Synthesis of C-14 labeled GABA_A α2/α3 selective partial agonists and the investigation of late-occurring and long-circulating metabolites of GABA_A receptor modulator AZD7325

Markus Artelsmair1 | Chungang Gu2 | Richard J. Lewis3 | Charles S. Elmore1

1 Early Chemical Development, Pharmaceutical Sciences, IMED Biotech Unit, AstraZeneca, Gothenburg, Sweden
2 DMPK, Oncology, IMED Biotech Unit, AstraZeneca, Boston, MA, USA
3 Medicinal Chemistry, Respiratory, Inflammation and Autoimmunity, IMED Biotech Unit, AstraZeneca, Gothenburg, Sweden

Correspondence
Markus Artelsmair, Early Chemical Development, Pharmaceutical Sciences, IMED Biotech Unit, AstraZeneca, Papparedsleden 1, 431 50 Mölndal, Sweden. Email: markus.artelsmair@astrazeneca.com

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1 | INTRODUCTION

γ-Aminobutyric acid (GABA) is a small, flexible molecule which can exist in a number of low-energy conformations. It is the main inhibitory neurotransmitter in the mammalian central nervous system. It has been estimated that GABA is used as a transmitter in between 20% and 50% of all central synapses, depending on the investigated brain region.1,2 GABA binding to a GABA receptor affects the receptor’s ability to conduct neural impulses, causing hyperpolarization. There are 3 classes of GABA receptors, which have been identified with the help of conformationally restricted analogues of GABA: GABA_A, GABA_B and GABA_C.3 GABA_A and GABA_C are transmembrane G-protein coupled receptors that activate secondary messengers as well as K⁺ and Ca²⁺ ion channels; this type of receptor is termed metabotropic receptor. On the other hand, GABA_A and GABA_C are ligand-gated transmembrane ion channels, called ionotropic receptors.3 Nineteen GABA_A receptor subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π and ρ1–3) have been identified.4 These subunits assemble into heterogeneous pentameric chloride ion channels with a large variety of possible combinations, which may explain the diverse range of physiological functions of GABA_A receptors. However, in...
the human brain, the predominant stoichiometry has been found to be 2 α units, 2 β units and 1 γ unit.\(^5,6\) There are a number of allosteric ligands that can modulate the response of GABA\(_\text{A}\) receptors to GABA. The most thoroughly studied and characterized site on the GABA\(_\text{A}\) receptor complex is the binding site of benzodiazepines (BZ) and BZ-like compounds, that is located on the interface of the α/β subunits.\(^7\) Ligands binding at this site can have 3 functional effects on the chloride current. The first of the 3 distinct modulatory modes is agonism or positive allosteric modulation, both leading to an increase in the GABAA induced chloride current. Secondly, binding at the BZ site can cause either inverse agonism or negative allosteric modulation and thereby decrease GABA induced chloride current. Thirdly, binding may produce neutral antagonism and have no effect on the chloride current.\(^8\) These modes of action at the α2 and α3 subunits lead to different behavioral effects. Positive allosteric modulators are anxiolytic, while negative allosteric modulations are anxiogenic and neutral antagonists cause no apparent physiological effect.\(^9,10\)

GABA\(_\text{A}\) receptors have been the target of numerous drugs. Most noticeably, BZs have been used for the treatment of anxiety and insomnia, among other diseases, for decades. Despite their rapid onset and their high efficacy, their adverse effects, including ataxia, amnesia, sedation as well as dependence and concomitant withdrawal, are significant and limit their applicability in the long-term treatment of anxiety disorders.\(^11\) As a result, they have recently been relegated to second-line treatment.\(^12\) Building on the information available from the first-generation BZs such as their pharmacological profile, safer, more effective and more tolerable alternatives are being sought.\(^13\) The fact that BZs bind non-selectively to GABA\(_\text{A}\) α1, α2, α3 and α5 has been proposed as the cause for some of the side effects of this class of drugs.\(^9\) Previous studies have shown that positive modulation of the α1 subunit correlates with sedation whereas modulation of α2 and α3 is linked to anxiolytic effects.\(^6,14,15\) It has also been suggested that the intrinsic activity of BZ modulators might be related to their side effects.\(^16,17\) Therefore, partial agonism could allow for separation of anxiolytic effects from unfavorable effects on the CNS. Selectively potentiating activity of GABA\(_\text{A}\) receptors containing α2 and α3 subunits with BZ site modulators could thus be a way to maximize therapeutic and reduce side effects.\(^11,18\)

Based on this information, AstraZeneca introduced a program to develop an orally bioavailable, positive modulator of the GABA\(_\text{A}\) α2 and α3 subunits in order to treat anxiety disorders. Several selective target compounds, including AZD7325 and AZD6280, were identified.\(^8\) AZD7325 has a much higher binding affinity for α1, α2, and α3 (Ki of 0.5, 0.3 and 1.3 nM, respectively) than for α5 (230 nM).\(^19\) Both compounds have been found to have potent anxiolytic-like effects without sedation in preclinical models and showed a distinct electro-encephalogram signature.\(^20\) Furthermore, recent PET studies confirmed that high GABA\(_\text{A}\) receptor occupancy by AZD7325 and AZD6280 could be reached without clear sedation or cognitive impairment.\(^11\) AZD7325 (4-amino-8-(2-fluoro-6-methoxyphenyl)-N-propylcinnoline-3-carboxamide, Figure 1B), the primary focus of this report, has undergone multiple clinical trials, including 2 Phase II proofs-of-concept in patients with General Anxiety Disorder.\(^8\) It is, however, worth noting that in a double-blind, randomized, 4-way crossover study with 16 healthy male volunteers, CNS effects were found to be modest at the administered dosage.\(^19\) This suggests that higher doses than those predicted by GABA-receptor occupancy may be warranted to achieve clinically effective concentrations of AZD7325.

Another study on the effect of the 2 positive GABA\(_\text{A}\) α2 and α3 receptor modulators on plasma prolactin levels reached similar conclusions.\(^23\) In the process of developing both drug candidates, studying their metabolism and pharmacokinetics was crucial to their understanding. In order to thoroughly investigate and comprehend the drug metabolism and pharmacokinetic (DMPK) properties of these compounds, C-14 labeled analogues were required.

## RESULTS AND DISCUSSION

During drug discovery efforts, a wide range of potential candidates were identified based on the substructure shown in Figure 1A. The highest priority compounds all contained a substituted cinnoline.\(^21,22\) Three compounds showing particular promise as selective GABA\(_\text{A}\) α2 and α3 positive modulators were selected for further studies on their DMPK properties, cf. Figure 1B.

In order to efficiently label all 3 compounds, a common intermediate was envisioned that could then be quickly partitioned into the targets shown in Figure 1B. The cinnoline moiety was common to the 3 compounds and analysis of the medicinal chemistry route leading to the 8-bromocinnoline made it appear readily amenable for synthesis with labeled compounds. The synthesis of the key intermediate was accomplished by coupling K\(^{14}\)CN with N-propyl bromoacetamide (1) to give the corresponding nitrile, N-propyl-2-[\(^{14}\)C]cyanoacetamide (2) in 76% yield and high purity. This was then converted to an advanced aromatic intermediate in 2 steps (cf. Figure 2). N-Propyl-2-[\(^{14}\)C]cyanoacetamide (2) was reacted with 2-bromodiadobenzene to give an isomeric mixture of E/Z- N’-(2-bromophenyl)-2-oxo-2-(propylamino) acetoxyhydrizonoyl [\(^{14}\)C]cyanoate (3) in a 3:1 ratio with a

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*NCT00807937 and NCT00808249*
yield of 61%. The isomers were separable, but this was more easily accomplished in the next step. The desired cinnoline (4) was afforded by an AlCl₃ catalyzed cyclization at 100 °C in toluene in 76% yield. At this point, the key intermediate was partitioned to prepare 3 target compounds via Suzuki coupling reactions (cf. Figure 3). The very poor yield of 31% was determined by the separation method.

FIGURE 1 (A) The general structural class of selective GABAₐ α2 and α3 positive modulators developed by AstraZeneca²¹,²²; (B) 3 drug candidates selected for C-14 labeling to study their DMPK properties.

FIGURE 2 Synthesis of the common precursor for the radiolabeling targets. Reaction conditions are shown.

FIGURE 3 Conversion of the key intermediate into the 3 target compounds via different Suzuki coupling reactions.
(12%) for the conversion to $^{14}$C$^{12}$AZ12449694 in Figure 3 was due to the poor stability of the boron reagent as well as difficulties with the purification procedure.

### 3 | METABOLISM

$^{12}$AZ12449694 was not selected to advance into pre-clinical development. The metabolism of AZD6280 and its investigation through a C-14 labeled analogue have previously been reported.24,25 The following sections will, therefore, primarily be concerned with the study of AZD7325. The metabolism of the majority of marketed drugs involves the cytochrome P450 (CYP) enzymes. CYP3A4 is the major hepatic and intestinal CYP isoform in humans and plays a role in approximately 50% of clinically used drugs.26 In previous studies, induction and drug-drug interactions for AZD7325 have been assessed.27,28 Herein, we report the preparation and use of radiolabeled compounds to understand the metabolism more thoroughly. For metabolites in safety testing analysis, detailed investigations on the metabolite profile were carried out. $^{14}$C\textsubscript{AZD7325} guided the profiling and identification of a great number of AZD7325 drug metabolites formed across species in vivo, revealing approximately 40 different metabolites. These were produced through various combinations of oxidations at 3 sites of the AZD7325 molecule as well as subsequent conjugation reactions. The rat metabolite profiles (for instance the plasma metabolite profile shown in Figure 4) and the resulting biotransformation scheme (shown in Figure 5) were determined using $^{14}$C\textsubscript{AZD7325}, highlighting the immense value of radio-labeling for DMPK studies. The full characterization of these metabolites is discussed by Gu et al.,29 noting that M9, M10 and M42 (cf. Figure 5) were only seen in trace or minor amounts in vitro or in vivo after a single dose; however, that all 3 became major circulating metabolites after repeated oral doses.29 Furthermore, this work includes the detailed investigations which led to the proposed tricyclic structure for M9, and thus also for M10 and M42, formed via cyclisation and successive aromatization (cf. Figure 6).

Two alternative mechanisms that can account for the formation of M9 and its subsequent transformation to M10 and M42 are proposed (shown below in Figure 6). In the first case (A), an oxidative rearrangement catalyzed by cytochrome P-450 followed by dehydrogenation and eventually ring closure leads to the formation of the novel tricyclic core of M9. Further oxidative metabolism then yields M42 via hydroxylation and M10 via O-demethylation. Alternatively, the dehydration step could also take place after the initial oxidation yet prior to the ring closure, which then proceeds via the resulting imine, followed by aromatization to M9 (B).

### 4 | SYNTHETIC STANDARD

In order to confirm the proposed structure of M9, a synthetic standard was prepared starting from AZD7325. The chemical synthesis was initiated by the hydrolysis of

**FIGURE 4** Metabolite profile of $^{14}$C\textsubscript{AZD7325} in female rat plasma pooled over the first 12 hours after dosage (2 mg/kg oral). Bold M# (i.e., M7, M13, M14 and M15) indicate major circulating phase-I metabolites in rat. “(s)” and “(g)” denote sulfate and glucuronide, respectively.
A biotransformation scheme for AZD7325 which was elucidated with $[^{14}C]$AZD7325 in rat in vivo. The unusual oxidative metabolites M9, M10 and M42 are shown. M42, absent from rat in vivo samples after a single oral dose of $[^{14}C]$AZD7325, was observed at trace amounts in liver microsomes across species.
the amide functionality using concentrated hydrochloric acid. The newly formed carboxylic acid was converted to acyl chloride 1 using thionyl chloride which was then transformed into primary amide 2 using ammonium hydroxide. In the final step, this precursor was reacted with propionyl chloride and cyclized to afford M9 in a 1-pot reaction. Interestingly, 2 products were formed in this reaction in a 5:2 ratio. Both compounds had the

FIGURE 6  The 2 proposed metabolic mechanisms for the formation of M9, M10 and M42. Option A proceeds via an initial oxidation, whereas B involves the loss of water prior to the intramolecular ring closure.
expected mass and very similar retention times on reversed-phase high performance liquid chromatography (RP-HPLC). They were separated using mass directed supercritical fluid chromatography mass spectrometry (SFC-MS) and thoroughly examined by NMR, in particular by 2D $^{15}$N HMBC spectroscopy. The minor component of the reaction was identified as M9 while the major product was nitrile 7 (Figure 7).

Given their structure and the reaction conditions employed, it appears likely that the pathways to the 2 compounds proceed through a common intermediate formed after the reaction of the primary amine with propionyl chloride. Subsequent cyclization then takes place either via the nitrogen atom of the primary amide, to afford M9, or via the oxygen atom. The latter leads to an oxygenated heterocycle which undergoes ring opening to give nitrile 7, for which a proposed mechanism is shown in Figure 8.

The synthetic standard was used to unambiguously confirm the structure of M9 using high resolution ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). In addition, the authentic standard was used to verify M10 and M42 by incubation of the standard in human liver microsomes.29

5  | CONCLUSION

In summary, C-14 labeled analogues of 3 AstraZeneca drug candidates, intended for the potential treatment of anxiety, were synthesized. The strategy of using an advanced intermediate to access this structural class allowed for the rapid generation of the target compounds. The diverse metabolite profile of the selective GABA$_A$ $\alpha_2$ and $\alpha_3$ receptor modulator AZD7325 was investigated and a wide range of metabolites were identified with the aid of [14C]AZD7325. In vivo studies in rat and in vitro studies on human, rat, mouse, rabbit and dog liver microsomes were performed with the radiolabeled compound, revealing approximately 40 metabolites. Three late-
occurring and long-circulating metabolites required further elucidation; thus, an authentic synthetic standard of M9 was prepared, confirming the proposed structure as well as those of its derivatives M10 and M42. A mechanism for the formation of M9 via metabolic cyclization and aromatization is presented. The results obtained in these studies have proven helpful for metabolites in safety testing analysis at steady state.

6 | EXPERIMENTAL SECTION

6.1 | General

All chemicals were purchased from Sigma-Aldrich or its subsidiaries. K14CN was obtained from American Radiolabeled Chemicals. 2-Bromo-N-propylacetamide (1) was prepared according to the procedure of Gathergood et al and was purified by bulb-to-bulb distillation.30 Anhydrous solvents were obtained from Aldrich and were used without further purification. Reactions were magnetically stirred unless otherwise noted. For reactions at elevated temperature, DrySyn temperature. Stated reaction temperatures refer to the external DrySyn temperature.

1H NMR and 13C NMR spectra were acquired on a Bruker Avance III spectrometer running at a proton frequency of 500.1 MHz and fitted with a cryogenic probe or on Bruker Avance Nanobay spectrometers operating at 400 MHz. Chemical shifts are reported in ppm (δ) relative to tetramethylsilane with the solvent resonance as internal standard (7.26 and 77.16 ppm for CDCl3, 3.31 and 49.00 ppm for CD3OD and 2.50 and 39.52 ppm for DMSO-d6, as reported in: H. E. Gottlieb, V. Kotlyar and A. Nudelman, J. Org. Chem., 1997, 62, 7512-7515). The signals derived from the 1H NMR spectra are reported with chemical shifts, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sext = sextet, dt = doublet of triplets, dq = doublet of quartets, ddd = double doublet of triplets, td = triplet of doublets, tt = triplet of triplets, qd = quartet of doublets or m = multiplet), coupling constant (Hz) and integration. Data for 13C-NMR are reported with chemical shifts and coupling to 19F where observed. Flash column chromatography was carried out using pre-packed silica gel columns supplied by Biotage and using a Biotage automated flash systems with UV detection.

LC/MS analysis was performed on an HP MSD-1100 using a Luna-C18 (2) column, with a 10% to 100% gradient over 10 minutes with MeCN-0.1% formic acid and electrospray ionization.

The reaction products were identified by HPLC comparison with commercially available materials or AstraZeneca Medicinal Chemistry intermediates.

Analytical HPLC was performed using a HP 1100 series HPLC system using either.

Method A : 10% to 100% MeOH-0.1% TFA
Method B : 30% to 60% MeOH-0.1% TFA
Method C : 10% to 40% MeCN-0.1% TFA

All HPLC analyses were conducted using a flow rate of 1 mL/min on 4.6 mm × 100 mm columns Phenomenex Luna C18 (2) heated to 30 °C over 20 minutes and concluded with a 5-minute wash of 100% MeCN or MeOH.

For the synthesis of M9, HPLC was performed using a system composed of a Gilson 322 Pump equipped with a Gilson UV/VIS-152 lamp with an Xbridge™ Prep C-18 10 μm OBD™ 19 × 250 mm Column.

For the synthesis of M9, LCMS was acquired on a Waters Acquity UPLC using a BEH C18 column (50 mm × 2.1 mm, 1.7 μm particles) with a 10% to 90% gradient over 2 minutes with MeCN-NH4/NH4CO3 or MeCN-formic acid and electrospray ionization.

6.2 | Radiochemistry—synthesis

6.2.1 | 2-[14C]Cyano-N-propylacetamide (2)

A solution of 2-bromo-N-propylacetamide (1) (0.526 g, 2.92 mmol) in THF (2 mL) and water (2 mL) was added to K14CN (156 mg, 2.32 mmol, 128 mCi) and the resulting solution was stirred overnight at room temperature. The reaction mixture was then diluted with water (10 mL) and CH2Cl2 (25 mL) and the layers were separated. The aqueous layer was extracted with CH2Cl2 (25 mL) and the organic layers were combined to give 68 mCi (96.9% radiochemical purity, HPLC method A). The organic layer was concentrated to give a yellow oil (343 mg) which consisted of 1H NMR (500 MHz, CDCl3): δ (ppm) 0.97 (t, J = 7.3 Hz, 11H), 1.60 (m, 7H), 3.28 (m, 7H), 3.38 (s, 3H), 3.90 (s, 4H).

6.2.2 | N-(2-Bromophenyl)-2-oxo-2-(propylamino)acetohydrazoneyl [14C]cyano-nitride (3)

A slurry of 2-bromoaniline (409 mg, 2.38 mmol) in acetic acid (0.6 mL), water (1.2 mL) and concentrated HCl (0.6 mL) was cooled to 0 °C as a solution of sodium nitrite (197 mg, 2.86 mmol) in water (1 mL) was added over 1 hour. The resulting solution was then stirred for 30 minutes at 0 °C. It was then added to a solution of 2-[14C]cyano N-propylacetamide (2) (158 mg, 1.24 mmol, 68 mCi) (contaminated with 185 mg [calculated] of 2-
propylcinnoline and sodium acetate (691 mg, 8.42 mmol) in ethanol (3 mL) and water (3 mL) at 0 °C. After stirring at 0 °C for 5 hours, the reaction mixture was partitioned between CH₂Cl₂ (25 mL) and saturated aqueous NaHCO₃ (15 mL) and the layers were separated. The aqueous layer was extracted twice with CH₂Cl₂ (2 × 15 mL) and the combined organic layers were dried over MgSO₄ and filtered to yield 53 mCi. The solvent was removed to afford N-(2-bromophenyl)-2-oxo-2-(propylamino)acetohydrazonoyl [¹⁴C]cyanide (3) as a yellow solid (78% radiochemical purity as a 3:1 mixture of 2 isomers, HPLC method A).

LC/MS (M + 1): 309 (11%), 311 (100%), 313 (86%).

6.2.3 | 4-Amino-8-bromo-N-propylcinnoline-4-[¹⁴C]-3-carboxamide (4)

A solution of (E and Z)-N’-(2-bromophenyl)-2-oxo-2-(propylamino)acetohydrazonoyl [¹⁴C]cyanide (3) (147 mg, 0.47 mmol, 26 mCi) in toluene (12 mL) was stirred under N₂ as AlCl₃ (292 mg, 2.19 mmol) was added. The solution was heated to 100 °C and stirred overnight. The solution was concentrated to dryness and the residue taken up with CH₂Cl₂ (25 mL) and water (25 mL). The layers were separated and the organic layer was extracted 3 times with 1 M HCl (3 × 25 mL) to leave 3 mCi in the organic layer which was discarded. The combined aqueous layers were basified with NaHCO₃ to pH >8 and the organic layer which was discarded. The combined aqueous layer was extracted with CH₂Cl₂ (5 × 25 mL). The combined organic layers were dried over MgSO₄ and filtered to give 21 mCi. The solvent was removed and the solid was taken up in CH₂Cl₂ (2.5 mL). The solution was cooled at 0 °C for 10 minutes and of 2.5 iPrOH (1 mL) was added. The solution was stirred at RT vigorously at 70 °C for 2 hours. Every 30 minutes during this period, (2-fluoro-6-methoxyphenyl)boronic acid (100 mg, 0.58 mmol) was added to the solution. The reaction mixture was poured into EtOAc (50 mL) and water (50 mL) and the layers separated. The aqueous layer was extracted with EtOAc (2 × 25 mL). The combined organic layers were concentrated to dryness to give 8.5 mCi (81% radiochemical purity, method C). Purification by preparative HPLC (15 to 50% MeCN-0.1% TFA over 40 minutes on a 21.2 × 250 mm Phenomenex Luna C-18(2) column, 30 mL/min) afforded 6.6 mCi of [¹⁴C]AZD7325.  4-amino-8-(2-fluoro-6-methoxyphenyl)-N-propylcinnoline-[¹⁴C]-3-carboxamide as the TFA salt in 98.7% radiochemical purity (method C) and a specific activity of 55 mCi/mmol.

LC/MS (M + 1): 355 (12%), 356 (3%), 357 (100%), 358 (19%), 359 (2%).

¹H NMR (500 MHz, CD₃OD): δ (ppm) 8.20 (dd, J = 8.5, 1.2 Hz, 1H), 7.66 (td, J = 8.4, 1.0 Hz, 1H), 7.60 (d, J = 8.3, 6.8 Hz, 1H), 7.35 (qd, J = 8.3, 6.8 Hz, 1H), 6.87 (d, J = 8.5 Hz, 1H), 6.77 (t, J = 8.5 Hz, 1H), 3.60 (s, 3H), 3.31 (t, J = 7.2 Hz, 2H), 3.21 (m, 2H), 1.58 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H).

¹⁹F NMR (470 MHz, CD₃OD): δ (ppm) -115.4, -76.9 (TFA).

¹³C NMR (125 MHz, CD₃OD): δ (ppm) 169.4, 162.2 (d, J = 2455 Hz), 160.2 (d, J = 7.3 Hz), 148.4, 135.7, 132.9, 131.3, 131.2, 129.5, 129.1, 122.9, 118.1, 116.9 (d, J = 20 Hz), 108.5 (d, J = 74.1 Hz), 108.6 (d, J = 98.8 Hz), 56.6, 41.9, 23.9, 11.8.

6.2.5 | [¹⁴C]AZD6280

A solution of 4-amino-8-bromo-N-propylcinnoline-4-[¹⁴C]-3-carboxamide (4) (55.4 mg, 0.18 mmol, 12 mCi, mmol) and Pd(PPh₃)₄ (18 mg, 0.02 mmol) in DME (5 mL) was degassed by bubbling N₂ through the solution for 5 minutes. Then, it was stirred vigorously at 70 °C for 2 hours. Every 30 minutes during this period, (2-fluoro-6-methoxyphenyl)boronic acid (100 mg, 0.58 mmol) was added to the solution. The reaction mixture was poured into EtOAc (50 mL) and water (50 mL) and the layers separated. The aqueous layer was extracted with EtOAc (2 × 25 mL). The combined organic layers were concentrated to dryness to give 8.5 mCi (81% radiochemical purity, method C). Purification by preparative HPLC (15 to 50% MeCN-0.1% TFA over 40 minutes on a 21.2 × 250 mm Phenomenex Luna C-18(2) column, 30 mL/min) afforded 6.6 mCi of [¹⁴C]AZD6280. 4-amino-8-(2,5-dimethoxyphenyl)-N-propylcinnoline-[¹⁴C]-3-
carboxamide with 99.8% radiochemical purity (0% to 30% MeCN-0.1% TFA over 20 minutes followed by a wash on Luna C-18 (2)) and a specific activity of 55 mCi/mmol.

LC/MS (M + 1): 367(12%), 368 (3%), 369 (100%), 370 (20.3%), 371 (2.7%), 372 (0.2%).

1H NMR (500 MHz, CD3OD): δ (ppm) 8.84 (s, 1H), 7.90 (s, 1H), 7.88 (s, 1H), 7.11 (m, 1H), 6.93 (d, 1H), 3.55 (s, 3H), 3.31 (q, 2H), 3.26 (s, 3H), 1.59 (m, 2H), 0.91 (t, 3H). (Note: 4 aromatic carbons could not be seen on the spectrum).

13C NMR (125 MHz, CD3OD): δ (ppm) 164.4, 153.3, 151.1, 129.0, 128.2, 122.9, 117.2, 116.8, 115.5, 112.9, 55.9, 55.6, 40.6, 22.2, 11.3. (Note: 4 aromatic carbons could not be seen on the spectrum).

6.2.6 | [14C]AZ12449694

A solution of 4-amino-8-bromo-N-propylcinnoline-4-[[14C]-3-carboxamide (4) (7.5 mCi), Pd(PPh3)4 (18 mg, 0.02 mmol), 2,4-dimethoxy-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyrimidine (72 mg, 0.27 mmol) and Na2CO3 (36 mg, 0.34 mmol) in DME (2.5 mL) and water (1 mL) was degassed by bubbling N2 through the solution for 5 minutes. The solution was then warmed to 92 °C and stirred under N2 overnight. LC/MS indicated the reaction to be 50% complete which was judged to be sufficient. The solution was diluted with water (5 mL) and extracted with CH2Cl2 (2 × 10 mL) to give 6 mCi of a yellow solution. The solution was concentrated to dryness and applied to preparative HPLC in 3 batches (20% to 40% MeCN-0.1% Formic acid over 35 minutes, 22.5 × 250 mm Luna C-18(2), 30 mL/min) to give 3.1 mCi of 4-amino-8-(2,4-dimethoxypyrimidin-5-yl)-N-propylcinnoline-4-[[14C]-3-carboxamide (I4C)AZ12449694. HPLC analysis showed the purity to be 98.8% (0 to 30% MeCN-0.1% TFA over 20 minutes followed by a wash on Luna C-18 (2)) and the specific activity to be 55 mCi/mmol.

LC/MS (M + 1): 369(13.7%), 370 (3.2%), 371 (100%), 372 (20%), 373 (2.5%), 374 (0.1%).

1H NMR (500 MHz, CDCl3): δ (ppm) 8.49 (t, J = 5.6 Hz, 1 H), 8.32 (s, 1 H), 8.04 (d, J = 7.0 Hz, 1 H), 7.67 - 7.83 (m, 2 H), 4.06 (s, 3 H), 3.93 (s, 3 H), 3.45 (q, J = 6.8 Hz, 2 H), 1.66 (tq, J = 7.5, 7.3 Hz, 2 H), 1.00 (t, J = 7.3 Hz, 3 H).

6.3 | Preparation of the M9 standard

6.3.1 | 4-Amino-8-(2-fluoro-6-methoxyphenyl)cinnoline-3-carboxylic acid (5)

Hydrogen chloride (12 M, 25 mL, 300 mmol) was added to 4-amino-8-(2-fluoro-6-methoxyphenyl)-N-propylcinnoline-3-carboxamide (500 mg, 1.41 mmol) and the mixture was refluxed at 130 °C overnight. The solvent was removed on the rotary evaporator to afford a yellow solid, 4-amino-8-(2-fluoro-6-methoxyphenyl)cinnoline-3-carboxylic acid-HCl (490 mg, 1.40 mmol, 99%) that was used directly in the next step.

LC/MS (M + 1): 314.1 (100%).

1H NMR (400 MHz, DMSO-d6): δ (ppm) 8.45 (d, J = 8.2 Hz, 1H), 7.61 - 7.82 (m, 2H), 7.39 - 7.5 (m, 1H), 6.99 (d, J = 8.4 Hz, 1H), 6.91 (t, J = 8.4, 8.4 Hz, 1H), 3.64 (s, 3H).

13C NMR (126 MHz, DMSO-d6): δ (ppm) 169.4, 161.4, 159.0, 158.4, 158.3, 146.4, 144.5, 133.5, 131.1, 130.0, 129.8, 129.7, 127.7, 122.8, 116.1, 116.0, 115.8, 107.6, 107.4, 107.3, 107.2, 56.0.

6.3.2 | 4-Amino-8-(2-fluoro-6-methoxyphenyl)cinnoline-3-carboxamide (6)

Thionyl chloride (12 mL, 165 mmol) was added to 4-amino-8-(2-fluoro-6-methoxyphenyl)cinnoline-3-carboxylic acid (460 mg, 1.47 mmol) and the yellow solution heated to reflux at 80 °C for 4 hours. The reaction mixture was cooled to RT and concentrated under reduced pressure. This afforded 4-amino-8-(2-fluoro-6-methoxyphenyl)cinnoline-3-carboxyl chloride as an orange solid that was used directly in the next step.

The orange residue was cooled to 0 °C and suspended in acetonitrile (5 mL). Aqueous ammonium hydroxide solution (25.0% – 30.0% NH3 basis, 5 mL) was added and the mixture was stirred at 0 °C for 30 minutes. Acetonitrile was removed on the rotovap and the aqueous solvent removed by freeze drying. The solid residue was further purified via HPLC (20% to 60% MeCN-0.2% NH3 over 25 minutes, Xbridge Prep C-18 10 μm OBD 19 × 250 mm Column, 20 mL/min). Acetonitrile was removed on the rotovap and the aqueous solvent removed by freeze drying. 4-Amino-8-(2-fluoro-6-methoxyphenyl)cinnoline-3-carboxamide (110 mg, 24.0%) was afforded as a white solid.

LC/MS (M + 1): 313 (100%).

1H NMR (500 MHz, CD3OD): δ (ppm) 8.32 (dd, J = 8.3, 1.4 Hz, 1H), 7.7 - 7.81 (m, 2H), 7.45 (td, J = 8.4, 6.7 Hz, 1H), 6.97 (d, J = 8.4 Hz, 1H), 6.87 (td, J = 8.4, 0.7 Hz, 1H), 3.70 (s, 3H).

13C NMR (101 MHz, CD3OD): δ (ppm) 172.4, 163.3, 160.9, 160.2, 160.1, 148.4, 146.6, 135.8, 132.9, 131.3, 131.3, 131.2, 129.5, 128.69, 122.9, 118.1, 109.0, 109.0, 108.7, 108.7, 108.1, 108.1, 56.5.

6.3.3 | M9

Propionyl chloride (237 mg, 2.56 mmol) was added to 4-amino-8-(2-fluoro-6-methoxyphenyl)cinnoline-3-
carboxamide (80 mg, 0.26 mmol), followed by pyridine (2 mL, 24.83 mmol). The mixture was heated to 80 °C and stirred for 3 hours. The solvent was removed on the rotary evaporator (co-evaporating pyridine with n-heptane twice). The solid residue was further purified via HPLC (20% to 60% MeCN-0.2% NH₃ over 25 minutes, Xbridge™ Prep C18 10 μm OBD™ 19 × 250 mm Column, 20 mL/min) followed by supercritical fluid chromatography (SFC) mass spectrometry (SFC-MS) (MeOH/NH₃ 20mM, Waters Prep 100q SFC-MS with a Phenomenex Luna Hilic 5 μ 30x250mm Column) to afford 2-ethyl-7-(2-fluoro-6-methoxyphenyl)pyrimido[5,4-c]cinnolin-4(3H)-one (M9) (7 mg, 0.02 mmol, 7.8%) and N-(3-cyano-8-(2-fluoro-6-methoxyphenyl)cinnolin-4-yl)propionamide (17 mg, 0.05 mmol, 18.9%) as solids.

2-Ethyl-7-(2-fluoro-6-methoxyphenyl)pyrimido[5,4-c]cinnolin-4(3H)-one (M9):

LC/MS (M + 1): 351 (100%).

1H NMR (500 MHz, DMSO-d6): δ (ppm) 8.86 (dd, J = 8.2, 1.3 Hz), 8.07 (t, J = 7.7 Hz), 7.99 (dd, J = 7.1, 1.2 Hz), 7.49-7.54 (m), 7.07 (d, J = 8.4 Hz), 6.99 (t, J = 8.6 Hz), 3.66 (s), 2.80 (q, J = 7.5 Hz), 1.36 (t, J = 7.5 Hz).

13C NMR (126 MHz, DMSO-d6): δ (ppm) 166.1, 161.2, 160.2 (d, J = 242.1 Hz), 158.4 (d, J = 7.2 Hz), 148.0, 142.1, 134.7, 134.3, 131.9, 131.3, 130.3 (d, J = 10.6 Hz), 123.4, 121.1, 115.0 (d, J = 19.2 Hz), 107.6 (d, J = 22.6 Hz), 107.4 (d, J = 2.4 Hz), 56.1, 28.9, 11.2.

N-(3-Cyano-8-(2-fluoro-6-methoxyphenyl)cinnolin-4-yl)propionamide (7):

LC/MS (M + 1): 351 (100%).

1H NMR (500 MHz, DMSO-d6): δ (ppm) 11.20 (s), 8.40 (dd, J = 8.4, 1.0 Hz), 8.10 (dd, J = 8.3, 7.2 Hz), 8.04 (dd, J = 7.1, 0.9 Hz), 7.50-7.55 (m), 7.06 (d, J = 8.5 Hz), 6.99 (d, J = 8.7 Hz), 3.66 (s), 2.62 (q, J = 7.6 Hz), 1.20 (t, J = 7.5 Hz).

13C NMR (126 MHz, DMSO-d6): δ (ppm) 173.0, 160.1 (d, J = 242.4 Hz), 158.3 (d, J = 7.1 Hz), 148.1, 138.7 (broad), 135.9, 132.1, 132.0, 130.5 (d, J = 10.6 Hz), 128.7, 123.5, 120.9, 116.0, 114.3 (d, J = 19.2 Hz), 107.6 (d, J = 22.6 Hz), 107.5 (d, J = 2.6 Hz), 56.1, 29.3, 9.5.

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CONFLICT OF INTEREST

None declared.

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