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Efficient tritiation of the translocator protein (18 kDa) selective ligand DPA-714

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DPA-714 (*N*,*N*-diethyl-2-(2-(4-(2-fluoroethoxy)phenyl)-5,7-dimethylpyrazolo[1,5-a]pyrimidin-3-yl)acetamide) is a recently discovered fluorinated ligand of the translocator protein 18 kDa (TSPO). Labelled with the short-lived positron emitter fluorine-18, this structure is today the radioligand of reference for *in vivo* imaging of microglia activation and neuroinflammatory processes with positron emission tomography. In the present work, an isotopically tritium-labelled version was developed ([^{3}H]DPA-714), in order to access high resolution *in vitro* and *ex vivo* microscopic autoradiography studies, repeated and long-lasting receptor binding studies and *in vivo* pharmacokinetic determination at late time points. Briefly, DPA-714 as reference, and its 3,5-dibrominated derivative as precursor for labelling, were both prepared from DPA-713 in nonoptimized 32% (two steps) and 10% (three steps) yields, respectively. Reductive debromination using deuterium gas and Pd/C as catalyst in methanol, performed at the micromolar scale, confirmed the regioselective introduction of two deuterium atoms at the meta positions of the phenyl ring. Tritiodebromination was analogously performed using no-carrier tritium gas. HPLC purification provided >96% radiochemically pure [^{3}H]DPA-714 (7 GBq) with a 2.1 TBq/mmol specific radioactivity. Interestingly, additional hydrogen-for-tritium exchanges were also observed at the 5-methyl and 7-methyl positions of the pyrazolo[1,5-a]pyrimidine, opening novel perspectives in the labelling of compounds featuring this heterocyclic core.

Keywords: isotopic labelling; tritium; DPA-714; TSPO; PBR; tritiodebromination; hydrogen-for-tritium exchange

Introduction

Well recognized as an early marker of neuroinflammation, the translocator protein 18 kDa (TSPO), a protein overexpressed at the outer mitochondrial membrane of microglial cells,[1,2] has occasioned extensive efforts towards the development of dedicated radiolabelled probes for positron emission tomography (PET) imaging.[3,4] Whilst dozens of radioligands have been developed or are still currently under development,[5] DPA-714, a pyrazolo[1,5-*a*]pyrimidine derivative designed with a fluorine atom in its structure and at a position allowing efficient labelling with the positron emitter fluorine-18 (Figure 1),[6,7] is today considered as the probe of reference.[8–11] As such, [^{18}F] DPA-714 has dethroned the historical and previously established radioligand [^{11}C]PK11195[12,13] and has been chosen as the radioligand of choice for imaging microglia activation within the INMiND consortium[14] both at the preclinical and clinical level.

Although labelled with a radioactive isotope, allowing thus for sensitive and quantitative studies such as *in vivo* PET imaging, [^{18}F]DPA-714 is not suitable for high resolution *in vitro* or *ex vivo* microscopic autoradiography studies, repeated and long-lasting receptor binding studies performed on tissues and cell or membrane extracts, or *in vivo* pharmacokinetic determination at late time points (e.g. >6 h, for example). Fluorine-18 is indeed a relatively high-energy ($E\beta^+_{max}$: 635 keV) and short-lived ($T_{1/2}$: 109.8 min) positron emitter, limiting from a practical point of view its use to a few hours. Also, as it is the case for all these radioisotopes, positron emission will give rise to two 511-keV antiparallel γ-rays, thus requiring specific handling precautions and infrastructures to limit the operator radiation dose. On the other hand, β-decaying long-lived radioisotopes, such as tritium ($T_{1/2}$: 12.32 years, $E\beta^-_{max}$: 18.6 keV), remains the radionuclide of choice for preparing isotopically radiolabelled compounds and then accessing drug absorption, distribution, metabolism and excretion parameters or performing selected binding assays.[15,16]

The aim of the present work was to prepare a tritium, isotopically labelled, version of DPA-714 ([^{3}H]DPA-714 (**1d**)), in order to support the development of the corresponding PET probe, seeking for alternative and additional information on this ligand and especially its binding to the TSPO, as well as bridging *in vitro* and *in vivo* studies performed with this tracer. A special care was given to the expected positioning of the tritium atoms in regard of the known *in vivo* metabolism of the tracer and to the selection of a tritiation synthesis pathway and process allowing the simultaneous

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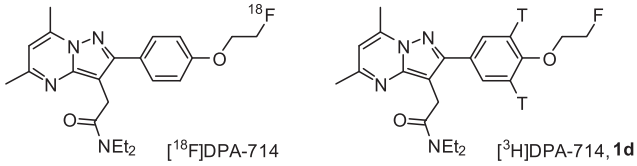


Figure 1. The positron emission tomography radioligand [^{18}F]DPA-714 and its tritiated analogue, [^{3}H]DPA-714 (**1d**).

introduction of theoretically two radioactive atoms, for an optimal value of specific radioactivity (SRA).

Results and discussion

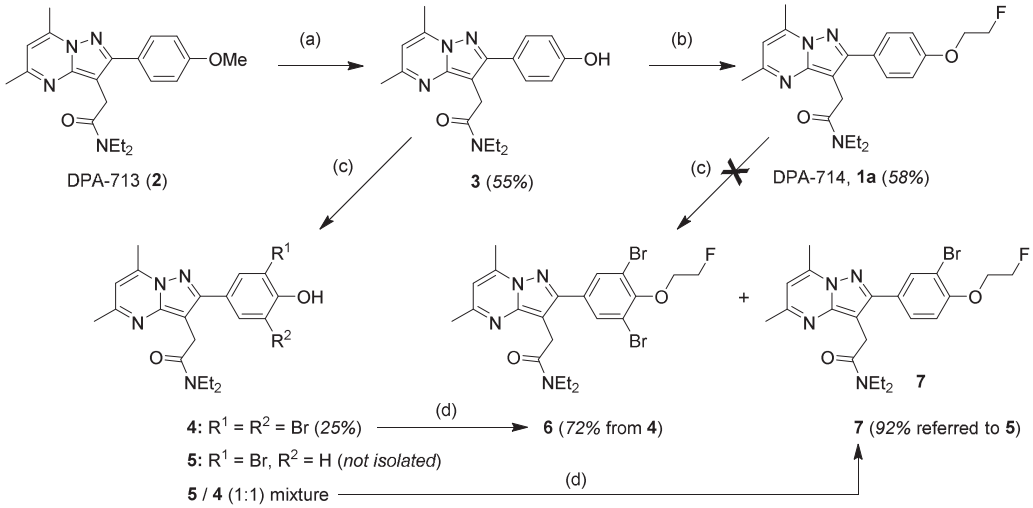
The radiolabelling strategy developed herein for the preparation of [^{3}H]DPA-714 (**1d**) involves a direct one-step, tritiodehalogenation reaction, performed with a heterogeneous catalyst (Pd/C), carrier-free tritium gas and a dibrominated derivative of DPA-714, compound **6** (coded Br_2-DPA-714) as the designed labelling precursor. The preparation of the latter (**6**) and DPA-714 (**1a**) as reference compound is illustrated in Scheme 1. Both compounds were synthesized from DPA-713 (**2**)[12] and obtained in nonoptimized 32% (2 steps for **1a**) and 10% (3 steps for **6**) yields, respectively.

Briefly, DPA-713 (**2**) was *O*-demethylated using a 1 M boron tribromide solution in dichloromethane at low temperature for 2 h, to give the expected phenol **3** in moderate yield (55%) (Scheme 1). Subsequent reaction with 1-fluoro-2-tosyloxyethane in tetrahydrofuran containing powdered sodium hydride for 16 h at 60 °C, afforded DPA-714 (**1a**) in 58% nonoptimized yield after recrystallisation.

Direct treatment of DPA-714 (**1a**) with bromine in acetic acid at room temperature to obtain the dibrominated derivative **6** was first investigated.[17] This approach failed, and complex mixtures of compounds resulting from overbromination were obtained. Thus, the following alternative route was developed. Phenol **3** was analogously dissolved in acetic acid and treated with bromine at room temperature. Bromine was carefully added dropwise, whilst the reaction was monitored by thin-layer chromatography (TLC) to avoid overbromination to tribromo or tetrabromo products. As a matter of fact, the bromination reaction was usually stopped before complete conversion of the monobromo product **5** because the reaction was difficult to control in batch mode. Using these conditions, the expected dibromophenol **4** was obtained in acceptable yield after purification (25%), together with a 1:1 mixture (according to ^{1}H-NMR) of **4** and the monobrominated analogue **5**. Conversion of pure dibromophenol **4** into its fluoroethoxy analogue **6** was then achieved using 1-fluoro-2-tosyloxyethane and sodium hydroxide as a base in a mixture of ethanol and water. The reaction mixture was stirred for 16 h at 65 °C to lead to the desired dibrominated analogue **6**, as precursor for tritium labelling, in 72% yield. When the reaction was carried out starting from the above mentioned mixture of **4** and **5**, compound **7** was also isolated (92% yield referred to **5**) and used as HPLC reference in the catalytic hydrodebromination reactions.

Prior to tritiation, reductive dehalogenation using gaseous deuterium (D_2) was performed in order to validate our labelling approach and to evaluate the exchange rate of the bromine atoms. Deuterations were performed at the micromolar scale, for example, 20 mg of Br_2-DPA-714 (**6**) and 10 mg of 10% palladium on charcoal (Pd/C) in 2 mL of solvent with a gaseous D_2 pressure of approximately 1 atm. The reaction was monitored to determine the optimal reaction time for efficient hydrodebromination. Follow-up was primarily carried out by TLC, but the reaction mixture was also analysed by ^{1}H-NMR to determine the sites of deuteration, by comparison with an authentic sample of DPA-714 (**1a**), after stirring at room temperature. Influence of the solvent was also rapidly evaluated. The main results are summarized in Scheme 2, and the ^{1}H-NMR signals, in the 6.9–8.2 ppm region, of the isolated compounds (or mixture of compounds) are depicted in Figure 2.

When the reaction was performed in methanol, after 30 min of stirring, [$^2H_2$]DPA-714 (**1b**) was formed. However, complete debromination was only observed after 1 h of reaction. At this time, no brominated compounds (neither the dibromo derivative **6** nor the monobromo derivative **7**) remained as indicated by ^{1}H-NMR. Nevertheless, the NMR spectra revealed



Reagents and conditions: (a) BBr_3, CH_2Cl_2, -20 °C, 2 h; (b) 1-fluoro-2-tosyloxyethane, NaH, THF, reflux, 16 h; (c) Br_2, AcOH, r.t., 2 h; (d) 1-fluoro-2-tosyloxyethane, NaOH 0.12 M, EtOH:H_2O 8:2, 65°C, 16 h.

Scheme 1. Synthesis of DPA-714 (**1a**) and its dibrominated derivative **6** (Br_2-DPA-714) as precursor for labelling with tritium.

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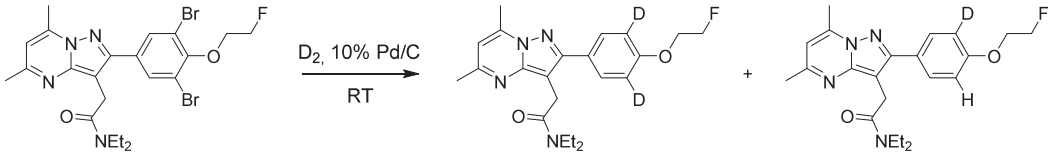


Br_2-DPA-714 (**6**)

solvent	*time*	[$^2H_2$]DPA-714 (**1b**)	[^{2}H]DPA-714 (**1c**)
MeOH	1 h	~ 70%	~ 30%
MeOD-d_4	30 min	> 95%	< 5%
Toluene	4 days	~ 5%	–

Scheme 2. Catalytic hydrodebromination and preparation of [$^2H_2$]DPA-714 (**1b**).

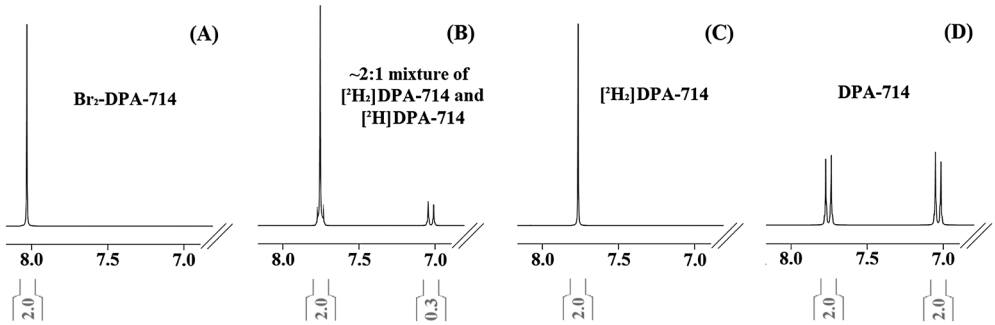
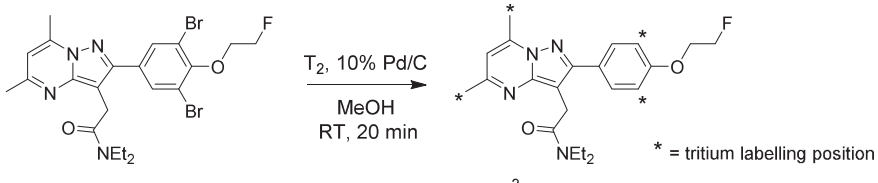


Figure 2. ^{1}H-NMR signals observed for (A) the starting material Br_2-DPA-714 (**6**), (B) the 2 : 1 mixture of **1b** and **1c** obtained when using methanol as solvent, (C) the [$^2H_2$] DPA-714 (**1b**) compound obtained when using methanol-d_4 as solvent and (D) the reference compound DPA-714 (**1a**).

the formation of [$^2H_2$]DPA-714 (**1b**) and [^{2}H]DPA-714 (**1c**) in a 2:1 ratio. Indeed, a singlet signal at 7.75 ppm was present as expected for the aromatic protons in the meta position to the oxygen, but a doublet signal at 7.02 ppm assigned to an aromatic proton ortho to the oxygen was also present showing the formation of a monodeuterated compound ([^{2}H]DPA-714, **1c**). When the reaction was carried out in a similar way but using methanol-d_4 instead of methanol, no formation of [^{2}H]DPA-714 (**1c**) was observed by ^{1}H-NMR, and complete conversion to [$^2H_2$]DPA-714 (**1b**) was achieved after 30 min of stirring at room temperature. This observation is in favour of a significant participation of the labile proton or deuterium atom born by the solvent to the reductive dehalogenation reaction. It should be noted that when the reaction time was prolonged, a mixture of polydeuterated compounds, resulting from deuteration at the methyl sites of the pyrazolo[1,5-a]pyrimidine, was observed as demonstrated by MS analysis. As an example, the ESI(+)-MS spectra recorded for the crude mixture, after 2 h of stirring in methanol in presence of D_2 and Pd/C, displayed the following peaks: 400 (32%), 401 (100%), 402 (62%), 403 (28%) and 404 (12%) showing that **1b** (*m/z* 401 $[M+H]^+$) is still the main compound (~50% of the mixture) but trideuterated (~20%) and tetradeuterated compounds (~8%) are formed. It should also be mentioned that when the reaction was performed in toluene, almost no conversion of the dibrominated compound **6** was observed after 4 days.

Catalytic tritiation was performed under standard conditions adapting the reaction time from the previously described observations. The reaction involved 9.3 μmoles of Br_2-DPA-714 (**6**) dissolved in 1 mL of methanol, 5.1 mg of 10% Pd/C (based on our experience, the use of less than 5 mg of catalyst usually results in low and unreproducible tritiation yields) and was performed at room temperature with a starting gaseous tritium pressure of approximatively 0.92 atm (Scheme 3). It should be noted that the amount of labelling precursor used for tritiation was reduced compared with the amount used for deuteration as reported above. This was deliberately performed in order to limit the production of [^{3}H]DPA-714—indeed, even a moderate tritiation yield would still largely exceed our needs in labelled product required for binding studies—and the associated radioactive wastes.

Using the conditions described earlier in the texts, tritium was rapidly consumed—as seen by the fast decrease monitored by the pressure system controller—and the reaction was stopped after 20 min only. The apparent faster reaction rate (compared with the deuteration reaction) is certainly not related to any isotopic effect but rather because of the following: (i) the lower ratio of precursor for labelling (**6**) over catalyst and (ii) the



Br_2-DPA-714 (**6**) [^{3}H]DPA-714 (**1d**)

Scheme 3. Tritiation and preparation of [^{3}H]DPA-714 (**1d**).

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particular microenvironment of the tritiation reaction (e.g. vessel configuration and difference in stirring). After removal of the heterogeneous catalyst by filtration, the reaction mixture was concentrated to dryness, and labile tritium was removed by codistillation with methanol *in vacuo*.

HPLC analyses of the crude mixture showed that the major tritiated product was the didebrominated compound **1d**. Indeed, the peak corresponding to [^{3}H]DPA-714 represented 53% of the radiochromatogram summed areas (HPLC C, t_R: ~17 min ± 1 min), whereas only one more polar peak was detected (t_R: ~14 min ± 1 min, 47%). The identity and preponderant formation of [^{3}H]DPA-714 was also confirmed by analytical HPLC (HPLC D, t_R: ~6.77 min, 50.2%), the latter surprisingly showing the formation of not one but three side products (t_R: ~5.32 min (4.8%), 4.19 min (29.9%) and 3.24 min (15.1%)). However, none of these compounds corresponded to the tritiated version of derivative **7** that would result from a monodebromination reaction.

Finally, HPLC purification was performed, the solvents removed and the final batch of [^{3}H]DPA-714 (**1d**) (7 GBq, 36%) characterized before being stored in pure methanol (±35 MBq/mL) at −20 °C.

Identity of **1d** was confirmed by analytical HPLC analysis (HPLC D, t_R: ~6.64 min) by coinjection with an authentic sample of DPA-714 (**1a**, reference), and the radiochemical purity of the radiotracer assessed (>96%). ^{3}H-NMR and MS spectra were in accordance with the chemical structure of **1d** and showed that catalytic hydrogen-for-tritium exchange also occurs at the methyl positions of the pyrazolo[1,5-a]pyrimidine core, thus confirming the observation made during the deuteration studies. Indeed, besides the expected singlet at 7.11 ppm (T-Ph, representing 47% of the tritium based on the integration), the proton-decoupled ^{3}H-NMR spectra also showed two groups (2.75–2.71 and 2.55–2.51) of three singlets (respectively, 26% and 27%), 10.6 Hz apart, corresponding to the CH_2T, CHT_2 and CT_3 species (tritium primary isotopic effect) for each methyl group.[18] Proton-coupled ^{3}H-NMR spectra also exhibited two sets of signals corresponding to overlapping tritium singlet, doublet and triplet for each methyl group[19] of the pyrazolopyrimidine core and a doublet at 7.11 ppm corresponding to the tritium placed on the phenyl moiety.

It should be noted that these hydrogen-for-tritium exchanges did occur in parallel or simultaneously with the tritiodebromination reactions and were unexpectedly rather fast when compared with the corresponding hydrogen-for-deuterium exchanges described earlier in the text. SRA was calculated from the MS spectra and was corrected from the contribution of natural isotopes. A value of 2.1 TBq/mmol was calculated, corresponding to an average of two tritium per molecule.

Conclusion

The TSPO ligand DPA-714 was successfully isotopically labelled with tritium. The process involved a direct, one-step, tritiodebromination reaction and afforded >96% radiochemically pure [^{3}H]DPA-714 (7 GBq) with an adequate SRA (2.1 TBq/mmol). Besides, the process herein described opens novel perspectives in the labelling of compounds featuring a 5,7-dimethylpyrazolo[1,5-a]pyrimidine core, because an easy incorporation of tritium atoms at the methyl functions by catalytic hydrogen-for-tritium exchanges has been observed.

Experimental

General

Chemicals, flash chromatography and thin-layer chromatography analysis

Chemicals were purchased from Sigma-Aldrich and were used without further purification. The Low-UV PIC® B7 reagent (% by weight:methanol (18–22%), heptane sulfonic acid – sodium salts (4–6%), phosphate buffer solution (3–7%), water (65–75%), pH 3) mentioned in the HPLC purification and analysis section in the following texts was purchased from Waters. TLCs were run on aluminium precoated plates of silica gel 60 F_{254} (VWR). The compounds were localized when possible at 254 nm using a UV lamp and/or by dipping the TLC plates in a 1% ethanolic ninhydrin solution, a basic $KMnO_4$ aqueous solution or a 1% MeOH/H_2O (50/50, v/v) $FeCl_3$ solution and developing with a heat gun. Flash chromatography was conducted on silica gel (0.63–0.200 mm, VWR) columns. Carrier-free tritium gas (T_2) was purchased from RC TRITEC (Teufen, Switzerland) and contained 98.9% tritium.

HPLC purification and analysis

HPLC A. Equipment: Waters Alliance 2690 (or a Waters binary HPLC pump 1525) equipped with a UV spectrophotometer (Photodiode Array Detector, Waters 996) and a Berthold LB509 radioactivity detector; column: analytical Symmetry-M® C-18, Waters (50 × 4.6 mm); porosity: 5.0 μm; conditions: isocratic elution with solvA/solvB: 40/60 (v/v) [solvent A: H_2O containing Low-UV PIC B7 reagent (20 mL for 1000 mL); solvent B: H_2O/CH_3CN: 30 : 70 (v/v) containing Low-UV PIC B7 reagent (20 mL for 1000 mL)]; flow rate: 2.0 mL/min; temperature: RT; absorbance detection at $\lambda = 265$ nm.

HPLC B. Equipment: Shimadzu system equipped with a SCL-10A VP Controller, a LC-10AT VP pump, a UV SPD-10A VP UV-detector and a Berthold Flowstar 513 radioactivity detector equipped with a dedicated 50 μL flow cell [Berthold YG50-S5P, solid scintillation counting, granularity 125–250 μm]; column: semipreparative Hypersil® hyPURITY™ C18, Thermo Scientific (250 × 10 mm); porosity: 5.0 μm; conditions: eluent: CH_3CN/H_2O: 50/50 (v:v); flow rate: 5 mL/min; temperature: RT; UV detection at $\lambda = 265$ nm.

HPLC C. Equipment: Shimadzu system equipped with a SCL-10A VP Controller, a LC-10AT VP pump, a UV SPD-10A VP UV-detector and a Berthold Flowstar 513 radioactivity detector equipped with a dedicated 50 μL flow cell [Berthold YG50-S5P, solid scintillation counting, granularity 125–250 μm]; column: semipreparative Hypersil hyPURITY C18, Thermo Scientific (250 × 10 mm); porosity: 5.0 μm; conditions: eluent: CH_3CN/H_2O: 40/60 (v:v); flow rate: 4 mL/min; temperature: RT; UV detection at $\lambda = 265$ nm.

HPLC D. Equipment: Shimadzu system equipped with a SCL-10A VP Controller, a LC-10 AD VP pump and a LDC analytical UV-detector (Monitor 3200) and a Berthold Flowstar 513 radioactivity detector equipped with a dedicated 500 μL flow cell [Berthod Z500-5, liquid scintillation counting, Perkin Elmer Ultima-FLO™ M]; column: analytical Hypersil hyPURITY C18, Thermo Scientific (250 × 4.6 mm); porosity: 5.0 μm; conditions: eluent: CH_3CN (containing 0.1% of HCO_2H)/H_2O (containing 0.1% of HCO_2H): 50/50 (v:v); flow rate: 1 mL/min; temperature: RT; UV detection at $\lambda = 265$ nm.

Spectroscopy

NMR spectra were recorded on a Bruker (Wissembourg, France) Avance (400 MHz) apparatus using the residual hydrogen resonance of the deuterated solvent $CDCl_3$ ($\delta = 7.24$ ppm) and CD_2Cl_2 ($\delta = 5.32$ ppm) as internal standards for ^{1}H-NMR and the deuterated solvents CD_2Cl_2 ($\delta = 54.0$ ppm) as internal standard for ^{13}C-NMR. Proton-decoupled ^{3}H-NMR spectra were recorded in D_2O using the same spectrometer as described earlier in the text. The chemical shifts are reported in ppm, downfield from TMS (s, d, t, q, m and b for singlet, doublet, triplet,

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quadruplet, multiplet and broad, respectively). The MS were measured on a Thermo Electron (Les Ulis, France) Ion Trap LCQ Deca XP+ spectrometer (ESI+) or on a Waters (Milford, MA, USA) Micromass ZQ 2000 spectrometer (ESI+).

Miscellaneous

Radiosynthesis involving tritium gas was performed in dedicated glovebox using a custom-built microhydrogenation apparatus.[20,21] Concentrations to dryness (solvent removal) and HPLC purifications of all reaction mixtures or solutions containing tritiated compound(s) were also performed in dedicated gloveboxes. All synthesized products were obtained with a chemical purity greater than 95% according the ^{1}H-NMR spectra. This purity was confirmed by HPLC analysis for the compounds used as references.

Chemistry

*N,N-Diethyl-2-(2-(4-methoxyphenyl)-5,7-dimethylpyrazolo[1,5-a] pyrimidin-3-yl)acetamide (DPA-713, **2**)*

Resynthesized according to Reference 6. Analytical data were in accordance with those reported.

*N,N-Diethyl-2-(2-(4-hydroxyphenyl)-5,7-dimethylpyrazolo[1,5-a] pyrimidin-3-yl)acetamide (**3**)*

To a solution of compound **2** (1 g, 2.73 mmol) in CH_2Cl_2 (12 mL) was added dropwise at −60 °C a 1 M solution of boron tribromide in THF (13.7 mL, 13.7 mmol). The reaction mixture was stirred for 2 h at −20 °C, then poured over ice and extracted twice with CH_2Cl_2 (100 mL). The organic layers were combined, washed with brine, dried over Na_2SO_4, filtered and evaporated to dryness. The residue was crystallized from Et_2O/EtOH to afford pure **3** (640 mg, 55%) as light yellow crystals. *Rf* (heptane/acetone 1/1): 0.20. ^{1}H-NMR (CD_2Cl_2) δ 8.62 (bs, 1H, OH), 7.54 (d, 2H, J = 8.4 Hz, Ph), 6.81 (d, 2H, J = 8.4 Hz, Ph), 6.54 (s, 1H, H-6), 3.90 (s, 2H, CH_2), 3.47 (q, 2H, J = 6.8 Hz, N($\underline{CH_2}CH_3$)), 3.37 (q, 2H, J = 6.8 Hz, N($\underline{CH_2}CH_3$)), 2.71 (s, 3H, 7-CH_3), 2.52 (s, 3H, 5-CH_3), 1.18 (t, 3H, J = 6.8 Hz, N($CH_2\underline{CH_3}$)), 1.09 (t, 3H, J = 6.8 Hz, N($CH_2\underline{CH_3}$)). ^{13}C-NMR (CD_2Cl_2) δ 171.2 [C], 158.7 [C], 158.2 [C], 155.8 [C], 148.2 [C], 145.8 [C], 130.4 [2xCH], 125.3 [C], 115.9 [2xCH], 108.9 [CH], 100.8 [C], 43.0 [CH_2], 41.4 [CH_2], 28.7 [CH_2], 24.7 [CH_3], 17.2 [CH_3], 14.4 [CH_3], 13.3 [CH_3]. MS (ESI+): *m/z* 353 $(M+H)^+$.

1-Fluoro-2-tosyloxyethane

Resynthesized according to Reference 6. Analytical data were in accordance with those reported.

*N,N-Diethyl-2-(2-(4-(2-fluoroethoxy)phenyl)-5,7-dimethylpyrazolo [1,5-a]pyrimidin-3-yl)acetamide (DPA-714, **1a**)*

To a suspension of NaH (12 mg, 0.50 mmol) in dry THF (8 mL) was added a dry solution of THF (1 mL) containing compound **3** (100 mg, 0.28 mmol). The reaction mixture was stirred for 10 min at 5 °C and then allowed to warm up to room temperature before addition of a solution of 1-fluoro-2-tosyloxyethane (200 mg, 0.92 mmol) in dry CH_2Cl_2 (1 mL). The reaction mixture was heated overnight at 60 °C with stirring. The mixture was then partitioned between 1 M aq. HCl (100 mL) and CH_2Cl_2 (100 mL). The organic layer was then separated, washed with brine, dried over Na_2SO_4, filtered and evaporated to dryness. The residue was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH: 98/2 to 95/5 (v:v)) to afford **1a** (65 mg, 58%) as white crystals after crystallization from Et_2O. *Rf* (heptane/acetone 2/1): 0.25. ^{1}H-NMR (CD_2Cl_2) δ 7.77 (d, 2H, J = 8.8 Hz, Ph), 7.01 (d, 2H, J = 8.8 Hz, Ph), 6.55 (s, 1H, H-6), 4.77 (dt, 2H, $J^2_{H\text{-}F}$ = 47.6 Hz and $J^3_{H\text{-}H}$ = 4.0 Hz, CH_2F), 4.26 (dt, 2H, $J^3_{H\text{-}F}$ = 28.8 Hz and $J^3_{H\text{-}H}$ = 4.0 Hz, OCH_2CH_2F), 3.88 (s, 2H, $CH_2(C=O)$), 3.50 (q, 2H, J = 7.2 Hz, NCH_2CH_3), 3.38 (q, 2H, J = 7.2 Hz, NCH_2CH_3), 2.72 (s, 3H, 7-CH_3), 2.53 (s, 3H, 5-CH_3), 1.22 (t, 3H, J = 7.2 Hz, NCH_2CH_3), 1.11 (t, 3H, J = 7.2 Hz, NCH_2CH_3). ^{13}C-NMR (CD_2Cl_2) δ 170.3 [C], 159.2 [C], 158.2 [C], 155.0 [C], 148.3 [C], 145.3 [C], 130.3 [2xCH], 127.6 [C], 115.0 [2xCH], 108.7 [CH], 101.3 [C], 82.7 [d, $J^1_{F\text{-}C}$ = 170 Hz, CH_2], 67.8 [d, $J^2_{F\text{-}C}$ = 20 Hz, CH_2], 42.8 [CH_2], 41.0 [CH_2], 28.5 [CH_2], 24.9 [CH_3], 17.1 [CH_3], 14.7 [CH_3], 13.4 [CH_3]. MS (ESI+): *m/z* 399 $(M+H)^+$. t_R (HPLC A): 1.98 min. t_R (HPLC B): 8.9 min. t_R (HPLC C): 16.9 min. t_R (HPLC D): 6.59 min.

*N,N-Diethyl-2-(2-(3,5-dibromo-4-hydroxyphenyl)-5,7-dimethylpyrazolo [1,5-a]pyrimidin-3-yl)acetamide (**4**)*

To a solution of compound **3** (500 mg, 1.42 mmol) in acetic acid (5 mL) was carefully added dropwise bromine at room temperature whilst stirring, and the reaction was monitored by TLC to stop the addition of bromine before tribrominated or tetrabrominated products appeared. When the starting material was almost completely converted, the reaction was stopped by addition of water, and the reaction mixture was extracted with ethyl acetate. The organic layer was separated, washed with brine, dried over Na_2SO_4, filtered and evaporated to dryness. The residue was purified on silica gel column chromatography (heptane/EtOAc 7/3) to afford pure compound **4** (181 mg, 0.35 mmol) as a white powder in 25% yield and a 1 : 1 mixture (<5% yield), according to ^{1}H-NMR, of **4** and its monobrominated counterpart **5**.

Compound **4**. *Rf* (heptane/acetone 1/1): 0.29. ^{1}H-NMR (CD_2Cl_2) δ 7.92 (s, 2H, Ph), 6.61 (s, 1H, H-6), 6.37 (bs, 1H, OH), 3.93 (s, 2H, CH_2), 3.55 (q, 2H, J = 7.2 Hz, N($\underline{CH_2}CH_3$)), 3.41 (q, 2H, J = 7.2 Hz, N($\underline{CH_2}CH_3$)), 2.74 (s, 3H, 7-CH_3), 2.55 (s, 3H, 5-CH_3), 1.28 (t, 3H, J = 7.2 Hz, N($CH_2\underline{CH_3}$)), 1.15 (t, 3H, J = 7.2 Hz, N($CH_2\underline{CH_3}$)). ^{13}C-NMR (CD_2Cl_2) δ 169.8 [C], 158.2 [C], 151.8 [C], 150.1 [C], 147.5 [C], 145.2 [C], 131.8 [2xCH], 128.3 [C], 110.3 [2xC], 108.7 [CH], 100.8 [C], 42.4 [CH_2], 40.8 [CH_2], 27.7 [CH_2], 24.1 [CH_3], 16.5 [CH_3], 14.0 [CH_3], 12.8 [CH_3]. MS (ESI+): *m/z* 509 (51%), 511 (100%), 513 (48%) $(M+H)^+$.

Compound **5**. *Rf* (heptane/acetone 1/1): 0.20. ^{1}H-NMR ($CDCl_3$) δ 7.84 (d, J = 1.6 Hz, 1H, Ph), 7.61 (dd, ^3J = 8.4 Hz, ^4J = 1.6 Hz, 1H, Ph), 6.98 (d, J = 8.4 Hz, 1H, Ph), 6.52 (s, 1H, H-6), 3.88 (s, 2H, CH_2), 3.47 (q, 2H, J = 7.2 Hz, N($\underline{CH_2}CH_3$)), 3.37 (q, 2H, J = 7.2 Hz, N($\underline{CH_2}CH_3$)), 2.71 (s, 3H, 7-CH_3), 2.52 (s, 3H, 5-CH_3), 1.22 (t, 3H, J = 7.2 Hz, N($CH_2\underline{CH_3}$)), 1.11 (t, 3H, J = 7.2 Hz, N($CH_2\underline{CH_3}$)). MS (ESI+): *m/z* 431 (100%), 433 (97%) $(M+H)^+$.

*N,N-Diethyl-2-(2-(3,5-dibromo-4-(2-fluoroethoxy)phenyl)-5,7-dimethylpyrazolo[1,5-a]pyrimidin-3-yl)acetamide (**6**)*

To a 0.12 M NaOH solution in a 8 : 2 mixture of ethanol/water was added compound **5** (120 mg, 0.24 mmol) and 1-fluoro-2-tosyloxyethane (153 mg, 0.71 mmol). The reaction mixture was stirred overnight at 65 °C before being partitioned between water and ethyl acetate. The organic layer was separated, washed with brine, dried over Na_2SO_4, filtered and evaporated to dryness. The residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH 100/0 to 97/3, v:v) to afford pure compound **6** (95 mg, 0.17 mmol) as a white solid in 72% yield. *Rf* (heptane/acetone 3/2): 0.38. ^{1}H-NMR (CD_2Cl_2) δ 8.06 (s, 2H, Ph), 6.60 (s, 1H, H-6), 4.82 (dt, 2H, $J^2_{H\text{-}F}$ = 47.6 Hz, $J^3_{H\text{-}H}$ = 4.0 Hz, CH_2F), 4.32 (dt, 2H, $J^3_{H\text{-}F}$ = 28.8 Hz, $J^3_{H\text{-}H}$ = 4.0 Hz, $OCH_2\underline{CH_2}F$), 3.90 (s, 2H, CH_2), 3.55 (q, 2H, J = 7.2 Hz, N($\underline{CH_2}CH_3$)), 3.39 (q, 2H, J = 7.2 Hz, N($\underline{CH_2}CH_3$)), 2.73 (s, 3H, 7-CH_3), 2.54 (s, 3H, 5-CH_3), 1.28 (t, 3H, J = 7.2 Hz, N($CH_2\underline{CH_3}$)), 1.13 (t, 3H, J = 7.2 Hz, N($CH_2\underline{CH_3}$)). ^{13}C-NMR (CD_2Cl_2) δ 169.3 [C], 158.2 [C], 152.5 [C], 151.1 [C], 147.7 [C], 145.0 [C], 133.0 [C], 132.3 [2xCH], 118.0 [2xC], 108.9 [CH], 101.5 [C], 82.5 [d, $J^1_{F\text{-}C}$ = 169 Hz, CH_2], 72.1 [d, $J^2_{F\text{-}C}$ = 20 Hz, CH_2], 42.3 [CH_2], 40.6 [CH_2], 27.7 [CH_2], 24.3 [CH_3], 16.5 [CH_3], 14.2 [CH_3], 12.9 [CH_3]. MS (ESI+): *m/z* 555 (51%), 557 (100%), 559 (48%) $(M+H)^+$. t_R (HPLC A): 8.86 min. t_R (HPLC B): 23.9 min. t_R (HPLC D): 15.42 min.

Note that the aforementioned protocol was also carried out on a 1 : 1 mixture of **4** and **5** (5 μmoles each) to give the corresponding equimolar mixture of **6** and its monobrominated counterpart **7** (92% yield, with respect to **5**).

Compound **7**. *Rf* (heptane/acetone 2/1): 0.30. ^{1}H-NMR ($CDCl_3$) δ 8.02 (d, J = 2.0 Hz, 1H, Ph), 7.84 (dd, J = 8.4 Hz, J = 2.0 Hz, 1H, Ph), 6.99 (d, J = 8.4 Hz, 1H, Ph), 6.52 (s, 1H, H-6), 4.81 (dt, $J^2_{H\text{-}F}$ = 47.2 Hz, $J^3_{H\text{-}H}$ = 4.0 Hz, 2H, CH_2F), 4.32 (dt, $J^3_{H\text{-}F}$ = 27.2 Hz, $J^3_{H\text{-}H}$ = 4.0 Hz, 2H, $OCH_2\underline{CH_2}F$), 3.92 (s, 2H, $CH_2(C=O)$), 3.53 (q, J = 7.2 Hz, 2H, NCH_2CH_3), 3.41 (q, J = 7.2 Hz, 2H, NCH_2CH_3), 2.73 (s, 3H, 7-CH_3), 2.54 (s, 3H, 5-CH_3), 1.24 (t, J = 7.2 Hz, 3H,

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$NCH_2\underline{C}H_3$), 1.13 (t, $J = 7.2$ Hz, 3H, $NCH_2\underline{C}H_3$). MS (ESI+): *m/z* 477 (50%), 479 (100%) $(M + H)^+$. t_R (HPLC A): 3.81 min. t_R (HPLC B): 13.3 min. t_R (HPLC D): 9.31 min.

*Catalytic deuterodebromination at the micromolar scale: formation of [$^2H_2$]DPA-714 (**1b**)*

To a solution of compound **6** (20 mg, 36.0 μmol) in methanol (MeOH or MeOD-d_4, 2 mL) was added 10 mg of 10% Pd/C, and the reaction mixture was degased and placed under D_2 atmosphere (1 atm) and stirred for 30 min to 1 h at room temperature. The reaction was monitored by TLC, and when no remaining starting material was observed, the catalyst was removed by filtration and the resulting filtrate analysed, after evaporation, by ^{1}H-NMR, MS and HPLC. *Rf* (heptane/acetone 2/1): 0.25. ^{1}H-NMR (CD_2Cl_2) δ 7.75 (s, 2H, Ph), 6.55 (s, 1H, H-6), 4.77 (dt, 2H, $J^2_{H\text{-}F} = 47.2$ Hz and $J^3_{H\text{-}H} = 4.0$ Hz, CH_2F), 4.26 (dt, 2H, $J^3_{H\text{-}F} = 28.8$ Hz and $J^3_{H\text{-}H} = 4.0$ Hz, $OC\underline{H}_2CH_2F$), 3.88 (s, 2H, CH_2 (C = O)), 3.50 (q, 2H, $J = 7.2$ Hz, $NC\underline{H}_2CH_3$), 3.38 (q, 2H, $J = 7.2$ Hz, $NC\underline{H}_2CH_3$), 2.72 (s, 3H, 7-CH_3), 2.53 (s, 3H, 5-CH_3), 1.22 (t, 3H, $J = 7.2$ Hz, $NCH_2\underline{C}H_3$), 1.11 (t, 3H, $J = 7.2$ Hz, $NCH_2\underline{C}H_3$). ^{13}C-NMR (CD_2Cl_2) δ 170.3 [C], 159.1 [C], 158.2 [C], 155.0 [C], 148.3 [C], 145.3 [C], 130.3 [2xCH], 127.6 [C], 115.0 [low intensity triplet, 2xCD], 108.7 [CH], 101.3 [C], 82.7 [d, $J^1_{F\text{-}C} = 169$ Hz, CH_2], 67.8 [d, $J^2_{F\text{-}C} = 20$ Hz, CH_2], 42.8 [CH_2], 41.0 [CH_2], 28.5 [CH_2], 24.9 [CH_3], 17.1 [CH_3], 14.7 [CH_3], 13.4 [CH_3]. MS (ESI+): *m/z* 401 $(M + H)^+$. t_R (HPLC A): 2.01 min.

*Catalytic tritiation: synthesis, purification and formulation of [^{3}H] DPA-714 (**1d**)*

In a dedicated, heat gun-dried, 5 mL glass vessel, was introduced a solution of compound **6** (5.2 mg, 9.3 μmol) in methanol (1 mL) followed by the addition of 5.1 mg of 10% Pd/C. The entire custom-built microhydrogenation apparatus was then exhaustively degassed by the application of three freeze (liquid nitrogen)–pump–thaw cycles. The vessel/reaction mixture was once more frozen and tritium gas (T_2, 18.65 mL, stored on a metal alloy from a homemade apparatus) was admitted into the system. The reaction vessel was then isolated, the solution allowed to warm up at room temperature and the pressure in the reaction vessel adjusted to 0.92 atm (0.93 bars). The reaction mixture was then vigorously stirred at room temperature for 20 min. Excess of tritium was then adsorbed back on the molecular sieve trap, and the reaction mixture passed through a 25 mm Acrodisc® Premium PN AP-4564 T syringe filter (Pall Corporation, 0.2 μm) in order to remove the catalyst (Pd/C). The filter unit was then rinsed twice with methanol (1 mL) and the filtrate diluted with more methanol (10 mL). The resulting solution was then concentrated to dryness under reduced pressure and the residue rapidly solubilized again in methanol (10 mL). This sequence was performed twice in order to remove all labile tritium atoms.

An aliquot (0.01%) of the crude solution (13 GBq) was submitted to HPLC analyses (HPLC C and D, see Results and Discussion Section) and the remaining solution concentrated once again to dryness.

The residue was resolubilized in CH_3CN (0.9 mL), the solution equally divided into three equal portions and each one was then independently purified using HPLC [HPLC C]. The fractions containing [^{3}H]DPA-714 (**1d**) were collected (t_R: ~17 min ± 1 min) and combined together. An aliquot (0.01%) was submitted to HPLC analysis (HPLC D, see Results and Discussion Section) and the remaining solution concentrated to dryness. Analytical data were collected at this stage (^{3}H-NMR, MS and HPLC purity), then the residue (7 GBq, 36%) was rapidly resolubilized in pure methanol (200 mL) and stored at -20 °C (±35 MBq/mL). ^{3}H-NMR (D_2O, proton-decoupled) δ 7.11 (s, T-Ph) [47%], 2.75 (s, 7-CTH_2), 2.73 (s, 7-CT_2H), 2.71 (s, 7-CT_3) [26%], 2.55 (s, 5-CTH_2), 2.53 (s, 7-CT_2H), 2.51 (s, 7-CT_3) [27%]. ^{3}H-NMR (D_2O, proton-coupled) δ 7.11 (d, $J^3_{T\text{-}H} = 9.1$ Hz, T-Ph), 2.75 (t, $J^2_{T\text{-}H} = 15.0$ Hz, 7-CTH_2), 2.73 (d, $J^2_{T\text{-}H} = 15.0$ Hz, 7-CT_2H), 2.71 (s, 7-CT_3), 2.55 (t, $J^2_{T\text{-}H} = 15.0$ Hz, 5-CTH_2), 2.53 (d, $J^2_{T\text{-}H} = 15.0$ Hz, 5-CT_2H), 2.51 (s, 5-CT_3). MS (ESI+): *m/z* $(M + Na)^+$: 423 (25.3%, 1 T), 425 (23.6%, 2 T), 427 (15.9%, 3 T), 429 (14.0%, 4 T), 431 (4.3%, 5 T). Peaks at 422, 424, 426, 428, 430 and 432 were also detected on the MS spectra, but these peaks were all associated with nonsignificant relative % values (0.2%, 0.4%, 1.3%, 0.4%, 0.5% and 0.1%, respectively). Note that a peak at 433 (6 T) was also observed but again, associated with a nonsignificant % value (0.1%).

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Conflict of interest

The authors did not report any conflict of interest.

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