Development of Radioligands for *In Vivo* Imaging of $GABA_A$-Benzodiazepine Receptors

Andrew Katsifis[1,*] and Michael Kassiou[2,3]

[1]*Radiopharmaceuticals Division R&D, Australian Nuclear Science and Technology Organisation, Private Mail Bag 1, Menai NSW 2234, Sydney, Australia*

[2]*Department of PET and Nuclear Medicine, Royal Prince Alfred Hospital, Missenden Road Camperdown NSW 2050, Sydney, Australia*

[3]*Department of Pharmacology, University of Sydney, NSW 2006, Sydney, Australia*

Abstract: Changes in the biochemical integrity and function of the $GABA_A$-benzodiazepine receptor (BZR) complex have been implicated in various neurological and psychiatric disorders. The development of specific radioligands for the $GABA_A$-BZR have not only contributed to the elucidation of the receptor's biochemical functions, but also provided a means by which these changes are correlated to disease states when studied with the functional imaging modalities of positron emission tomography (PET) and single photon emission computed tomography (SPECT).

Keywords: Benzodiazepines, CNS receptors, radioligands, PET, SPECT.

INTRODUCTION

Positron emission tomography (PET) and single photon emission computed tomography (SPECT) are powerful tools for the non-invasive study of physiological, biochemical and pharmacological functions at the molecular level in living beings, whether in health or disease. This development, also termed "molecular imaging" has largely been possible through the rapid technological advances in imaging technology, the availability of specific "physiological tracers" and small medical cyclotrons. During the last two decades numerous biochemical processes have been probed in neuroscience, oncology and cardiology using radiolabelled metabolic tracers such as 2-[^{18}F]-fluorodeoxyglucose, radiolabelled amino acids and nucleotides, receptor ligands, fatty acids, enzyme inhibitors, DNA probes and immuno-conjugates.

Of enormous significance is the ability of these techniques to detect and measure functional receptors and binding sites at sub-nanomolar concentrations. As a consequence, monitoring the changes in receptor concentration or binding sites may provide significant insights into the aetiology and progress of disease at the molecular level. Critical to this, is the availability of specific radiolabelled molecules produced in high purity and specific activity that do not perturb the receptor system it is designed to measure. Another requirement is that the radioisotope incorporated into the drug does not modify the biochemical and pharmacological properties of the original molecule. This means that the radioligand must contain either a radioisotope of one of the chemical elements constituting the drug or when a different radioisotope is required it is incorporated into a position which will retain the drug's interaction with the receptor.

*Address correspondence to this author at the Radiopharmaceuticals Division R&D, Australian Nuclear Science and Technology Organisation Private Mail Bag 1, Menai NSW 2234, Sydney, Australia; Tel: +612 9717 9094; Fax: +612 9717 9262; E-mail: akx@ansto.gov.au

The cyclotron produced radioisotope carbon-11 ($t_{1/2}$ = 20 min) is an ideal radionuclide for the radiolabelling of many drugs and biochemical intermediates. However, its short half-life, short imaging times and the need for close proximity to cyclotrons have promoted the development of longer-lived radiolabelled drugs bearing the PET radionuclides fluorine-18 ($t_{1/2}$ = 109 min), and bromine-76 ($t_{1/2}$ = 16 h) and the SPECT radionuclide iodine-123 ($t_{1/2}$ = 13.2 h). In addition to high specificity and selectivity, a radiolabelled ligand should display other essential properties including high specific activity, low non-specific binding, slow metabolism, receptor saturability, blood brain barrier permeability and safety for human use.

This review will detail the current status of benzodiazepine receptor (BZR) radioligands in basic pharmacological studies and in imaging disease.

THE CENTRAL BENZODIAZEPINE RECEPTOR (BZR)

Since their discovery and introduction into clinical use in the late 1950's, benzodiazepines have established themselves amongst the most widely prescribed drugs in modern medicine. In addition to their anti-anxiolytic, anticonvulsant and sedative effects, benzodiazepines have made major contributions in drug treatment of mental illnesses as well as contributing to the basic understanding and mechanisms of inhibitory neurotransmission. Even more remarkable, the major advances in the therapeutic and pharmacological applications of these compounds were achieved long before their mechanism of action and the target site "the benzodiazepine receptor" was determined [1]. Subsequent, research on the mechanism of action of these drugs revealed specific binding to a single population of recognition sites on the $GABA_A$-BZR complex with the ability to modulate the gating of the chloride ion channel. More recently, it was found that many other ligands; some with diverse chemical structures were able to bind to the $GABA_A$-BZR complex. Therefore a wide range of ligands producing a continuum of

1389-5575/04 $45.00+.00

intrinsic activity ranging from full agonists to antagonists and inverse agonists have been developed.

BZR's bind benzodiazepines and other ligands with high specificity and affinity. Radiolabelled analogues of these ligands have been studied with neuronal cell membrane preparations expressing the BZR providing basic information such as the affinities of individual ligands and the number of binding sites in a given amount of tissue to be determined. The binding and distribution of the BZR in CNS tissue could also be "visualised" by autoradiography, after exposing tissue slices to a radioligand or after systemic administration of a radioligand to a living animal followed by postmortem examination of brain slices. Today the BZR can be made visible in the intact brains of animals and man [2] using the intravenous injection of a trace amount of a suitably labelled ligand with either PET or SPECT [3].

MOLECULAR BIOLOGY AND PHARMACOLOGY OF THE $GABA_A$-BZR

The $GABA_A$-BZR is a macromolecular protein complex composed of specific combinations of various protein subunits assembled in pentameric structures to form a functional gated ion channel. Multiple isoforms of these subunits exist ($\alpha_1-\alpha_6$, $\beta_1-\beta_4$, $\gamma_1-\gamma_3$, δ, ε, π and $\rho_1-\rho_3$) resulting in the heterogeneity of the $GABA_A$-BZR's in the CNS [4]. Histochemical analysis indicate the presence of discrete $GABA_A$-BZR subunits in different parts of the brain, suggesting that specific physiological or behavioural effects may be associated with stimulation of specific populations of BZR's [5, 6].

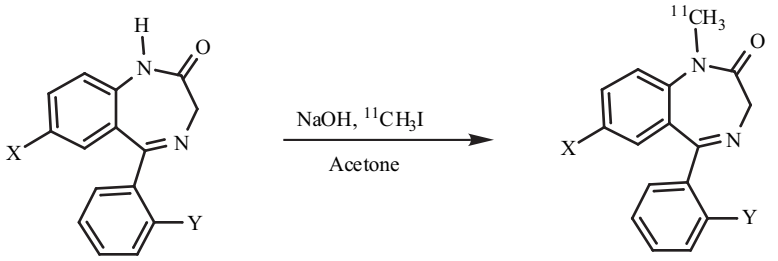
Pharmacological studies on recombinant receptors indicate that it is the subunit composition, particularly the α-subunit, which influences the physical, biochemical and pharmacological properties of the receptor complex [7]. In addition, the discovery of a full spectrum of activities exhibited by agonists, antagonists and inverse agonists has suggested the existence of a variety of receptor conformations corresponding to the different functional states of the receptor complex [8, 9]. This suggests that selective interactions between agonists, antagonists and inverse agonists with the receptor may be possible [10, 11] and that the pharmacological differences may correspond to variations in the α-subunit composition of the receptor [12-14]. Pharmacologically, the $GABA_A$-BZR mediates the major inhibitory synaptic events in the brain and is involved in the regulation of anxiety, vigilance, memory, epileptogenic activity and muscle tension. Changes in the biochemical integrity and function of the $GABA_A$-BZR complex have also been implicated in various neurological and psychiatric disorders. Therefore studies in which changes in the BZR density and function can be measured and monitored in disease with PET [15, 16] and SPECT [17] may be of clinical value. In particular, radiolabelled benzodiazepine receptor ligands which are selective for $GABA_A$-BZR subtypes or which can provide information on the conformational state of the receptors in disease may be of potential value in studying the role of the $GABA_A$-BZR complex in disease.

A number of ligands (both agonists and antagonists) with high affinity for the BZR have been radiolabelled with a variety of isotopes for PET and SPECT imaging studies of the central BZR's. To date, the antagonists [^{11}C]flumazenil (**[^{11}C]1**) and [^{123}I]iomazenil (**[^{123}I]2**) have been used in the majority of imaging studies in humans as the binding affinity of antagonists is unaffected by the presence of other modulatory ligands. These radioligands have been widely used to investigate changes in BZR density in normals and in a number of neurodegenerative and psychiatric diseases using PET and SPECT [15-19]. As antagonists such as flumazenil (**1**) and iomazenil (**2**) are able to block or antagonise the effects of agonists and inverse agonists it is assumed that they competitively bind to the same binding domain of the BZR. However, the exact pharmacophoric descriptors such as hydrogen donors, hydrogen acceptors and lipophilic groups must differ if they are to induce the different receptor conformations associated with the appropriate intrinsic efficacy. Hence during the last two decades considerable effort has been directed towards the radiolabelling, characterisation and elucidation of BZR-ligand interactions.

RADIOLABELLED LIGANDS FOR THE $GABA_A$-BZR

The earliest studies involving radiolabelled benzodiazepines for imaging date back to the early eighties. The first benzodiazepines to be radiolabelled with carbon-11 were diazepam (**3**) and flunitrazepam (**4**), both by *N*-methylation (Fig. **1**). [20, 21]. However, their clinical utility was limited due to the lack of selectivity for the central BZR *vs* the peripheral BZR, reduced BZR affinity *in vivo* and fast dissociation from the receptor at 37 ^{o}C. In fact 4, a potent BZR agonist displayed (K_D = 1 nM at 0 ^{o}C) a ten-fold decrease in affinity at 37 ^{o}C. Consequently only partial displacement of **[^{11}C]4** was observed in primate studies and the ratio of specific to non-specific binding was too low to allow accurate determination of BZR binding *in vivo*. The 2'-fluoro derivative fludiazepam (**5**) was also radiolabelled by ^{11}C-methylation at the amide nitrogen using [^{11}C]iodomethane [22], but, similar reduced binding was reported *in vivo*.

The widespread availability of iodine-125 for basic biochemical studies has seen the development of radiolabelling techniques suitable for adaptation to iodine-123 for SPECT imaging. In addition, the large numbers of well characterised BZR ligands developed by the pharmaceutical industry incorporate halogens, hence suitable for development as imaging agents. Initially 1,4-benzodiazepine-5-phenyl derivatives (Fig. **2**) were radiolabelled with no carrier added (n.c.a) bromine-75/77 [23]. In these studies the radiobromine was introduced into the 7-position of the benzene ring via triazine decomposition resulting in radiolabelled products with specific activity of 20,000 mCi/μmol and radiochemical yield of 20 %. Although biodistribution studies of **7b** in mice demonstrated rapid brain uptake with a brain to blood ratio of 2 at 10 minutes post injection (p.i.) no human studies were reported with these radioligands. In a similar manner, decomposition of the piperidinyl triazine **6b** in carbon tetrachloride at 70 ^{o}C for 60 minutes in the presence of iodine-123 gave **[^{123}I]8b** in a radiochemical yield of 25-30 % and a specific activity of 900-1100 mCi/μmol. The



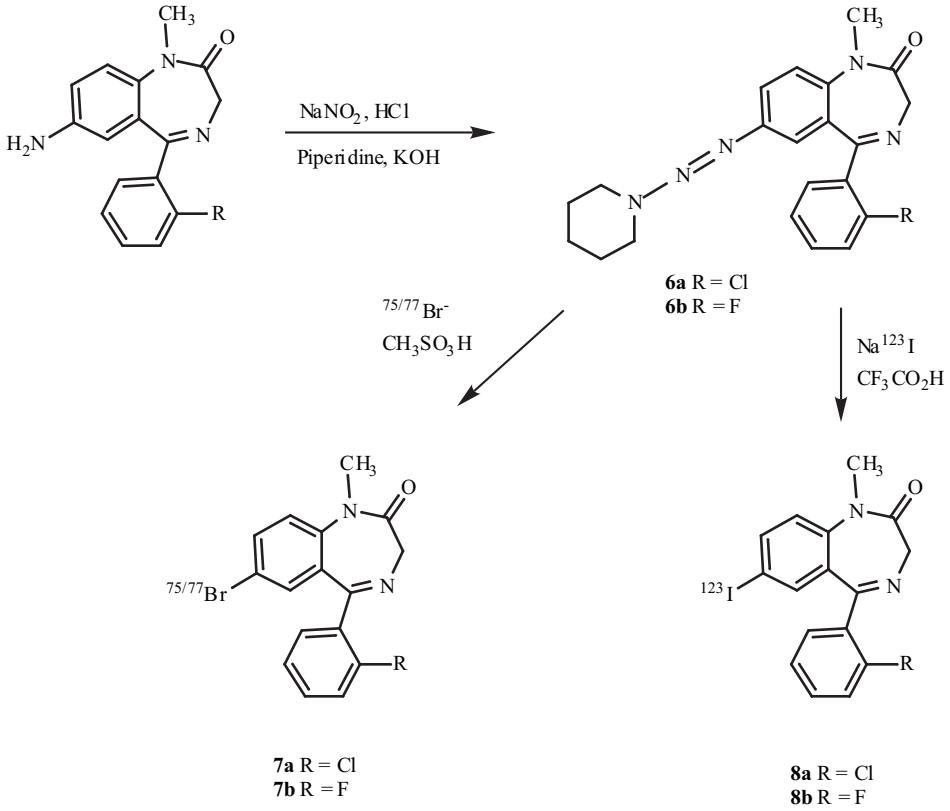
3 Diazepam X = Cl, Y = H
4 Flunitrazepam X = NO_2, Y = F
5 Fludiazepam X = Cl, Y = F

Fig. (1).

inhibition constant for this molecule was IC_{50} = 2.0 nM indicating no loss of affinity by substituting chlorine or nitro groups for iodine. Biodistribution studies of **[^{123}I]8b** in mice indicated a peak brain uptake of 0.35 % of the injected dose at 15 minutes which declined to 0.2 % at 120 minutes [24]. Alternatively 1,4-benzodiazepine-5-phenyl derivatives have been radiolabelled with iodine-123/125 in the 2'-position of the 5-phenyl ring (Fig. **3**). The regioselective radioiodination of 2'-iododiazepam (**9)** in high specific activity was carried out by a bromine-iodine exchange reaction followed by HPLC purification [25]. The *in vitro* binding affinity of **9** was reported to have a higher affinity for the BZR than diazepam (**3**), while *in vivo* distribution studies in mice indicated a higher uptake and higher brain/blood ratio than that reported for **[^{11}C]3**. The uptake of **[^{123}I]9** in the cortical regions was specific as demonstrated by its displacement by pharmacologically active benzodiazepines.

The substitution pattern on the imidazobenzodiazepine skeleton is critical for high affinity and selective binding to the BZR. This is especially important when substituting halogens for protons or even iodine for chlorine or bromine. The incorporation of halogens such as chlorine or iodine in the 4'-position of diazepam (**10)** produces compounds devoid of central BZR affinity but with high affinity for the peripheral BZR.

When investigating ligands with either PET or SPECT it is essential that they possess acceptable metabolic stability for ligand-receptor modelling and subsequent image reconstruction and analysis. However, *N*-[^{11}C]methylation of the amide nitrogen in the above molecules (**8a**, **9**, **10**) resulted in the incorporation of the radiolabel at a metabolically or hydrolytically unstable position leading to radioligands with short biological half-lives and a poor, non-useful, signal. As a result, the potent agonists alprazolam (**11**) (IC_{50} = 14 nM) and triazolam (**12**) (IC_{50} = 15.5 nM)

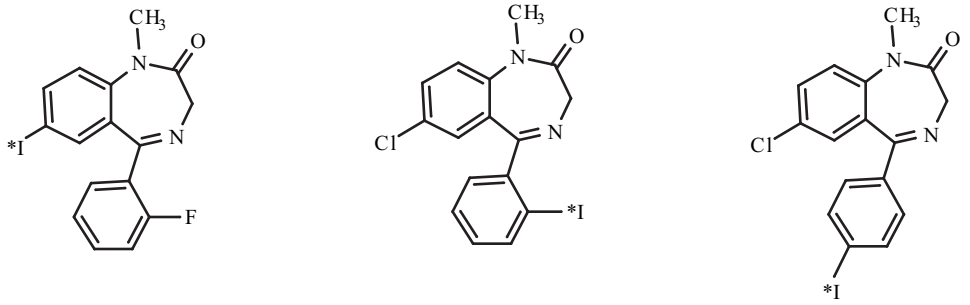


6a R = Cl
6b R = F

7a R = Cl
7b R = F

8a R = Cl
8b R = F

Fig. (2).



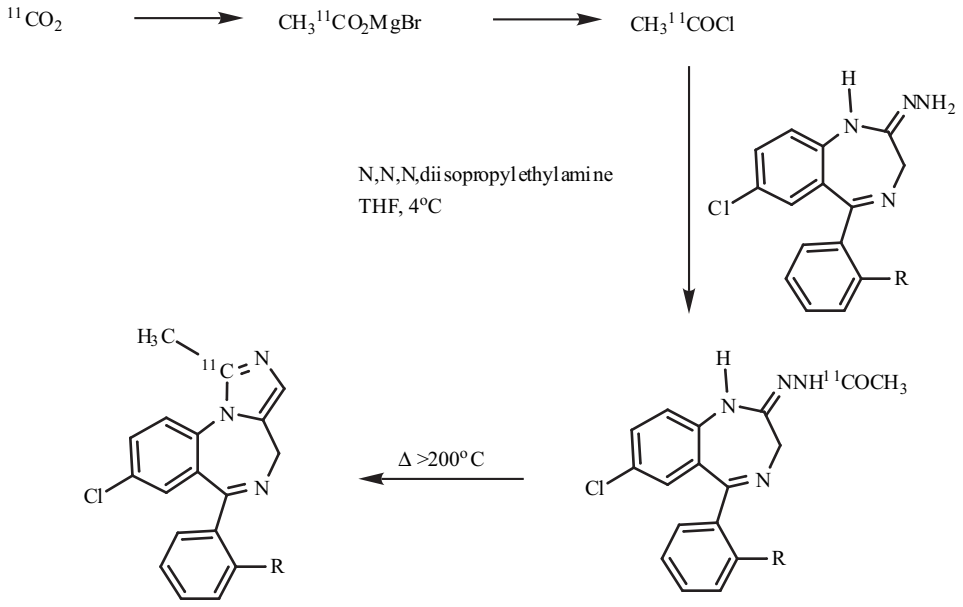
8b 5-iododiazepam **9** 2'-iododiazepam **10** 4'-iododiazepam

Fig. (3).

were labelled with carbon-11 at the C-1 position of the triazole ring leading to more metabolically stable molecules. In addition, the synthesis of **[^{11}C]11** and **[^{11}C]12** presented opportunities to visualise and study the interaction of agonists with improved specificity for the BZR using PET. The synthesis involved the conversion of $^{11}CO_2$ to its corresponding magnesium bromide 1-[^{11}C]acetate salt by the Grignard reaction with methyl magnesium bromide, followed by conversion into 1-[^{11}C]acetyl chloride using phthaloyl dichloride. Condensation with the amidrazones, 7-chloro-5-phenyl-3H-1, 4-benzodiazepine-2-yl hydrazine or 7-chloro-5-(2'-chlorophenyl)-3H-1,4-benzodiazepine-2-yl hydrazine in the presence of the hindered base N,N-diisopropylethylamine gave the corresponding diazepine-2-yl hydrazines which were pyrolysed at 200 ^{o}C to give **[^{11}C]11** in 43-65 % yield, in 40-55 minutes and specific activity of 0.93-2.18 Ci/μmol or **[^{11}C]12** in 55-65 minutes with specific activity of 100-400 mCi/μmol (Fig. **4**) [26, 27]. The radiosynthesis of **[^{11}C]11** was also automated for ease of large scale repetitive synthesis [28].

The PET *in vivo* pharmacokinetics of **[^{11}C]12** in primates [27] indicated rapid uptake of radioactivity in regions of the brain expressing BZR's, reaching a maximum 25 minutes p.i. followed by slow clearance (reported $t_{1/2}$ = 190 min). The uptake of activity could be blocked by pre-administration of the BZR antagonist flumazenil (1 mg/kg) 5 minutes prior to administration of **[^{11}C]1**, indicating 30 % non-specific binding at saturation. In separate experiments the activity in the brain decreased rapidly after injecting three different benzodiazepine ligands: triazolam (0.03 mg/kg), flumazenil (0.008 mg/kg) and the BZ_1 specific agonist zolpidem (2.5 mg/kg) 20 minutes p.i. demonstrating that **[^{11}C]12** binding was displaceable.

Subsequently **[^{11}C]11,** when prepared with specific activity >12,000 mCi/μmol, was studied in six male healthy volunteers [28]. The **[^{11}C]11** uptake in the brain displayed characteristics of receptor mediated binding in brain regions of known BZR density that was displaceable in the presence of unlabelled agonists. The uptake however was relatively low (less than 1 % of the injected activity) but



11 Alprazolam R =H
12 Triazolam R = Cl

Fig. (4).

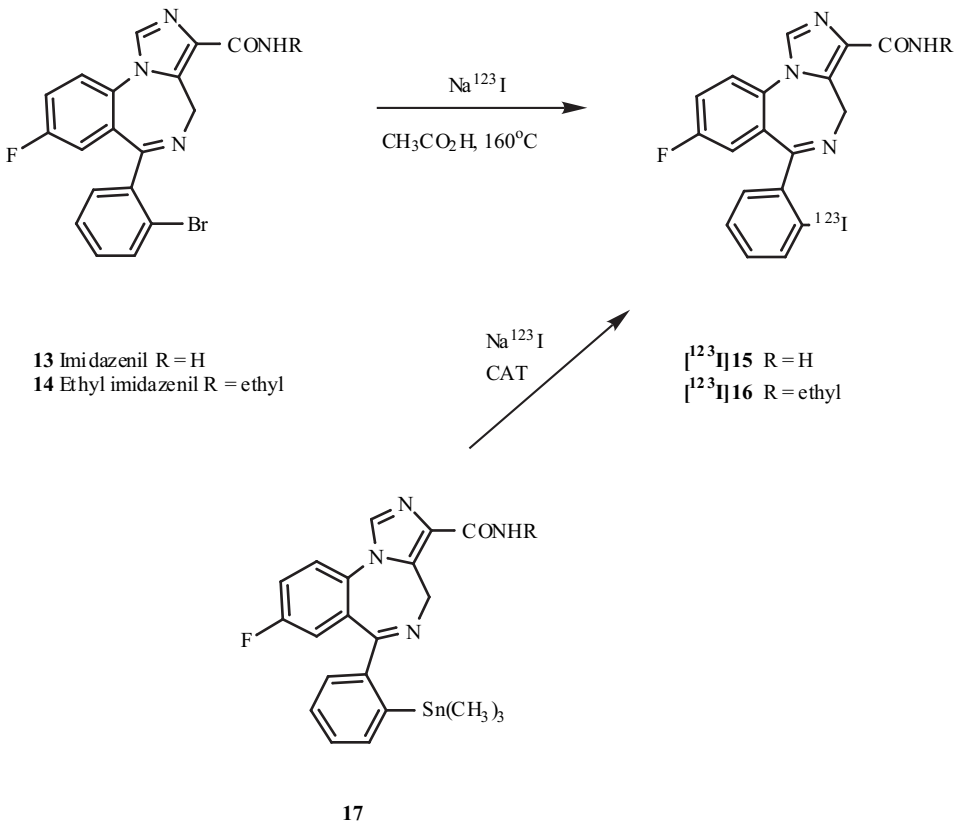


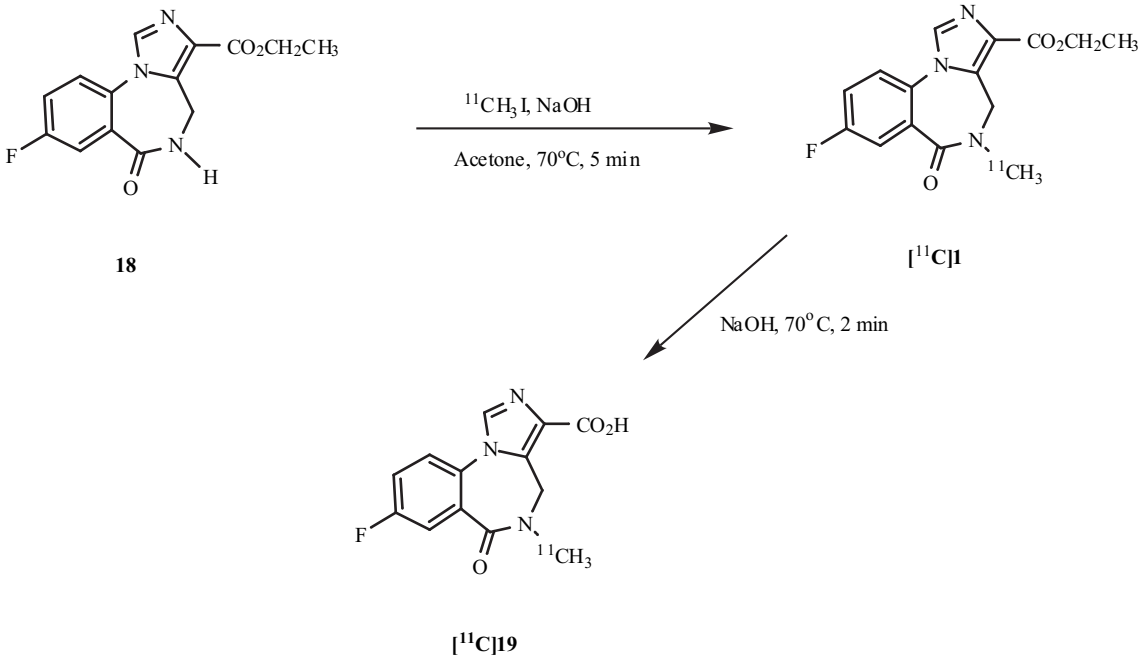
Fig. (5).

more interestingly it exhibited unusual pharmacokinetics during blocking studies. The authors attributed this to a "depot effect" where the radioligand is trapped at a peripheral saturable binding site that is then slowly released for brain uptake.

More recently, the highly selective imidazobenzodiazepine partial agonist imidazenil (**13)** (Ki = 0.5 nM) and its ethyl analogue (**14)** (Ki = 0.47 nM) were radiolabelled in the 2'-phenyl position with iodine-123 and evaluated in rodents [29, 30]. The radiosynthesis of both compounds was carried out by both nucleophilic bromine-iodine-123 exchange followed by HPLC purification as well as via electrophilic iododestannylation of the corresponding trimethyl tin precursor **17** in the presence of chloramine-T (Fig. **5**). In both cases the product was formed in very high specific activity. *In vitro* binding of the iodinated derivative of ethylimidazenil indicated a higher affinity than the parent bromo-compound. Biodistribution studies of both 2'-[^{123}I]iodoimidazenil (**[^{123}I]15**) (Ki = 1.1 nM) and 2'-[^{123}I]iodoethylimidazenil (**[^{123}I]16**) (Ki = 0.03 nM) in rats indicated high uptake in the brain regions associated with BZR density (0.7-0.8 % ID/g and 0.9-1.0 % ID/g respectively). Pretreatment with a number of benzodiazepine agonists, partial agonists and inverse agonists partially reduced the radioactivity in these brain regions indicating selectivity for the BZR. No BZR-subtype selectivity for these ligands was found.

The pharmacological problems encountered with carbon-11 labelled diazepam (**3**) and flunitrazepam (**4**) prompted the radiolabelling of the antagonist flumazenil (**1**) (Ro 15-1788). Ligand **1** was a new benzodiazepine type ligand that lacked the 5-phenyl substituent but incorporated an imidazo ring system. The preparation of **[^{11}C]1** was readily achieved by *N*-[^{11}C]methylation of the desmethyl precursor **18** in the presence of a strong base such as trimethylbenzylammonium hydroxide or sodium hydroxide (Fig. **6**) [16]. The product was obtained in greater than 70 % radiochemical yield and with specific activity of 1100-1700 mCi/μmol. PET imaging studies of **[^{11}C]1** in primates indicated high uptake of activity in the regions of high BZR density such as the cerebellum and cortical regions. The tracer displayed an equilibrium phase in 10 minutes which lasted for another 10 minutes before slowly decreasing. The uptake of activity in the BZR rich regions could be displaced by the administration of cold flumazenil (0.5 mg/kg) suggesting specific binding greater than 80 % *in vivo*. An automated synthesis of **[^{11}C]1** was reported soon after enabling its large scale and routine preparation for clinical studies [31].

As **[^{11}C]1** is rapidly metabolised *in vivo*, any form of image analysis and kinetic modelling requires a knowledge of the radioactivity distribution between the parent compound and metabolites. As a result, flumazenil (**1**) was also radiolabelled at the 2-ethyl ester position by treatment of the free acid **19** (Ro 15-3890) with [^{11}C]ethyl iodide resulting in **[^{11}C]20** (Fig. **7**) [32]. The main metabolite, the carboxylic acid derivative **[^{11}C]19** (Ro 15-3890), was prepared by simple de-esterification of **[^{11}C]1** with NaOH at 70 °C for 2 minutes (Fig. **6**). The favourable pharmacological properties and reasonable pharmacokinetics have made **[^{11}C]1** the most widely studied carbon-11 labelled BZR ligand. Since its development almost 20 years ago, it has been extensively used to study the central BZR



18 **[^{11}C]1**

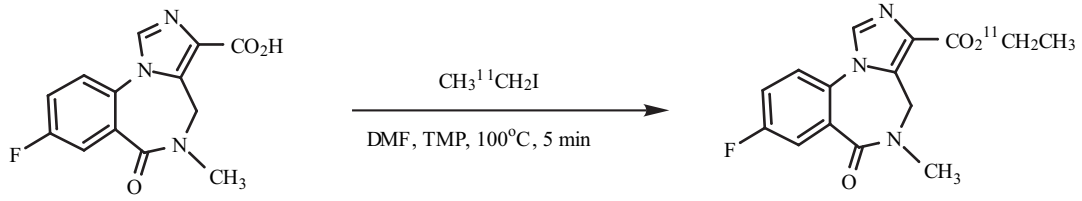
[^{11}C]19

Fig. (6).

and has become the "gold standard" in PET studies. Today it is still routinely used to study the role of the BZR in a number of clinical conditions including epilepsy.

The success of **[^{11}C]1** has encouraged the development of a SPECT derivative. The antagonist iomazenil (**2**) (Ro 16-0154) was therefore radiolabelled with iodine-123 in the 7-position (Fig. **8**) [17]. Ligand **2** was originally radiolabelled with iodine-123/125 via a nucleophilic bromine-iodine substitution reaction followed by HPLC purification to separate the bromo derivative. The presence of the activating carbonyl group ortho to the bromine atom on the aromatic ring facilitates the exchange reaction in the absence of copper catalysts ensuring almost quantitative yields in the relatively short reaction time of 15 minutes. More recently, radiolabelled **2** has been produced by electrophilic reactions involving the corresponding tributylstannane intermediate **22** [33]. Iododestannylation is carried out in an organic solvent in the presence of an oxidising agent such as chloramine-T or peracetic acid in radiochemical yields of 50-70 % with specific activity of 180,000 mCi/μmol. Radioligand **[^{123}I]2** displayed high affinity and selective binding to the BZR. *In vivo* it has good blood brain barrier permeability as demonstrated by it's high uptake in the brain regions expressing the BZR in rodents, primates and humans and could be displaced by known BZR ligands. To establish the molecule's pharmacokinetic profile and assist in quantitative measurements, **2** has also been radiolabelled with carbon-11 by *N*-methylation of the amide precursor for PET analysis [34]. Even though **[^{123}I]2** displayed favourable imaging properties, the ethyl ester function makes it relatively labile *in vivo* resulting in rapid metabolism, with less than 10 % of the injected activity remaining unchanged in blood plasma after 10 minutes. Although this drawback has been somewhat addressed by slow infusion of the tracer to provide a constant input of activity to the brain to reach a steady state, a metabolically more stable radioligand would be of benefit. Nevertheless, this molecule has been extensively studied in normal subjects and in a number if disease states using SPECT.

The rapid metabolism of **[^{11}C]1** and **[^{123}I]2** has prompted the development of other BZR ligands for *in vivo* studies. NNC 13-8241 (**26**) [35] and iodobretazenil (**29**) [36, 37] are both imidazobenzodiazepines bearing more stable "ester analogues" for enhanced metabolic stability. NNC 13-8199 (**24**), is an imidazo benzodiazepine derivative bearing a 5-cyclopropyl-1,2,4-oxadiazole group in the 3-position and was found to display partial agonist properties with potent affinity and selectivity for the BZR. In addition, the 5-cyclopropyl-1,2,4-oxadiazole group is less sensitive to metabolism compared to the ethyl ester of either flumazenil (**1**) or iomazenil (**2**) resulting in more favourable count rates during image analysis. The presence of the *N*-methyl group



19 **[^{11}C]20**

Fig. (7).

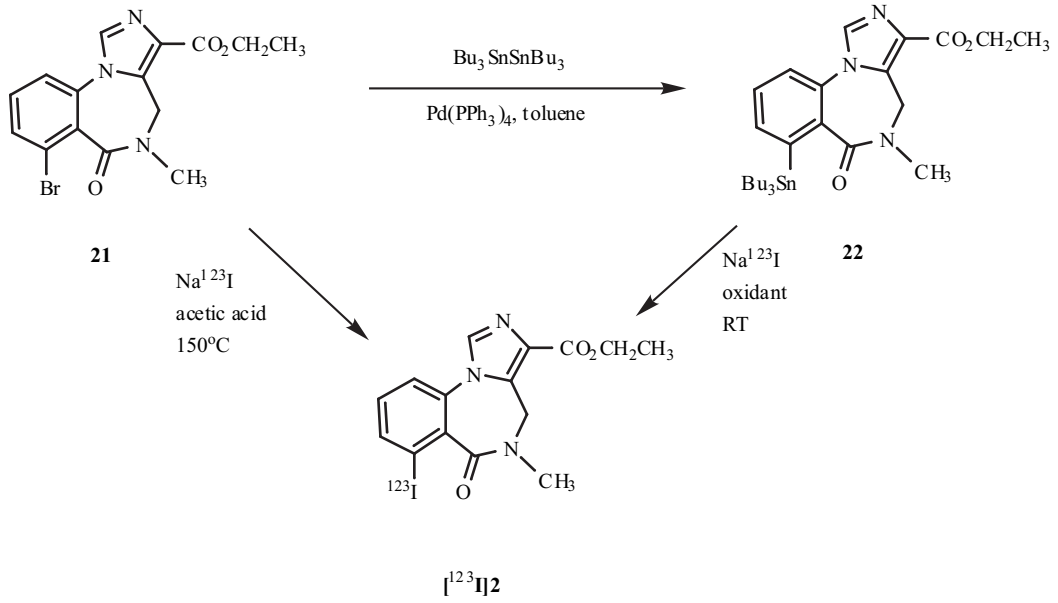


Fig. (8).

and a bromine-atom in the 7-position also allows alternative radiolabelling with either bromine-76 or iodine-123 as well as *N*-[^{11}C]methylation. [^{11}C]NNC 13-8199 (**[^{11}C]24)** was prepared in approximately 50 % radiochemical yield and a specific activity of 800 mCi/μmol (Fig. **9**) [35]. Studies in primates indicated high brain uptake, peaking at about 50 minutes p.i. with a total uptake of 8 % of the injected dose. The radioligand bound to neuronal BZRs with the neocortex displaying the highest uptake and the pons the lowest as expected. The activity in all brain regions could be displaced by 1.4 mg/kg flumazenil. HPLC analysis of plasma radioactivity in primates demonstrated very slow metabolism with no labelled metabolites detected within the time of measurement (95 minutes) confirming the enhanced stability of the 5-cyclopropyl-1,2,4-oxadiazole group compared to the ethyl ester of flumazenil (**1**). As the short half life of carbon-11 did not permit extensive studies of this compound the authors radiolabelled this compound with the PET radionuclide bromine-76.

NNC 13-8199 (**24)** and NNC 13-8241 (**26)** are BZR partial agonist bearing the halogens iodine and bromine in the 7-position of the benzodiazepine ring respectively making them excellent candidates for radiolabelling with the PET and SPECT isotopes bromine-76 and iodine-123. Both ligands displayed selective and potent (subnanomolar) affinity for the BZR receptor *in vitro*. As indicated above the 5-cyclopropyl-1,2,4-oxadiazole group in the 3-position imparts enhanced stability compared to the ethyl esters of flumazenil (**1**) and iomazenil (**2**). Radioligand **[^{123}I]26** was prepared in approximately 50 % radiochemical yield by the reaction of the trimethyl tin precursor **25** and sodium [^{123}I]iodide in the presence of chloramine-T as the oxidant (Fig. **10**) [36]. *In vivo* studies in rats indicated high uptake in brain regions known to express high BZR density. SPECT studies in primates indicate high uptake of activity in the occipital and frontal cortex which could be displaced with flumazenil. Four hours p.i. 80 % of the radioactivity in plasma was found to be unchanged radioligand. Radioligand **[^{76}Br]24** was obtained in an analogous manner via the same trimethyl tin precursor, ammonium [^{76}Br]bromide and chloramine-T [35]. The product was obtained in a radiochemical yield of 60 % with radiochemical purity greater than 98 %. PET studies with **[^{76}Br]24** in primates indicated continuous uptake of activity in the brain with time until it was displaced with flumazenil at 215 minutes p.i. As for the iodine-123 analogue (**[^{123}I]26**) the activity in the cortical brain regions were markedly reduced with flumazenil. Metabolite analysis of **[^{76}Br]24** indicated that more than 98 % of the radioactivity in plasma represented unchanged 40 minutes p.i. It appears that the 5-cyclopropyl-1,2,4-oxadiazole group imparts a high degree of metabolic stability on these benzodiazepine ligands compared to **1** and **2**. However, the question of slow dissociation from the BZR and continued accumulation remains a concern for quantitative analysis.

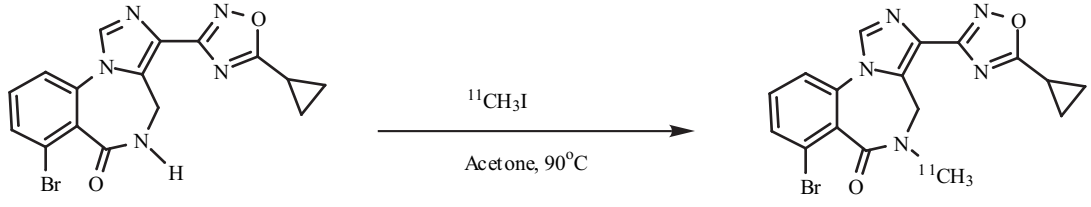


Fig. (9).

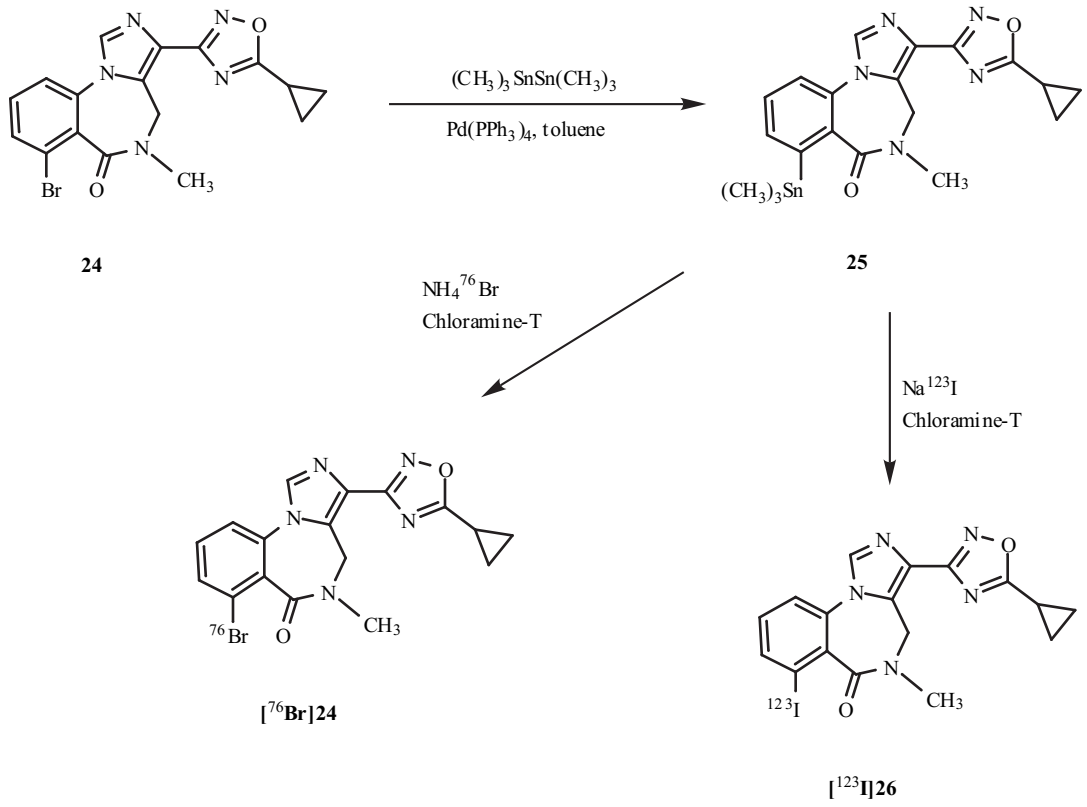


Fig. (10).

Bretazenil, (**27**) (Ro 16-6028) (Fig. **11**)**,** is a high affinity (Ki = 1.1 nM) BZR partial agonist. The presence of the tert-butyl ester and the fused pyrrole ring in **27** not only imparts enhanced metabolic stability on the molecule compared to iomazenil (**2**) and flumazenil (**1**) but also permits radiolabelling with iodine-123 [37, 38] and bromine-76 for SPECT and PET imaging of a partial agonist. However, **27** is a chiral molecule existing in two enantiomeric forms with all of the pharmacological activity residing on the (*S*)-enantiomers **27** and **29**, while the (*R*)-enantiomers **28** and **30** were devoid of any activity *in vitro* and *in vivo*. **27** has been radiolabelled with both iodine-123 for SPECT studies as well as with the PET radionuclide bromine-76 (Fig. **11**). Radiolabelling in both cases was achieved by halodestannylation of the corresponding tributyl tin precursor **31** in an organic solvent in the presence of either sodium [^{123}I]iodide or ammonium [^{76}Br]bromide and chloramine-T or peracetic acid as the oxidants. The reactions are generally high yielding, are carried out at room temperature and are extremely rapid (1-2 minutes). This reaction is however pH sensitive as a competing side reaction occurs at higher pH leading to the formation of [^{123}I]iodobutane [39]. The formation of volatile [^{123}I]iodo-butane could be explained by competitive electrophilic reaction at the aliphatic butyl groups of the stannane compared to the aryl site enhanced by the neighbouring ortho electron-withdrawing amide group [37]. Presumably at the lower pH, the electron withdrawing effect of the amide group is reduced by protonation effectively enhancing reaction at the aryl site. This was further supported by the comparative reaction of the 7-substituted tributylstannyl benzodiazepine **32** with the electron withdrawing group meta to the stannane. In the latter case reaction of **32** with sodium [^{123}I]iodine using chloramine-T or peracetic acid at various pH levels and solvents proceeded smoothly in high yields to give the desired product **33** without any side reaction and loss of activity. *In vivo* studies of **[^{123}I]29** and **[^{76}Br]27** in rodents indicated a distribution pattern consistent with BZR distribution and which could be blocked with flumazenil and other BZR ligands. Metabolite studies of **[^{123}I]29** in rodent blood plasma indicated 60-70 % intact radioligand at 3 hours p.i. [38]. For **[^{76}Br]27**, metabolite analysis showed that at 30 minutes and 2.5 hours p.i., 60 % and 40 % of the radioactivity represented unchanged radioligand (Katsifis et al unpublished data). Despite the similarities between the two radioligands, **[^{123}I]29** displayed high and prolonged retention of activity in the brain regions even after 24 hours. **[^{76}Br]27** on the other hand indicated high initial uptake (30-60 minutes) but followed by clearance after 2-3 hours. Primate studies of **[^{76}Br]27** indicated high uptake in the brain with a distribution profile similar to that of specific BZR ligands and could be displaced using flumazenil.

Although a large number of compounds of non-benzodiazepine structure have been described with potent affinity for the BZR, very few have been radiolabelled and evaluated *in vivo*. 2-Phenylpyrazolo[4,3-c]-quinolin-3(5H)-one is one such derivative with potent affinity for the BZR. Whereas it is a potent antagonist, its chloro derivative exhibits potent anxiolytic activity without apparent sedative liability. Two derivatives of this compound have been radiolabelled with radioiodine to determine its tissue distribution and investigate its pharmacological activity [40]. Radioiodination was carried out via the classical triazine decomposition reaction of **34** and **36** in acetonitrile and in the presence of methanesulfonic acid to give **[^{131}I]35**

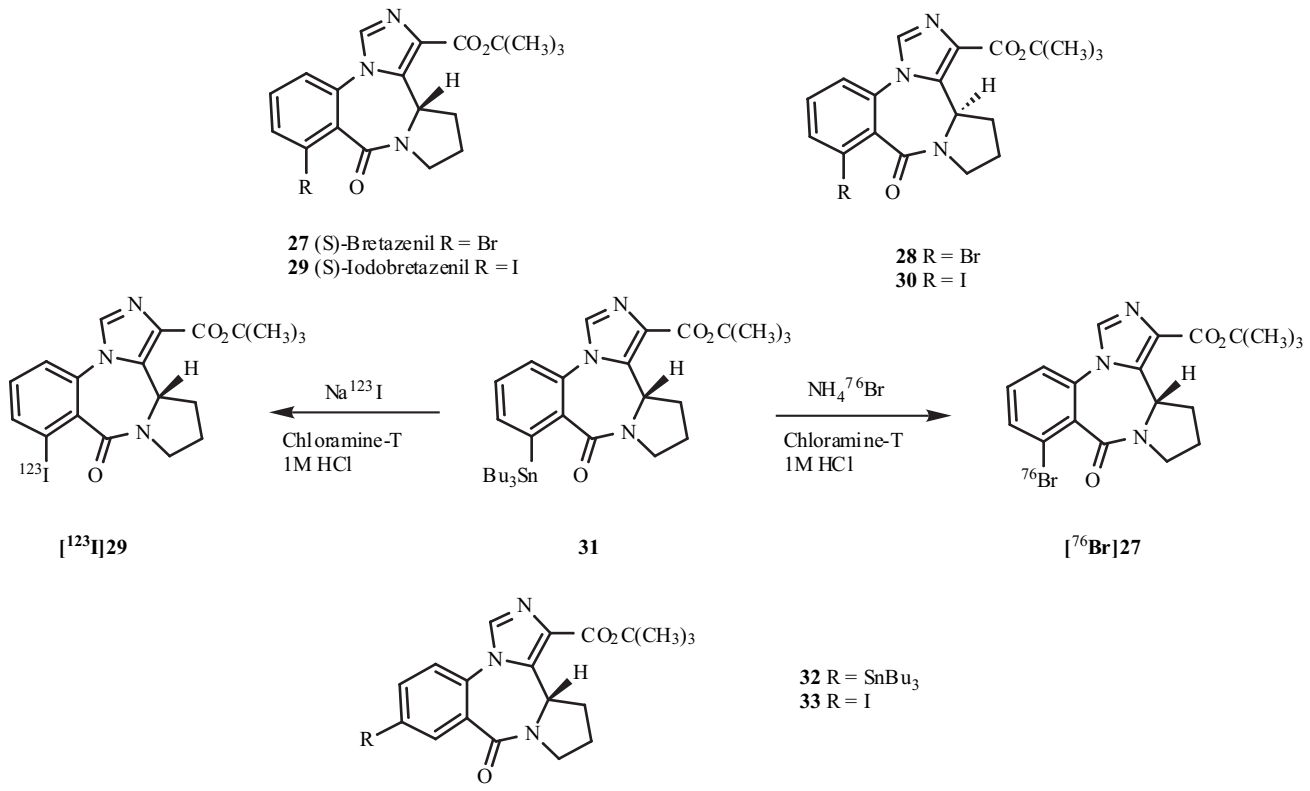


Fig. (11).

and **[^{131}I]37** in high specific activity with low isolated yields of 5 and 11 % respectively (Fig. **12**). Modifications to this reaction involving solid phase decomposition reactions increased the isolated radiochemical yields to 15-35 %. No biological results were reported.

Of all the ligands that have been labelled with carbon-11 for imaging the BZR using PET, [^{11}C]suriclone (**[^{11}C]39**) has been the most significant compound that does not possess the typical 1,4-benzodiazepine structure. Compound **39** is a cyclopyrrolone with similar pharmacological properties to that of the classical BZ agonists and displays potent activity in animals and man. Even though at 0^{o}C it has a similar affinity for the GABA$_A$-BZR as flunitrazepam, its affinity at 37 ^{o}C is about ten-fold higher, a marked contrast to the classical 1,4-benzodiazepines. In addition, it also appears that **39** and other cyclopyrrolones bind to a site on the GABA$_A$-BZR complex quite different to that of the conventional BZR ligands. **[^{11}C]39** was prepared by standard *N*-methylation of the desmethyl precursor **38** using [^{11}C]methyl iodide in 40-60 % radiochemical yield and with a specific activity of 750 mCi/μmol (Fig. **13**) [41, 42]. An alternative radiosynthetic procedure involving the reductive alkylation of the desmethyl derivative RP 35489 with [^{11}C]formaldehyde in the presence of sodium cyanoborohydride gave **[^{11}C]39** in only 5-30 %

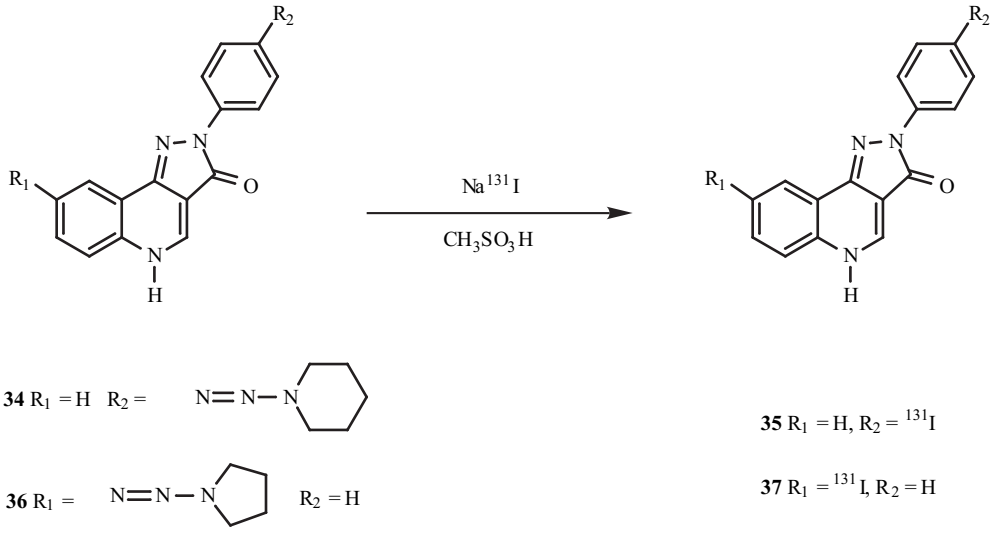


Fig. (12).

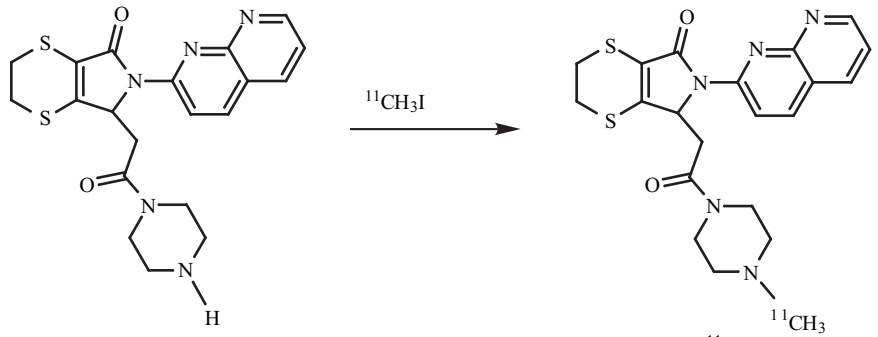


Fig. (13).

radiochemical yield. The low yield was attributed to the difficulty in trapping the [^{11}C]formaldehyde in the reaction mixture.

PET studies with **[^{11}C]39** in primates indicated high and persistent uptake in brain areas consistent with the known distribution of $GABA_A$-BZR, with the most intense uptake observed in the cerebellum and cortical areas approximately 1 hour p.i. Pretreatment with flumazenil (1 mg/kg) 10 minutes prior to the administration of **[^{11}C]39** decreased total brain radioactivity four-fold while it had no effect on the uptake of activity in the peripheral tissue indicating the specificity of the tracer for the central BZR. The distribution pattern of **[^{11}C]39** in human subjects was similar and resembled that of **[^{11}C]1**. Binding was again highest in the cerebellum and cerebral cortex with low levels in the striatum-caudate nucleus. In contrast to **[^{11}C]1**, **[^{11}C]39** displayed slow clearance from the regions of high uptake suggesting a slow dissociation of the ligand from the receptor. Although **[^{11}C]39** appeared to be an excellent ligand for imaging the $GABA_A$-BZR's no further work has since been reported with this ligand.

The partial inverse agonist Ro 15-4513 (**40**) and its analogues were found to possess partial selectivity for the α_6 subtype or the "diazepine insensitive" receptors found in the granule cells of the cerebellum. These inverse agonists were found to display unique properties including enhance cognitive functions, anxiogenesis and the ability to antagonise the effects of ethanol. Ligand **40** is an azido derivative of **1** and as such was radiolabelled with [^{11}C]methyl iodide in 30-40 % radiochemical yield and with specific activity of about 1000 mCi/μmol (Fig. **14**) [43]. Autoradiographic analysis on human post-mortem brain tissue indicated specific binding in the neocortex, basal ganglia and cerebellar cortex with the predominant uptake being in the neocortex. Although flumazenil and clonazepam were able to inhibit the uptake of activity in the cerebral regions, a significant portion of activity in the cerebellar regions could not be inhibited. This portion was attributed to the α_6 or "diazepam-insensitive" component. Although some basic PET examinations in primates indicated uptake in BZR regions which could be displaced with high doses of cold **40** or clonazepam, no further studies on the significance of this receptor subtype were reported. The availability of several benzodiazepine derivatives of **40** has encouraged the development of halogenated analogues suitable for SPECT imaging. Replacement of the azido group in the 7-position with iodine makes it possible for radiolabelling with iodine-123. Two iodine-123 derivatives **41** and **42** bearing different ester groups were prepared and evaluated *in vivo* [44, 45].

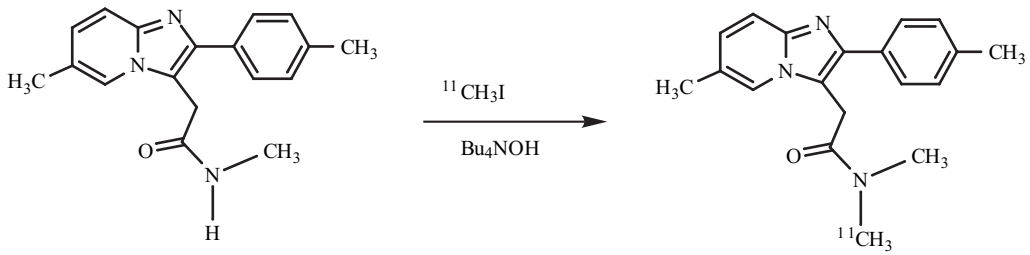
The imidazopyridine zolpidem (**44**) has been reported to exhibit BZ1 subtype selectivity and as a consequence has been deployed in numerous pharmacological studies to elucidate BZR function including blocking or displacement studies. Recently it has been radiolabelled with carbon-11 on the acetamide side chain (Fig. **15**). Despite its detailed pharmacological characterisation and well established use as a hypnotic, the uptake of **[^{11}C]44** in the brain of primates was actually quite low [46]. Attempts to make a SPECT derivatives by replacing the 4'-methyl group *via* the stannane **45** with iodine led to **[^{123}I]46** with low affinity for the central BZR and high affinity for the peripheral BZR (Fig. **16**) [47].

Despite the significant advantages of the PET radionuclide fluorine-18 for imaging relatively little has been undertaken in the field of BZR research. This has been attributed to the difficulty in introducing high specific activity fluorine-18 *via* nucleophilic methods onto 1,4-benzodiazepine systems. For example, fluorination of **3** with either "carrier added" [^{18}F]fluorine or "carrier added" [^{18}F]acetyl hypofluorite [48] led to products of low specific

[^{11}C]40, ([^{11}C]Ro 15-4513)

[^{123}I]41 R = CH_2CH_3
[^{123}I]42 R = $C(CH_3)_3$

Fig. (14).

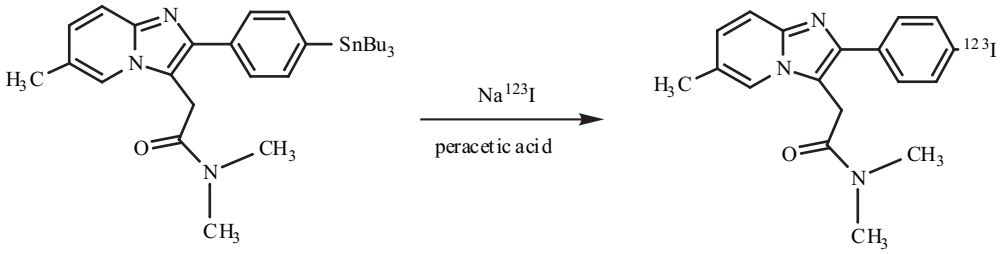


43 **[^{11}C]44**

Fig. (15).

activity unsuitable for central BZR measurements. No carrier added (nca) **[^{18}F]3** was prepared [48, 49] *via* [^{18}F]fluoride-chlorine displacement in the 3-position leading to a molecule more potent than diazepam (**3**) and also more resistant to metabolism by blocking the site of hydroxylation. However, as this molecule is also racemic it proved difficult in PET data analysis.

^{18}F]fluoropropyl ester of Ro 15-3890 did not possess suitable properties for receptor binding studies [53]. *N*-[^{18}F]Fluoroethyl flumazenil (**[^{18}F]50**) was initially synthesised by reaction of 1-bromo-2-[^{18}F]fluoro ethane and the precursor **49** (Ro 15-5528) to give **[^{18}F]50** in 24-29 % yield and specific activity >1000 mCi/μmol in 80 minutes of preparation time. Increased radiochemical yields of about



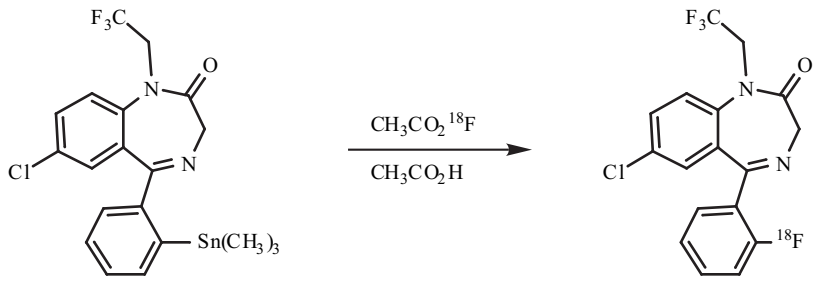
45 **[^{123}I]46**

Fig. (16).

The potent and BZ_1 selective antagonist oxaquazepam (**48**) (IC_{50} = 14 nM for BZ_1 and 111 nM for BZ_2) was fluorinated in the 2'-position of the 5-phenyl ring by electrophilic fluorination via a trimethyl tin precursor **47**, however, the low specific activity of this compound was also unsuitable for PET receptor studies (Fig. **17**) [50].

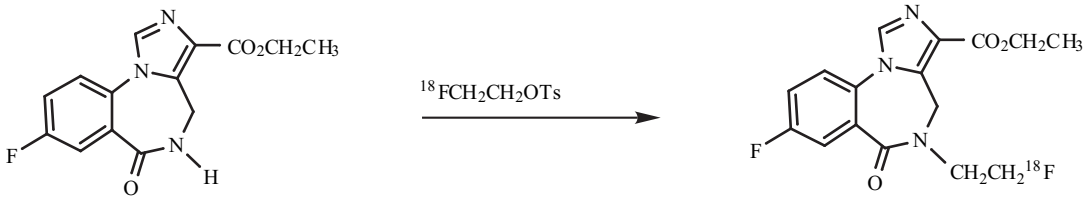
As the incorporation of fluorine-18 in the natural position of flumazenil (**1**) is not feasible, fluorine-18 labelling can be achieved via fluoroalkylation at the 5 or *N*-methyl position. Alkylation with a [^{18}F]fluoroethyl derivative gave a ligand with appropriate affinity, selectivity and specific activity suitable for human studies [51, 52]. The *N*-(3-[^{18}F]fluoropropyl) derivative and the [3-

90% could be achieved when using the [^{18}F]fluoroethyltosylate in DMSO and NaH as the base at 90 ^{o}C after 10 minutes of reaction time. (Fig. **18**) [54]. *In vitro* studies of **[^{18}F]50** revealed a Ki of 5.2 nM with the same $GABA_A$-BZR subtype selectivity as **1**. PET studies in human brains indicated identical distribution to known BZR density with significantly reduced uptake in all regions after pre-treatment with cold flumazenil. Peak uptake occurred at 4 minutes followed by rapid clearance resulting in 50 % and 20 % of maximum uptake after 10 and 20 minutes respectively. Metabolites represented 50-60 % of the blood plasma activity after 5 minutes and 80-90 % at 20 minutes p.i. The radioligand **[^{18}F]50** therefore displayed suitable



47 **[^{18}F]48**

Fig. (17).



49 **[^{18}F]50**

Fig. (18).

characteristics for imaging central BZR's *in vivo* using PET. However, despite the longer imaging times possible with the use of fluorine-18 compared to carbon-11, the similar metabolic profile of **[^{18}F]50** does not offer significant advantages over its carbon-11 analogue **[^{11}C]1** except in its production and distribution to centres remote from cyclotron centres. Recently flumazenil was labelled at the 2-ethyl ester position (2-[^{18}F]fluoroethylflumazenil) however no biological results were reported [55].

SUMMARY

Over the past 20 years ligands not only with improved metabolic stability but also with different interactions with the $GABA_A$-BZR complex have been prepared which may be of value in studying BZR subtypes and function in various parts of the brain. The ability to fine tune the pharmacological activity of benzodiazepines through partial activation of the BZR has overcome the undesirable side effects of many of the early BZ ligands. However, all of the ligands developed so far lack appropriate selectivity for the BZR subtypes. An alternative approach to achieving selective pharmacological profiles is through the development of such type of ligands. Therefore, the further refinement and development of $GABA_A$-BZR subtype selective PET and SPECT radioligands will play an important role towards understanding selective $GABA_A$-BZR disturbances as they occur in various human pathologies.

REFERENCES

[1] Squires, R. F.; Braestrup, C. *Nature*, **1977**, *266*, 732-734.
[2] Hantraye, P.; Brouillet. E; Fukuda, H.; Chavoix, C.; Guibert, B.; Dudd, R. M.; Prenant, C.; Crouzel, C.; Naquet, R.; Maziere, M. *Eur. J. Pharmacol.*, **1988**, *153*, 25-32.
[3] Pike, V. W.; Halldin, C.; Crouzel, C.; Barre, D.; Nutt, D. J.; Osman, S.; Shah, F.; Turton, D. R.; Waters, S. L. *Nucl. Med. Biol.*, **1993**, *20*, 503-525.
[4] Metha, A. K.; Ticku, M. K. *Brain Res. Rev.*, **1999**, *29*, 196-217.
[5] Sieghart, W. *J. Psychiatr. Neurosci.*, **1994**, *19*, 24-27.
[6] Sieghart, W. *Pharm. Rev.*, **1995**, *47*, 181-234.
[7] Griebel, G.; Perrault, G.; Letang, V.; Granger, P.; Avenet, P.; Schoemaker, H.; Sanger, D. *Psychopharmacology*, **1999**, *146*, 205-213.
[8] Mohler, H. F.; Knofflach, H.; Paysan, J.; Motejlek, K.; Benke, D.; Luscher, B.; Fritschy, J.-M. *Neurochemical Research*, **1995**, *20*, 631-636.
[9] Kenakin, T. *Trends in Pharmacological Sciences*, **1995**, *16*, 188-192.
[10] Haefely, W. E.; Martin, J. R.; Richards, J. G.; Schoch, P. *Can. J. Psychiatry*, **1993**, *38*, S102-S107.
[11] Zhang, W.; Koehler, K. F.; Zhang, P.; Cook, J. M. *Drug Design and Discovery*, **1995**, *12*, 193-248.
[12] Finn, D. A.; Gee, K. W. *Eur. J. Pharmacol.*, **1993**, *247*, 233-237.
[13] Puia, G.; Vicini, S.; Seeburg, P. H.; Costa, E. *Mol. Pharmacol.*, **1991**, *39*, 691-696.
[14] Puia, G.; Ducic, I.; Vicini, S.; Costa, E. *Proc. Natl. Acad. Sci. USA*, **1991**, *89*, 3620-3624.
[15] Pappata, S.; Samson, Y.; Chavoix, C.; Prenant, C.; Maziere, M.; Baron, J. C. *J. Cereb. Blood Flow Metab.*, **1988**, *8*, 304-313.
[16] Maziere, M.; Hantraye, P.; Prenant, C.; Sastre, J.; Comar, D. *Int. J. Appl. Radiat. Isot.*, **1984**, *35*, 973-976.
[17] Beer, H. F.; Blauenstein, P. A.; Hasler, P. H.; Delaloye, B.; Ricabona, G.; Bangerl, I.; Hunkeler, W.; Bonetti, E. P.; Piere, L.; Richards, J. G. *J. Nucl. Med.*, **1990**, *31*, 1007-1014.
[18] Bartenstien, P.; Ludolph, A.; Schober, O.; Lottes, G.; Scheidhauer, K.; Scuik, J.; Beer, H. F. *Eur. J. Nucl. Med.*, **1991**, *18*, 111-118.
[19] Johnson, E. W.; Woods, S. W.; Zoghbi, S.; McBride, B. J.; Baldwin, R. M.; Innis, R. B. *Life Sci.*, **1990**, *47*, 1535-1546.
[20] Comar, D.; Maziere, M.; Godot, J. M.; Berger, G.; Soussaline, F.; Menini, C.; Arfel, G.; Naquet, R. *Nature*, **1979**, *280*, 329-331.
[21] Maziere, M.; Godot, J. M.; Berger, G.; Prenant, C.; Comar, D. *J. Radioanal. Chem.*, **1980**, *56*, 229-235.
[22] Ishiwata, K.; Yanai, K.; Ido, T.; Miura-Kanno, Y.; Kawashima, K. *Nucl. Med. Biol.*, **1988**, *15*, 365-371.
[23] Scholl, H.; Kloster, G.; Stocklin, G. *J. Nucl. Med.*, **1983**, *24*, 417-422.
[24] Zecca, L.; Ferrario, P. *Appl. Radiat. Isot.*, **1988**, *39*, 353-356.
[25] Saji, H.; Nakastuka, I.; Iida, Y.; Magata, Y.; Yoshitake, A.; Yonekura, Y.; Konishi, J.; Yokoyama, A. *J. Nucl. Med.*, **1989**, *30*, 803-804.
[26] Banks, W.; Tewson, T. J.; Digenis, G. A. *Appl. Radiat. Isot.*, **1990**, *41*, 719-725.
[27] Le Breton, C.; Bottaender, M.; Crouzel, C.; Varastet, M.; Sitbon, R.; Maziere, B.; Maziere, M. *J. Lab. Compds and Radiopharm.*, **1993**, *32*, 525-527.
[28] Dobbs, F. R.; Banks, W.; Fleishaker, J. C.; Valentine, A. D.; Kinsey, B. M.; Franceschini, M. P.; Digenis, G. A.; Tewson, T. J. *Nucl. Med. Biol.*, **1995**, *22*, 459-466.
[29] Katsifis, A.; Mardon, K.; Kassiou, M.; Mattner, F.; Dikic, B. *J. Lab. Compds and Radiopharm.*, **1995**, *20*, 396-399.
[30] Katsifis, A.; Mattner, F.; Dikic, B.; Nadjovski, L.; Kassiou, M. *J. Lab. Compds and Radiopharm.*, **1996**, *38*, 1121-1132.
[31] Suzuki, K.; Inoue, K.; Yamasaki, M.; Kuchiki, M.; Tamate, K. *Int. J. Appl. Radiat. Isot.*, **1985**, *36*, 971-976.
[32] Halldin, C.; Stone-Elander, S.; Thorell, J.-O.; Persson, A.; Sedvall, G. *Int. J. Appl. Radiat. Isot.*, **1988**, *39*, 993-997.
[33] McBride, B. J.; Baldwin, R. M.; Kerr, J. M.; Wu, J.-L. *Appl. Radiat. Isot.*, **1991**, *42*, 173-175.
[34] Westra, G.; Buck, A.; Burger, C.; Leenders, K. L.; Von Schulthess, G. K.; Schubiger, A. P. *Eur. J. Nucl. Med.*, **1996**, *23*, 5-12.
[35] Foged, C.; Halldin, C.; Loc'h, C.; Maziere, B.; Pauli, S.; Maziere, M.; Hansen, H.; Suhara, T.; Swah, C.-G.; Karlsson, P.; Farde, L. *Eur. J. Nucl. Med.*, **1997**, *24*, 1261-1267.
[36] Foged, C.; Halldin, C.; Hiltunen, J.; Braestrup, C.; Thompson, C.; Hansen, H.; Suhura, T.; Pauli, S.; Swahn, C.-G.; Karlsson, P.; Larsson, S.; Farde, L. *Nucl. Med. Biol.*, **1996**, *23*, 201-209.
[37] Katsifis, A.; Mattner, F.; McPhee, M. E.; Kassiou, M.; Nadjovski, L.; Dikic, B. *J. Lab. Compds and Radiopharm.*, **1996**, *38*, 835-845.

[38] Katsifis, A.; Mardon, K.; Mattner, F.; Loc'h, C.; McPhee, M. E.; Dikic, B.; Kassiou, M.; Ridley, D. D. *Nucl. Med. Biol.*, **2003**, *30*, 191-198.
[39] Zea Ponce, Y.; Baldwin, R. M.; Zoghbi, S. S.; Innis, R. B. *Appl. Radiat. Isot.*, **1994**, *454*, 63-68.
[40] Maeda, M.; Komori, H.; Dohmoto, H.; Kojima, M. *J. Lab. Compds and Radiopharm.*, **1985**, *22*, 487-501.
[41] Frost, J. J.; Wagner, H. N.; Dannals, R. F.; Ravert, H. T.; Wilson, A. A.; Links, J. M.; Rosenbaum, A. E.; Trifiletti, R. R.; Snyder, S. H. *Eur. J. Pharmacol.*, **1986**, *122*, 381-383.
[42] Boullais, C.; Oberdorfer, F.; Sastre, J.; Prenant, C.; Crouzel, C. *J. Lab. Compds. Radiopharm.*, **1985**, *22*, 1081-1086.
[43] Halldin, C.; Farde, L.; Litton, J.-E.; Hall, H.; Sedvall, G. *Psychopharmacology*, **1992**, *108*, 16-22.
[44] He, X.; Matecka, D.; Lee, K. S.; Gu, Z.-Q.; Rice, K. C.; Wong, G.; Skolnick, P.; de Costa, B. R. *J. Lab. Compds and Radiopharm.*, **1993**, *34*, 27-32.
[45] Katsifis, A.; Mardon, K.; McPhee, M. E.; Mattner, F.; Dikic, B.; Ridley, D. D. *Nucl. Med. Biol.*, **1999**, *26*, 641-649.
[46] Dommont, F.; Waterhouse, R.; Montoya, J.; Mattner, F.; Katsifis, A.; Kegeles, L.; Laurelle, M. *Nucl. Med. Biol.*, **2003**, 30, 435-439.
[47] Katsifis, A.; Mattner, F.; Nadjovski, L.; Dikic, B. *J. Lab. Compds and Radiopharm.*, **2000**, *43*, 385-394.
[48] Luxen, A.; Barrio, J. R.; Bida, G. T.; Satyamurthy, N.; Phelps, M. E. *J. Nucl. Med.*, **1985**, *26*, P38.
[49] Luxen, A.; Barrio, J. R.; Satyamurthy, N.; Bida, G. T.; Phelps, M. E. *J. Fluorine Chem.*, **1987**, *36*, 83-92.
[50] Duelfer, T.; Johnstrom, P.; Stone-Elander, S.; Holland, A.; Halldin, C.; Haaparanta, M.; Solin, O.; Bergman, J.; Steinman, M.; Sedvall, G. *J. Lab. Compds and Radiopharm.*, **1991**, *29*, 1224-1239.
[51] Moerlein, S. M. *J. Nucl. Med.*, **1990**, *31*, 902.
[52] Moerlein, S. M.; Perlmutter, J. S. *Eur. J. Pharmacol.*, **1992**, *218*, 109-115.
[53] Teng, R.; Bai, L.-Q.; Shiue, C.-Y.; Dewey, S.; Arnett, C. D.; Wolf, A. P.; Hitzemann, R. J. *Nucl. Med. Biol.*, **1990**, *17*, 811-817.
[54] Hamkens, W.; Oiel, M.; Schirrmacher, R.; Schmii, U.; Kleinz, R.; Luddens, H.; Grunder, G.; Rosch, F. *J. Lab. Compds and Radiopharm.*, **1999**, *42*, 510-512.
[55] Jeong, J. M.; Hong, S. H.; Lee, D. S.; Chung, J.-K.; Lee, M. C.; Cho, J.-H.; Oh, C. H.; Lee, S. J. *J. Lab. Compds and Radiopharm.*, **1999**, *42*, 517-519.