The CPCU vessel was rinsed with HPLC solvent (3 mL) and this was added to the conical tube. The solution was drawn into a 10-mL HPLC injector loop and then purified by semi-preparative HPLC (Waters XTerra, 70:30:0.1 v/v/v MeOH/H_2O/NEt_3, 9 mL/min, t_R (range) = 21–24 min). The desired fractions were combined, diluted 1:1 v/v with H_2O, loaded onto a

Waters tC_{18} Sep-Pak, and rinsed with 0.9% $NaCl_{(aq)}$ (25 mL). The radiotracer was eluted from the Sep-Pak with EtOH (1.5 mL) and collected in a sealed sterile vial containing 0.9% $NaCl_{(aq)}$ (13.5 mL) to give [^{18}F]**29** (178 mCi (6.6 GBq), 39% rcy decay-corrected). The solution was passed successively through a 1 mm filter and then a 0.2 mm filter (Acrodisc PTFE) under $Ar_{(g)}$ pressure and collected in a sealed sterile dose vial (22.4 mCi (0.8 GBq), 13% recovery, decay-corrected; pH = 5.3). Radioactivity remaining on filters: 151 mCi (5.6 GBq). The total synthesis time was ~107 min from transfer of the $H^{18}F_{(aq)}$ to the CPCU. The radiotracer was analyzed by analytical HPLC (Waters Nova-Pak, 75:25:0.1 v/v/v $MeOH/H_2O/NEt_3$, 1 mL/min, t_R = 11.3 min) to determine the radiochemical purity (97.3%) and the specific activity (>2.8 × 10^5 Ci/mol).

4.33. Log$P_{7.4}$ procedure

The log$P_{7.4}$ value of each radiotracer was measured by adapting the procedure of Wilson and Houle.[104,105] 1-Octanol (10 mL) and 0.02 M/pH 7.4 phosphate buffer (5 mL) were placed in a 60-mL separatory funnel and briefly shaken. Radiotracer (~75–125 mCi, formulated in 10% EtOH/saline) was added, the mixture was shaken by hand for 1 min, allowed to stand for 5 min, and then the aqueous layer was separated and discarded. Aliquots of the 1-octanol layer (2 mL) were pipetted into each of four 15-mL Corning polypropylene centrifuge tubes which contained phosphate buffer (2 mL). The tubes were shaken mechanically for 10 min and then centrifuged for 5 min. Aliquots (1 mL) of each layer were pipetted into separate glass culture tubes and counted on a gamma counter. Each culture tube was counted 3× (decay-corrected) and the average value for each culture tube was calculated using Microsoft Excel. The log$P_{7.4}$ value for each of the four centrifuge tubes was calculated as log$P_{7.4}$ = log (average culture tube radioactivity counts in octanol/average culture tube radioactivity counts in phosphate buffer). The average value and standard deviation of the four centrifuge tubes were calculated using Microsoft Excel.

4.34. CRF receptor binding assays

4.34.1. Materials

[^{125}I]-Tyr^0-sauvagine and [^{125}I]antisauvagine-30 radiotracers were purchased from Perkin Elmer (San Jose, California). Unlabeled sauvagine and antisauvagine-30 were purchased from the American Peptide Company (San Diego, CA).

4.34.2. Cell culture and membrane preparation

Plasmids for human CRF_1 receptors were a generous gift from Dr. Bryan Roth (University of North Carolina, Chapel Hill, NC). Plasmids were sequenced to verify gene delivery of the desired receptor. HEK293T cells (ATCC, Manassas, VA) were grown in culture media (DMEM supplemented with 4.5 g/L glucose, L-glutamine, sodium pyruvate, 10% fetal bovine serum, 1% penicillin and streptavidin) in T-75 cm^2 sterile culture flasks to 75% confluency. Cells were transiently transfected by Fugene 6 lipofection (Roche, Mannheim, Germany). A negative control transfection with pUC19 DNA (Invitrogen, Carlsbad, CA) was used to assure a lack of endogenous receptor binding. The media was aspirated 24 h after transfection and replaced with fresh media free of lipofectamine. The media was again aspirated 48 h after transfection and the cell monolayer was washed once with sterile PBS. Cells were dislodged by triturating with 30% PBS, 0.53 mM EDTA and 70% culture media. Suspended cells were centrifuged at 200*g* for 5 min at 23 °C. The supernatant was aspirated and the cell pellets were frozen at −20 °C until the day of the assay.

On the day of the assay, cell pellets were washed once in binding assay buffer (55 mM HEPES, 10 mM $MgCl_2$, 2.2 mM EGTA, 0.1% bovine serum albumin, 1 μg/mL aprotinin, pH 7.2). Pellets were homogenized in binding assay buffer with a polytron homogenizer on a speed setting of 1 for 30 s (Kinematica, Lucerne, Switzerland). A radioligand binding assay of the membrane homogenate was conducted to determine total binding and nonspecific binding with unlabeled sauvagine. The membrane homogenate was titrated based on the preliminary assay so that total binding would approximate 3,000 CPM per reaction.

4.34.3. Radioligand binding assays

Sauvagine was dissolved in degassed and sonicated 10 mM acetic acid. Sauvagine stocks (100 μM) were aliquoted and frozen at −80 °C. Compounds were dissolved in DMSO (1–3 mg/mL) and serially-diluted with binding assay buffer in silanized glass tubes. Radiotracers were diluted in binding assay buffer and counted on a Wallac LKB gamma counter (Perkin Elmer, Waltham, MA). Radiotracer concentrations were titrated based on gamma counts so radioligand assays would use approximately 80 pM of [^{125}I]sauvagine or 80 pM of [^{125}I]antisauvagine-30.

Binding assay buffer (250 μL), unlabeled ligand over a final concentration range of 10^{-13} to 10^{-5} M (50 μL), radiotracer (100 μL of [^{125}I]sauvagine or [^{125}I]antisauvagine-30), and membrane homogenate (100 μL) were added sequentially to a 96 deep well plate (Fisher Scientific, Hampton, NH). Plates were vortexed and gently centrifuged at 200*g* for 5 min at 23 °C to bring all liquids together in the well. Plates were incubated for two hours at 23 °C. During the incubation, 96-position FilterMat B filters were soaked in 0.1% polyethyleneamine. Binding reactions were terminated by adding 1 mL of 4 °C PBS and 0.01% Triton X-100. Membranes were filtered over FilterMat B filters with a 96-well FilterMate Universal Harvester (Perkin Elmer, Waltham, MA). Filters were washed 5 times with 1 mL of 4 °C PBS and 0.01% Triton X-100. Filters were dried overnight at 23 °C, placed in plastic bags with a MeltiLex solid scintillator, and melted at 70 °C for 30 min. Filters were then counted on a MicroBeta TriLux Microplate Scintillation Counter (Perkin Elmer, Waltham, MA). The counting efficiencies of the MicroBeta detector and LKB gamma counter are 49% and 42%, respectively. Nonspecific binding (~5–8% of total binding) was defined as 80 pM of [^{125}I]sauvagine or 80 pM of [^{125}I]antisauvagine-30 in the presence of 1 μM of sauvagine. Assays were performed in triplicate and independent experiments were performed for statistical replicates.

4.34.4. Data analysis

Equilibrium dissociation constants were determined with a one-site competition nonlinear regression curve analysis in GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA). The concentration of labeled ligand was set at 80 pM and the K_d was set at 0.5 nM.

4.35. MicroPET

Animal imaging procedures were approved by the Emory University IACUC. Non-human primate handling was performed by the Yerkes National Primate Research Center veterinary staff.

4.35.1. Rat MicroPET imaging

MicroPET imaging was performed in a male Sprague-Dawley rat on a Siemens Inveon microPET/CT scanner. The rat was initially anesthetized with 3–4% isoflurane in an anesthesia chamber and then maintained with 1–1.5% isoflurane via nose cone. The rat was placed in the prone position on the microPET scanner bed and body temperature was maintained with a warm air-circulating heating pad. A transmission scan was performed with a Co-57 point source for attenuation correction. The radiotracer was

administered via tail vein injection and the rat was imaged dynamically for 60 min.

4.35.2. Non-human primate microPET imaging

MicroPET imaging was performed in a male cynomolgus monkey using a Siemens MicroPET Focus 220. The monkey was fasted for 12 h prior to receiving anesthesia. The monkey was initially anesthetized with 3–4 mg/kg Telazol, intubated, and then maintained with 1–2% isoflurane. Body temperature was maintained with a warming blanket. Respiratory rate, oxygen saturation, expired carbon dioxide, heart rate, blood pressure, and temperature were continuously monitored using a SurgiVet monitor. A transmission scan was performed with a Co-57 point source for attenuation correction. The radiotracer was administered via saphenous vein injection and imaging was performed dynamically for 120 min.

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Supplementary data

Supplementary data (microPET images and TACs) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.06.036.

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