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 Artelsmair,[1*] Chungang Gu,[2] Richard J. Lewis[3] and Charles S. Elmore[1]

[1] *Early Chemical Development, Pharmaceutical Sciences, IMED Biotech Unit, AstraZeneca, Gothenburg, Sweden;*

[2] *DMPK, Oncology, IMED Biotech Unit, AstraZeneca, Boston, USA;*

[3] *Medicinal Chemistry, Respiratory, Inflammation and Autoimmunity, IMED Biotech Unit, AstraZeneca, Gothenburg, Sweden.*

* *Correspondence: Markus Artelsmair. AstraZeneca, Pepparedsleden 1, 431 50 Mölndal, Sweden. Email: markus.artelsmair@astrazeneca.com*

Abstract

Anxiolytic activity has been associated with $GABA_A$ α2 and α3 subunits. Several target compounds were identified and required in C-14 labeled form to enable a better understanding of their DMPK properties. **AZD7325** is a selective $GABA_A$ α2 and α3 receptor modulator intended for the treatment of anxiety through oral administration. A great number of **AZD7325** metabolites were observed across species *in vivo*, whose identification was aided by [^{14}C]**AZD7325**. An interesting metabolic cyclization and aromatization pathway leading to the tricyclic core of **M9** and the oxidative pathways to **M10** and **M42** are presented.

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Introduction

GABA (γ-aminobutyric acid) 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 to 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 three 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] $GABA_B$ are transmembrane G-protein coupled receptors that activate secondary messengers as well as K^+ and Ca^{2+} 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 two α units, two β units and one γ unit.[5,6] There are a number of allosteric ligands that can modulate the response of $GABA_A$ receptors to GABA. The most thoroughly studied and characterized site on the $GABA_A$ receptor complex is the binding site of benzodiazepines (BZ) and benzodiazepine-like compounds, that is located on the interface of the α/β subunits.[7] Ligands binding at this site can have three functional effects on the chloride current. The first of the three distinct modulatory modes is agonism or positive allosteric modulation (PAM), both leading to an increase in the GABA induced chloride current. Secondly, binding at the BZ site can cause either inverse agonism or negative allosteric modulation (NAM) and thereby decrease GABA induced chloride current. Third, binding may produce neutral antagonism (NA) 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 NAMs are anxiogenic and neutral antagonists cause no apparent physiological effect.[9,10]

$GABA_A$ receptors have been the target of numerous drugs. Most noticeably, benzodiazepines 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 benzodiazepines such as their pharmacological profile, safer, more effective and more tolerable alternatives are being sought.[13] The fact that benzodiazepines bind non-selectively to $GABA_A$ 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 benzodiazepine 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 $GABA_A$ receptors containing α2 and α3 subunits with benzodiazepine 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 $GABA_A$ 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 (EEG) signature.[20] Furthermore, recent PET studies confirmed that high $GABA_A$ 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 two Phase II proofs-of-concept in patients with General Anxiety Disorder (GAD).[*] It is, however, worth noting that in a double-blind, randomized, four 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 two positive $GABA_A$ α2 and α3 receptor modulators on plasma prolactin levels reached similar conclusions.[21] 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.[22,23] Three compounds showing particular promise as selective $GABA_A$ α2 and α3 positive modulators were selected for further studies on their DMPK properties, cf. Figure 1b.

In order to efficiently label all three 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 three 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-bromodiazobenzene to give an isomeric mixture of E/Z-*N*'-(2-bromophenyl)-2-oxo-2-(propylamino)acetohydrazonoyl [^{14}C]cyanide (**3**) in a 3:1 ratio with a 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 $AlCl_3$ catalyzed cyclization at 100 °C in toluene in 76% yield. At this point, the key intermediate was partitioned to prepare three target compounds *via* Suzuki coupling reactions (cf. Figure 3). The very poor yield (12%) for the conversion to [^{14}C]**AZ12449694** in Figure 3 was due to the poor stability of the boron reagent as well as difficulties with the purification procedure.

Metabolism

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 (MIST) analysis, detailed investigations on the metabolite profile were carried out. [^{14}C]**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 three sites of the

[*] NCT00807937 and NCT00808249

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]**AZD7325**, highlighting the immense value of radio-labeling for DMPK studies. The full characterisation of these metabolites is discussed by Gu *et al.*, 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 three 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).[29]

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**).

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 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 one-pot reaction. Interestingly, two products were formed in this reaction in a 5:2 ratio. Both compounds had the expected mass and very similar retention times on Reversed-Phase HPLC (RP-HPLC). They were separated using mass directed supercritical fluid chromatography (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**.

Given their structure and the reaction conditions employed, it appears likely that the pathways to the two 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]

Conclusion

In summary, C-14 labeled analogues of three 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 $GABA_A$ α2 and α3 receptor modulator **AZD7325** was investigated and a wide range of metabolites were identified with the aid of [^{14}C]**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 MIST analysis at steady state.

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The authors declare no conflict of interest.

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Experimental Section

General

All chemicals were purchased from Sigma-Aldrich or its subsidiaries. $K^{14}CN$ 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 temperatures, DrySyns and electric heating plates were used. Stated reaction temperatures refer to the external DrySyn temperature.

^{1}H NMR and ^{13}C 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 (TMS) with the solvent resonance as internal standard (7.26 ppm and 77.16 ppm for $CDCl_3$, 3.31 ppm and 49.00 ppm for CD_3OD and 2.50 ppm and 39.52 ppm for DMSO-d_6 as reported in: H. E. Gottlieb, V.Kotlyar and A. Nudelman, J. Org. Chem., 1997, 62, 7512-7515). The signals derived from the ^{1}H NMR spectra are reported with chemical shifts, multiplicity (s = singlet, d= doublet, t = triplet, q = quartet, p = pentet, sxt = sextet, dt= doublet of triplets, dq = doublet of quartets, ddd = double doublet of doublets, ddt = 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 ^{13}C-NMR are reported with chemical shifts and coupling to ^{19}F 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 10-100% gradient over 10 min 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 x 100 mm columns Phenomenex Luna C18(2) heated to 30 °C over 20 min and concluded with a 5 min wash of 100% MeCN or MeOH.

For the synthesis of **M9**, high performance liquid chromatography (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™ 19x250 mm Column.

For the synthesis of **M9**, LCMS was acquired on a Waters Acquity UPLC using a BEH C18 column (50mm×2.1mm, 1.7µm particles) with a 10-90% gradient over 2 min with MeCN-NH_4/NH_4CO_3 or MeCN-formic acid and electrospray ionization.

Radiochemistry - Synthesis

*2-[^{14}C]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 K^{14}CN (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 CH_2Cl_2 (25 mL) and the layers were separated. The aqueous layer was extracted with CH_2Cl_2 (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 a 4:3 mixture (as observed by ^{1}H NMR) of 2-bromo-*N*-propylacetamide: 2-[^{14}C]cyano-*N*-propylacetamide.

LC/MS (M+1): 127 (12%), 129 (100%), 130 (7.2%).

^{1}H NMR (500 MHz, $CDCl_3$): 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).

*N-(2-Bromophenyl)-2-oxo-2-(propylamino)acetohydrazonoyl [^{14}C]cyanide (**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 h. The resulting solution was then stirred for 30 min at 0 °C. It was then added to a solution of 2-[^{14}C]cyano *N*-propylacetamide (**2**) (158 mg, 1.24 mmol, 68 mCi) (contaminated with 185 mg [calculated] of 2-bromo-*N*-proylacetamide) 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 h, the reaction mixture was partitioned between CH_2Cl_2 (25 mL) and saturated aqueous $NaHCO_3$ (15 mL) and the layers were separated. The aqueous layer was extracted twice with CH_2Cl_2 (2 x 15 mL) and the combined organic layers were dried over $MgSO_4$ and filtered to yield 53 mCi. The solvent was removed to afford *N*-(2-bromophenyl)-2-oxo-2-(propylamino)acetohydrazonoyl [^{14}C]cyanide (**3**) as a yellow solid (78% radiochemical purity as a 3:1 mixture of two isomers, HPLC method A).

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

*4-Amino-8-bromo-N-propylcinnoline-4-[^{14}C]-3-carboxamide (**4**)*

A solution of (E and Z)-*N*'-(2-bromophenyl)-2-oxo-2-(propylamino)acetohydrazonoyl [^{14}C]cyanide (**3**) (147 mg, 0.47 mmol, 26 mCi) in toluene (12 mL) was stirred under N_2 as $AlCl_3$ (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 CH_2Cl_2 (25 mL) and water (25 mL). The layers were separated and the organic layer was extracted three times with 1 M HCl (3x 25 mL) to leave 3 mCi in the organic layer which was discarded. The combined aqueous layers were basified with $NaHCO_3$ to pH >8 and the aqueous slurry was then extracted with CH_2Cl_2 (5 x 20 mL). The combined organic layer was dried over $MgSO_4$ and filtered to give 21 mCi. The solvent was removed and the solid was taken up in CH_2Cl_2 (2.5 mL). The solution was cooled at -30 °C for 2 h and the solid removed to give 4-amino-8-bromo-*N*-propylcinnoline-4-[^{14}C]-3-carboxamide (**4**) (112 mg, 0.36 mmol, 76%) measured at 19 mCi (94% radiochemical purity, HPLC method B).

LC/MS (M+1): 309 (11.2%), 311 (100%), 313 (96.2%).

^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 0.96 (t, *J* = 7.5 Hz, 3 H) 1.62 (sxt, *J* = 7.3 Hz, 2 H) 3.13 - 3.24 (m, 0 H) 3.35 - 3.47 (m, 2 H) 7.43 (t, *J* = 7.9 Hz, 1 H) 7.77 (d, *J* = 7.6 Hz, 1 H) 8.05 (d, *J* = 7.3 Hz, 1 H) 8.50 (br. s., 1 H).

^{13}C NMR (CD_3Cl, 125 MHz): δ (ppm) 167.1, 145.9, 135.4, 129.1, 128.8, 125.6, 119.8, 117.8, 40.9, 22.6, 11.5.

[^{14}C]AZD7325

A solution of 4-amino-8-bromo-*N*-propylcinnoline-4-[^{14}C]-3-carboxamide (**4**) (47 mg, 0.15 mmol, 8.4 mCi), of 2-methoxy-6-fluorobenzeneboronic acid (142 mg, 0.83 mmol), Pd(dppf)Cl_2•CH_2Cl_2 (7.7 mg, 0.011 mmol) and Na_2CO_3 (259 mg, 2.44 mmol) in water (1 mL), THF (2 mL) and iPrOH (1 mL) was de-oxygenated by bubbling N_2 through the solution for 5 min. Then it was stirred vigorously at 70 °C for 2 h. 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 x 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 min on a 21.2 x 250 mm Phenomenex Luna C-18(2) column, 30 mL/min) afforded 6.6 mCi of [^{14}C]**AZD7325***,* 4-amino-8-(2-fluoro-6-methoxyphenyl)-*N*-propylcinnoline-[^{14}C]-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%).

^{1}H NMR (500 MHz, CD_3OD): 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).

^{19}F NMR (470MHz, CD_3OD): δ (ppm) -115.4, -76.9 (TFA).

^{13}C NMR (125 MHz, CD_3OD): δ (ppm) 169.4, 162.2(d, *J* = 245.5 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* = 20Hz), 108. 5(d, 74.1 Hz), 108.6 (d, *J* = 98.8 Hz), 56.6, 41.9, 23.9, 11.8.

[^{14}C]AZD6280

A solution of 4-amino-8-bromo-*N*-propylcinnoline-4-[^{14}C]-3-carboxamide (**4**) (55.4 mg, 0.18 mmol, 12 mCi, mmol) and $Pd(PPh_3)_4$ (18 mg, 0.02 mmol) in DME (5 mL) was degassed by bubbling N_2 through the solution for 15 min and of 2,5-bismethoxybenzeneboronic acid (51 mg, 0.28 mmol) and sodium carbonate (48 mg, 0.45) in water (1 mL) were added. The solution was stirred at RT under N_2 for 10 min and then at 85 °C overnight. The reaction was diluted with CH_2Cl_2 (20 mL) and sat. aqueous $NaHCO_3$ (10 mL). The layers were separated and the aqueous layer extracted with CH_2Cl_2 (10 mL). The combined organic layers contained 12 mCi with a radiochemical purity of 89% (15 to 30% MeCN-0.1% formic acid over 10 min, 22.5x250 mm Luna C-18(2), 30 mL/min). The sample was concentrated to dryness and then purified by preparative HPLC (25 to 50% MeCN-0.1% TFA over 35 min, 20x250 mm Phenomenex Luna C-18(2), 30 mL/min) in three batches to give 7.5 mCi of [^{14}C]**AZD6280**, 4-amino-8-(2,5-dimethoxyphenyl)-*N*-propylcinnoline-4-[^{14}C]-3-carboxamide with 99.8% radiochemical purity, (0 to 30% MeCN-0.1% TFA over 20 min followed by a wash on LunaC-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%).

^{1}H NMR (500 MHz, CD_3OD): δ (ppm) 8.84 (t, 1H), 8.63 (t, 1H), 7.90 (t, 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: J-values not available)

^{13}C NMR (125 MHz, CD_3OD): 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)

*[^{14}C]**AZ12449694***

A solution of 4-amino-8-bromo-*N*-propylcinnoline-4-[^{14}C]-3-carboxamide (**4**) (7.5 mCi), $Pd(PPh_3)_4$ (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 Na_2CO_3 (36 mg, 0.34 mmol) in DME (2.5 mL) and water (1 mL) was degassed by bubbling N_2 through the solution for 5 min. The solution was then warmed to 92 °C and stirred under N_2 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 CH_2Cl_2 (2 x 10 mL) to give 6 mCi of a yellow solution. The solution was concentrated to dryness and applied to preparative HPLC in three batches (20 to 40% MeCN-0.1% Formic acid over 35 min, 22.5x250 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-[^{14}C]-3-carboxamide ([^{14}C]**AZ12449694**). HPLC analysis showed the purity to be 98.8% (0 to 30% MeCN-0.1% TFA over 20 min followed by a wash on LunaC-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%).

^{1}H NMR (500 MHz, $CDCl_3$): 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).

Preparation of the M9 standard

*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%).

^{1}H 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).

^{13}C 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.

*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-carbonyl 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% NH_3 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% NH_3 over 25 min, Xbridge™ Prep C-18 10 µm OBD™ 19x250 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%).

^{1}H NMR (400 MHz, CD_3OD): 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).

^{13}C NMR (101 MHz, CD_3OD): 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, 131.2, 129.5, 128.69, 122.9, 118.1, 109.0, 109.0, 108.7, 108.7, 108.1, 108.1, 56.5.

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% NH_3 over 25 min, Xbridge™ Prep C-18 10 µm OBD™ 19x250 mm Column, 20 mL/min) followed by Supercritical Fluid Chromatography Mass Spectrometry (SFC-MS) (MeOH/NH3 20 mM, Waters Prep 100q SFC-MS with a Phenomenex Luna Hilic 5µ 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%).

^{1}H 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).

^{13}C 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%).

^{1}H 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).

^{13}C 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.

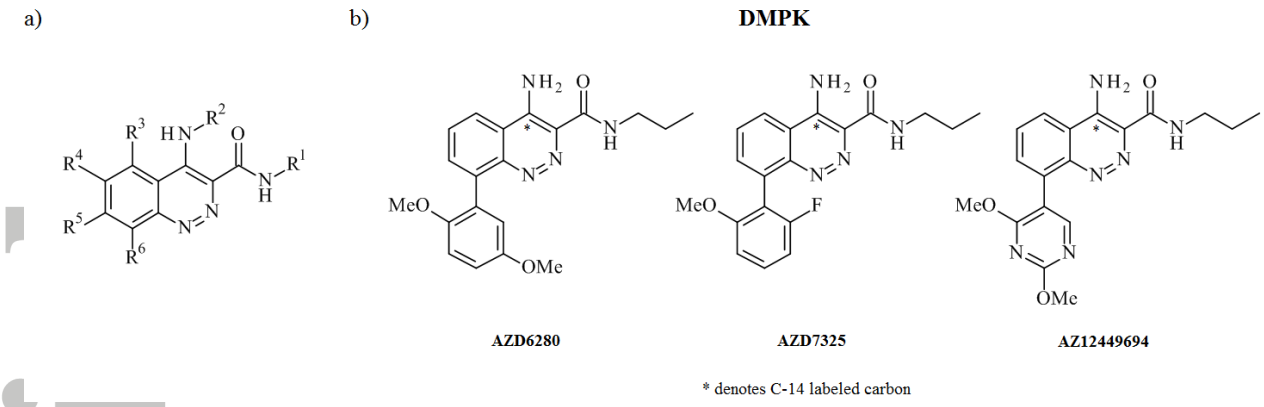


Figure 1 *a) The general structural class of selective $GABA_A$ α2 and α3 positive modulators developed by AstraZeneca;[22,23] b) Three drug candidates selected for C-14 labeling to study their DMPK properties.*

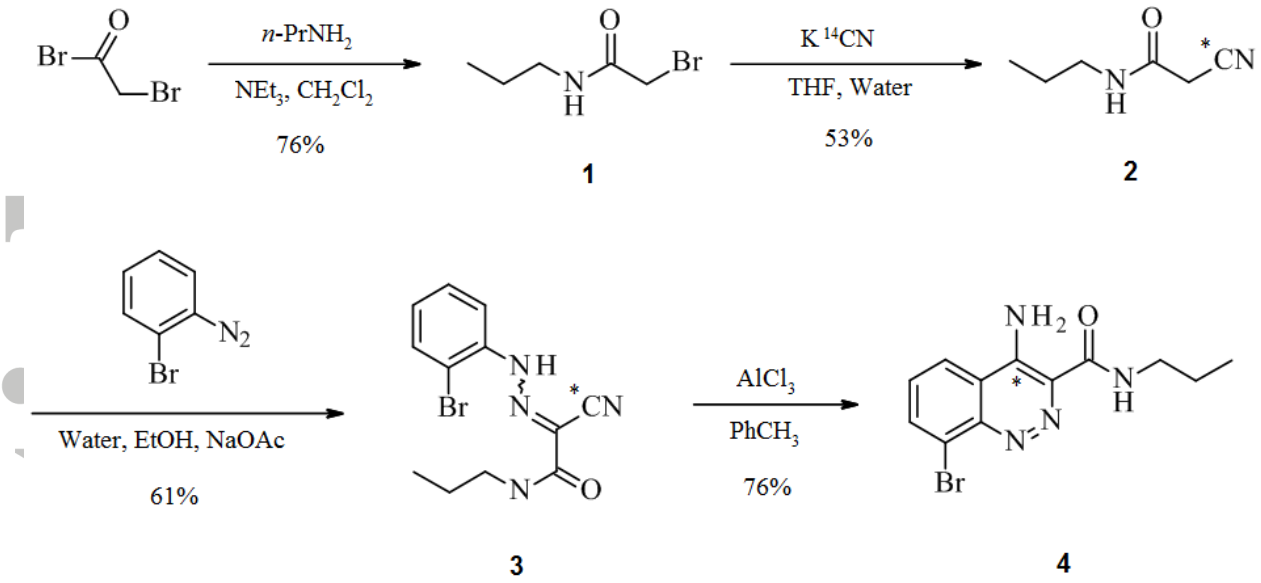


Figure 2 *Synthesis of the common precursor for the radiolabeling targets. Reaction conditions are shown.*

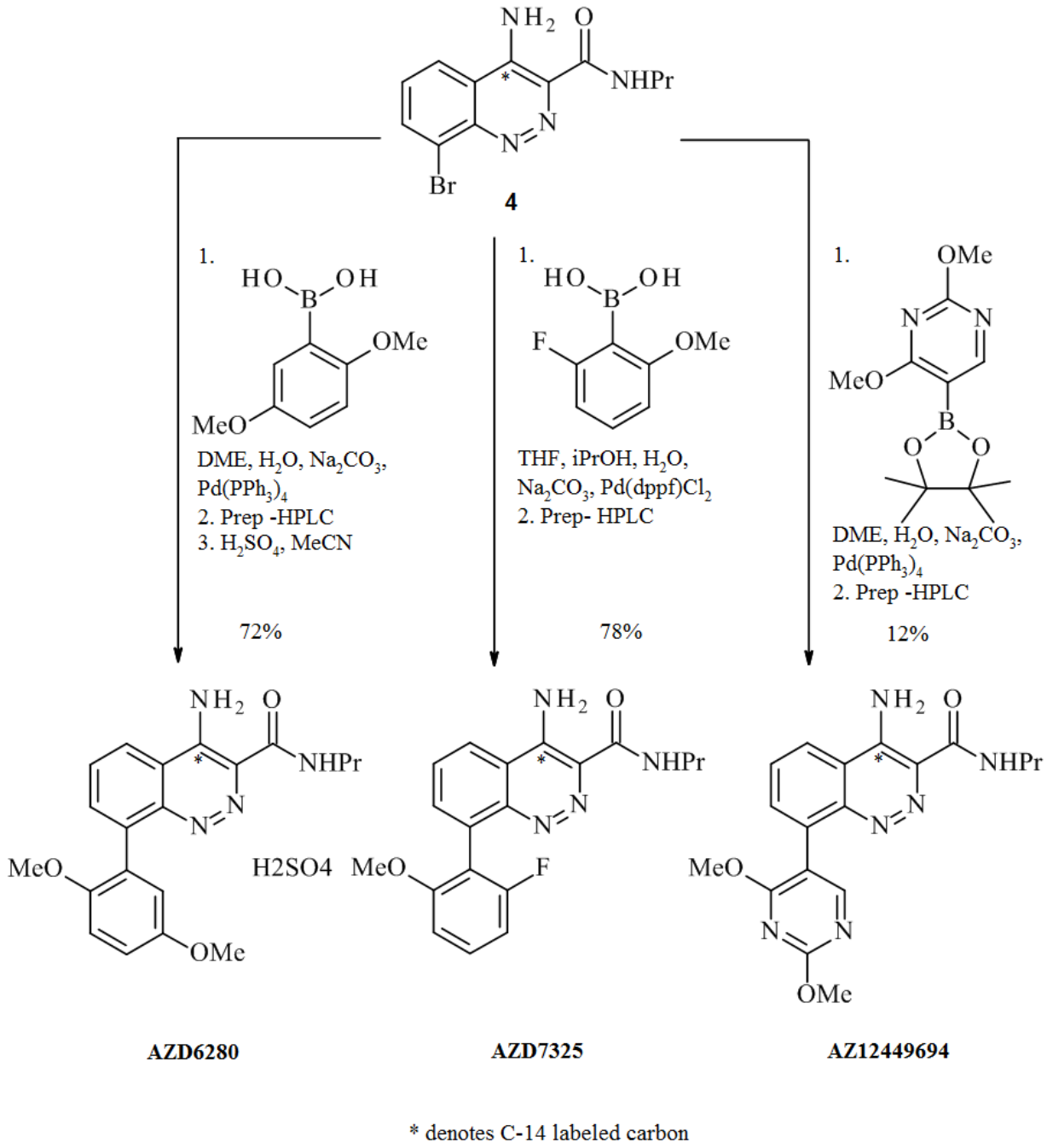


Figure 3 *Conversion of the key intermediate into the three target compounds via different Suzuki coupling reactions.*

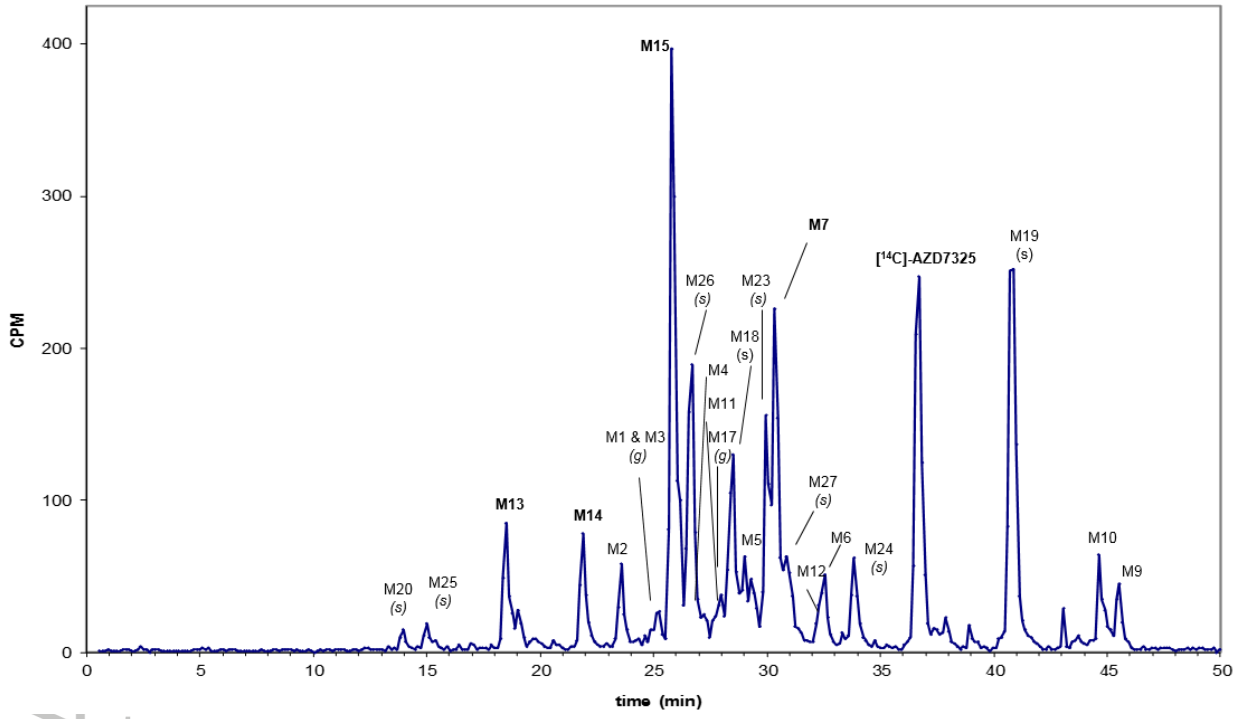


Figure 4 *Metabolite profile of [^{14}C]**AZD7325** in female rat plasma pooled over the first 12 hours after dosage (2 mg/kg oral). Bold **M#** (i.e. **M7**, **M13**, **M14** & **M15**) indicate major circulating phase-I metabolites in rat. "(s)" and "(g)" denote sulfate and glucuronide, respectively.*

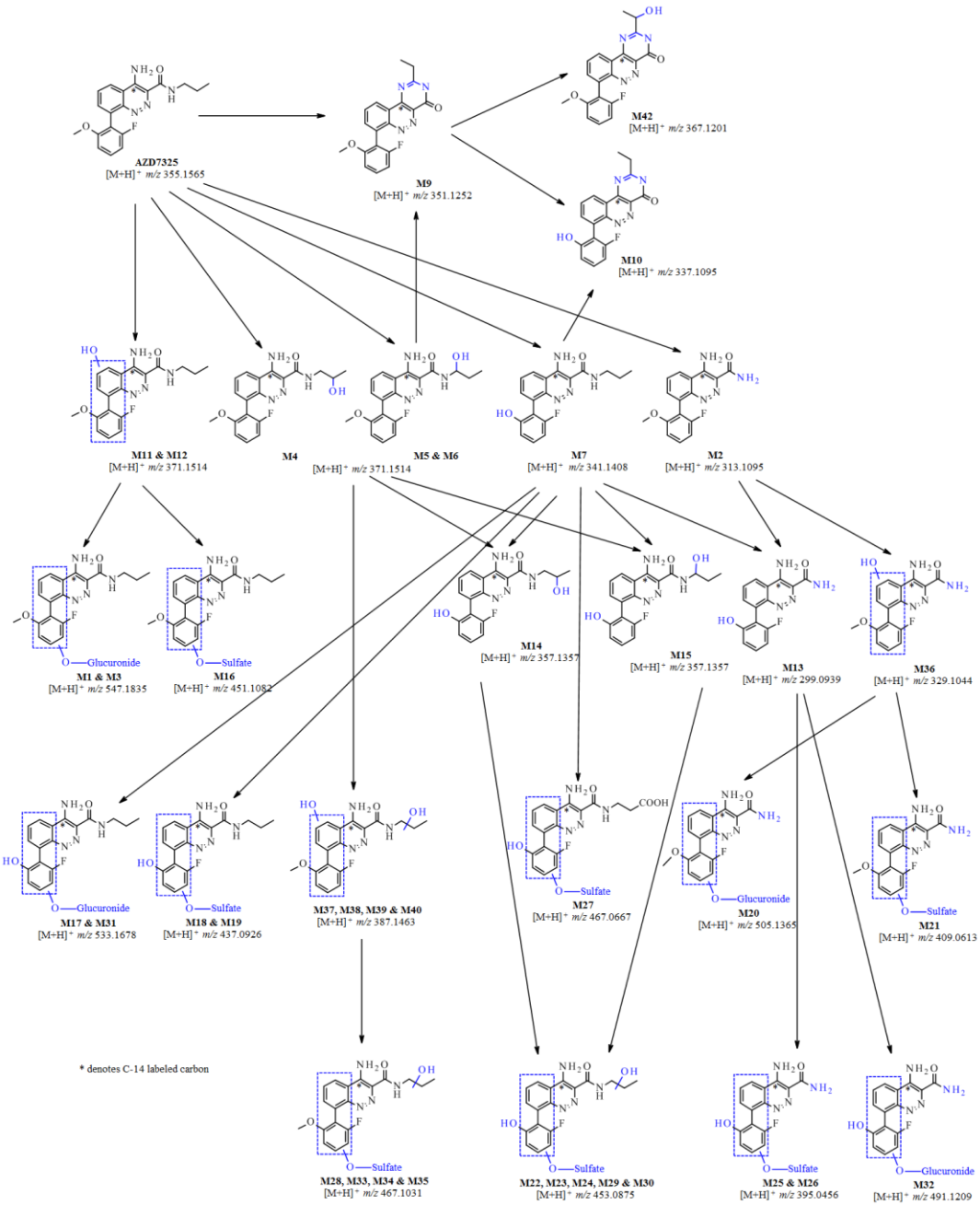


Figure 5 *A biotransformation scheme for **AZD7325** which was elucidated with [^{14}C]**AZD7325** in rat in vivo. The unusual oxidative metabolites **M9**, **M10** and **M42** are shown. **M42**, absent from rat in vivo samples after a single oral dose of [^{14}C]**AZD7325**, was observed at trace amounts in liver microsomes across species.*

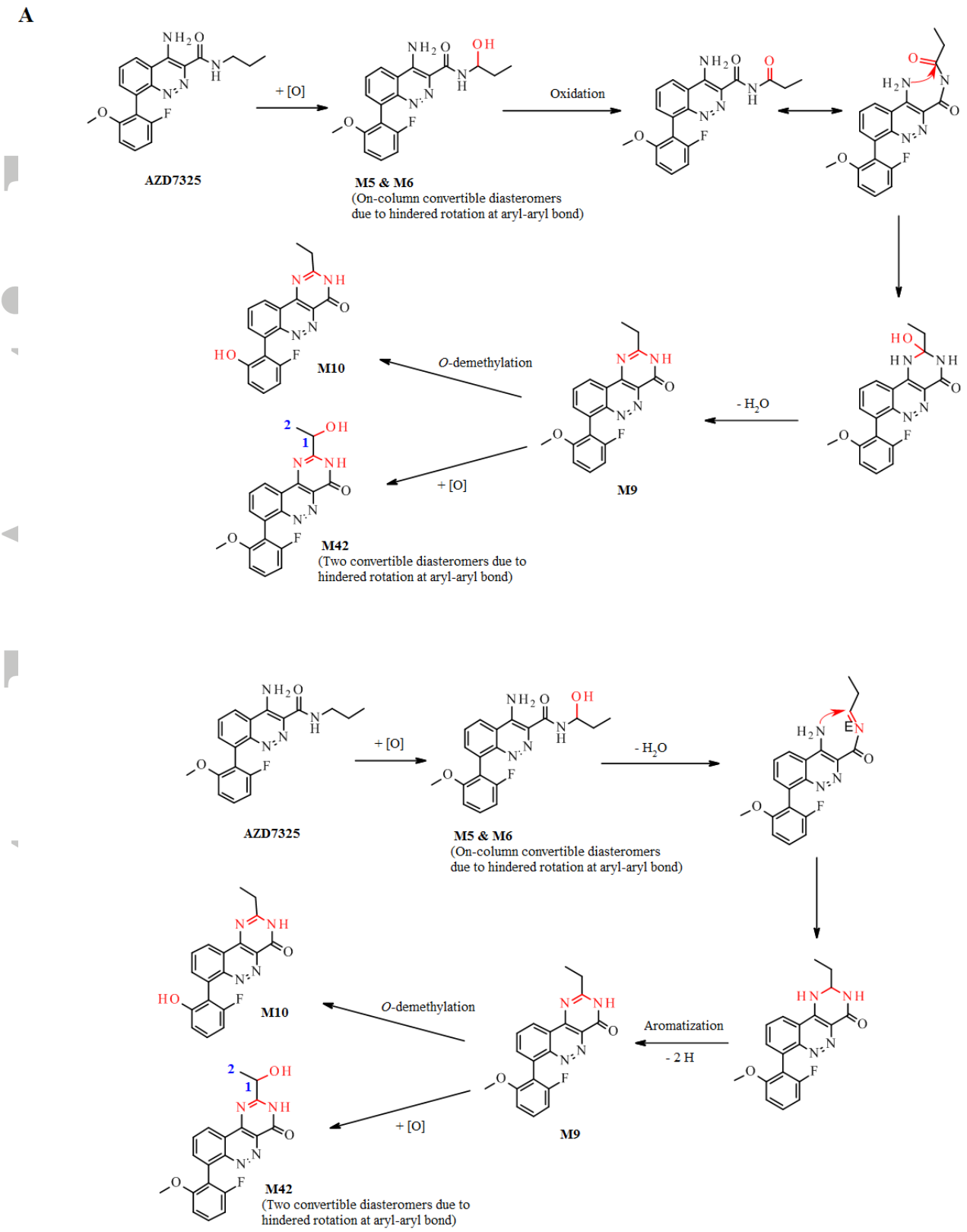


Figure 6 *The two proposed metabolic mechanisms for the formation of **M9**, **M10** and **M42**. Option **A** proceeds via an initial oxidation whereas **B** involves the loss of water prior to the intramolecular ring closure.*

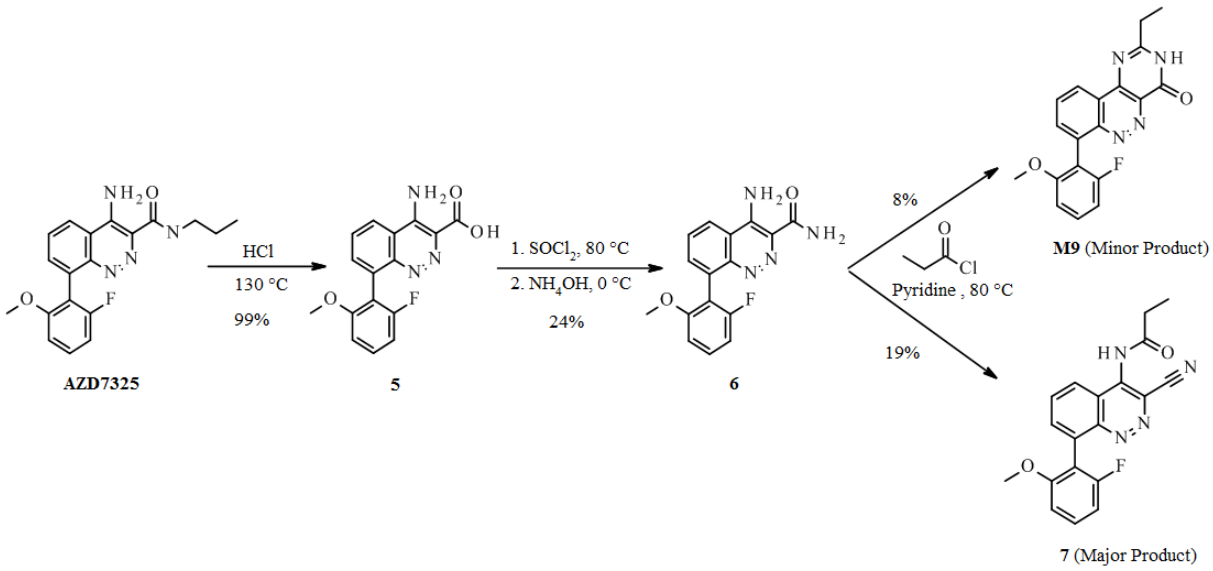


Figure 7 *The synthetic route to the authentic standard for **M9**. Reaction conditions are shown. The major product of the route to **M9**, the nitrile **7**, is formed in a 5:2 ratio to the tricyclic product.*

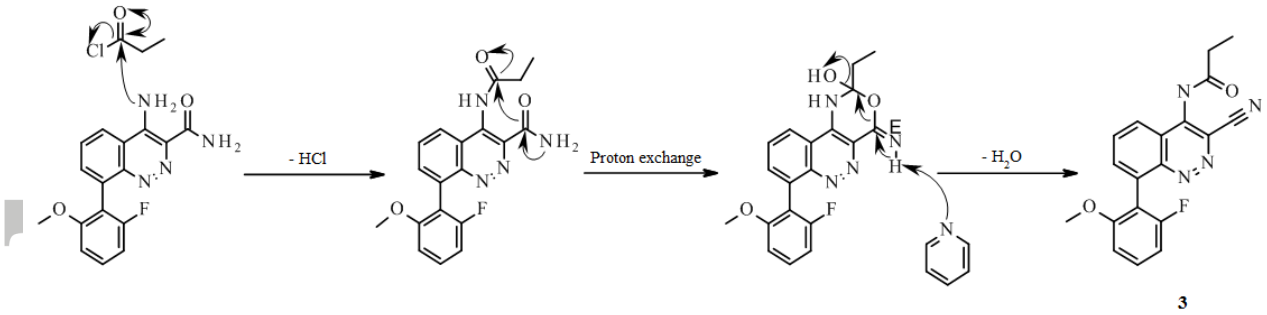
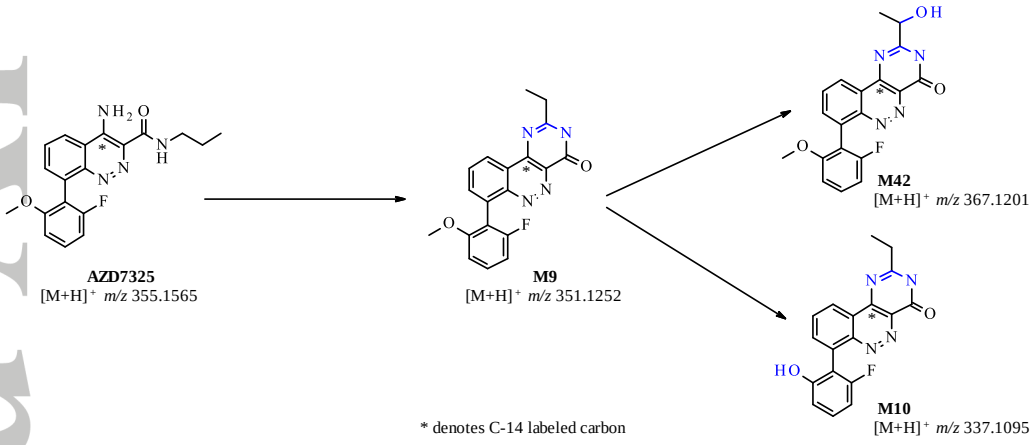


Figure 8 *A proposed mechanism for the formation of the major reaction product* ***7****.*

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 Artelsmair,* Chungang Gu, Richard Lewis and Charles S. Elmore

Three selective $GABA_A$ α2 and α3 positive modulators were labeled with C-14 to study their DMPK properties. For **AZD7325**, a drug candidate intended for the treatment of anxiety, a vast number of metabolites were observed across species *in vivo*, whose identification was aided by [^{14}C]**AZD7325**. An interesting metabolic cyclization and aromatization pathway for the formation of the tricyclic core of **M9** and the oxidative pathways to **M10** and **M42,** all of which were late-occuring and long circulating metabolites, are presented.



*Graphical Abstract: Identification of key metabolites of **AZD7325**, a selective $GABA_A$ α2 and α3 receptor modulator.*