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Pharmacokinetics and metabolism studies on (3-*tert*-butyl-7-(5-methylisoxazol-3-yl)-2-(1-methyl-1*H*-1,2,4-triazol-5-ylmethoxy) pyrazolo[1,5-*d*][1,2,4]triazine, a functionally selective $GABA_A$ α5 inverse agonist for cognitive dysfunction

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Abstract—(3-*tert*-Butyl-7-(5-methylisoxazol-3-yl)-2-(1-methyl-1*H*-1,2,4-triazol-5-ylmethoxy)pyrazolo[1,5-*d*][1,2,4]triazine (**1**) was recently identified as a functionally selective, inverse agonist at the benzodiazepine site of $GABA_A$ α5 receptors and enhances performance in animal models of cognition. The routes of metabolism of this compound in vivo in rat have been well characterised, the identities of the major metabolites are confirmed by synthesis and their biological profiles were evaluated. An unusual oxidation of the pyrazolo[1,5-*d*][1,2,4]triazine core to the corresponding pyrazolo[1,5-*d*][1,2,4]triazin-4(5*H*)-one scaffold by aldehyde oxidase has been observed.

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system and $GABA_A$ receptors are GABA-gated chloride ion channels which can be modulated by simultaneous binding of chemical entities to allosteric sites on the pentameric ion channel complex, the most important of which is the benzodiazepine (BZ) site.[1,2] The major $GABA_A$ receptor subtypes which possess a BZ binding site present in the brain are α1βγ2, α2βγ2, α3βγ2 and α5βγ2,[3] and BZ inverse agonists have been shown to decrease the frequency of ion channel opening in the presence of GABA, resulting in increased neuronal excitability. Non-selective BZ inverse agonists have been shown to improve cognitive function in animal models,[4] but are anxiogenic,[5] convulsant,[6] and proconvulsant.[7] Recently, (3-*tert*-butyl-7-(5-methylisoxazol-3-yl)-2-(1-methyl-1*H*-1,2,4-triazol-5-ylmethoxy)pyrazolo[1,5-*d*][1,2,4]triazine (**1**) has been identified as a functionally selective, inverse agonist at the BZ site of $GABA_A$ α5 receptors which enhances performance in animal models of cognition and does not exhibit convulsant, proconvulsant or anxiogenic activity (see Fig. 1).[8] This manuscript describes metabolic studies carried out on this compound, the identification of the routes of metabolism and the characterisation of the major metabolites.

Compound **1** displays a short half-life in rats $t_{1/2} = 0.5$ h, oral bioavailability F = 52% and modest clearance Cl = 27 ± 11 mL/min/kg, whereas in dog $t_{1/2} = 0.5$ h, F = 8% and Cl = 39 ± 10 mL/min/kg and rhesus $t_{1/2} = 0.3$ h, F < 2% and Cl = 33 ± 2 mL/min/kg. Initial analysis of plasma samples from the rat in vivo studies by mass spectroscopy showed the presence of three M + 16 metabolites. The same metabolites were detected in incubations with rat hepatocytes. Analysis of mass

Keywords: GABA; $GABA_A$ α5; Inverse agonist; Cognition; Metabolism; Aldehyde oxidase; Radiolabel.
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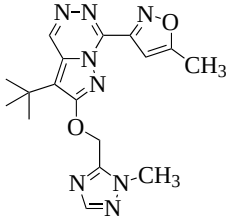
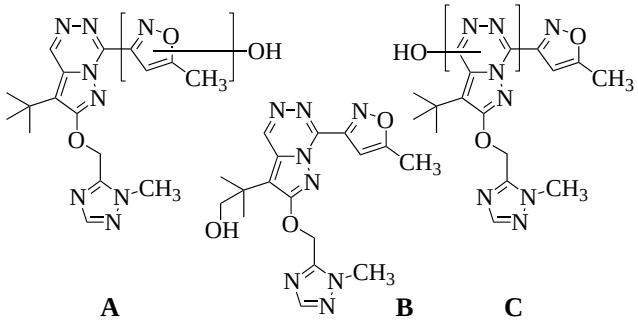


Figure 1. (3-*tert*-Butyl-7-(5-methylisoxazol-3-yl)-2-(1-methyl-1*H*-1,2,4-triazol-5-ylmethoxy)pyrazolo[1,5-*d*][1,2,4]triazine (**1**).



	A and **B**	**C**
Plasma	Rat, Dog, Rhesus	Rat, Rhesus
Microsomes	Rat, Dog, Rhesus	Not formed
Hepatocytes	Rat, Dog, Rhesus	Rat, Rhesus

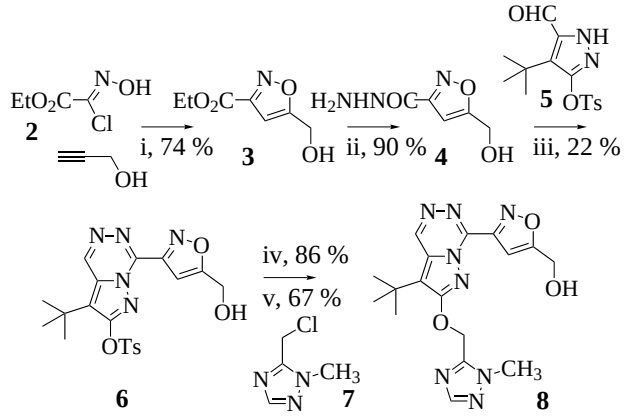
Figure 2. Potential metabolites.

spec fragmentation patterns tentatively assigned these as shown in Figure 2: **A**, oxidation, of the 5-methylisoxazole; **B**, oxidation of the *tert*-butyl group; and **C**, oxidation of the pyrazolotriazine core. Metabolite **C** is observed in rat and rhesus plasma and following incubations with rat hepatocytes, but not microsomes. However, it is seen in rat liver cytosol and S9 incubations, where formation does not require NADPH. It is not observed in the corresponding dog samples. These species differences, the subcellular localisation and cofactor requirements, and the nature of the biotransformation (adjacent to a nitrogen on an electron-poor aromatic ring), point to the involvement of a relatively unstudied enzyme, aldehyde oxidase.[9]

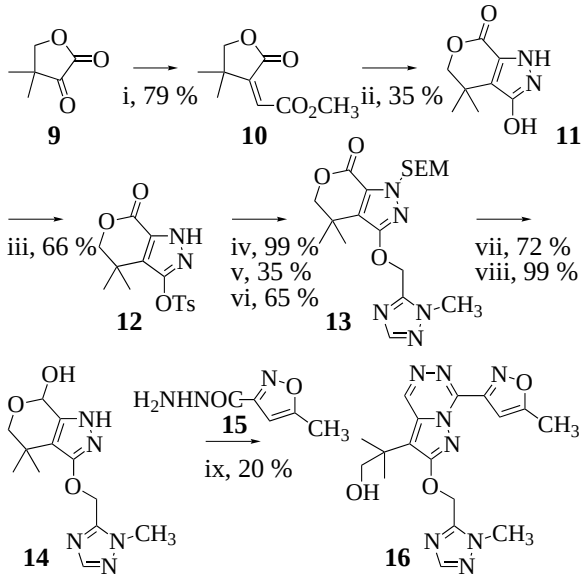
Synthetic chemistry was then deployed to synthesise these compounds with a view to confirming the metabolic routes and ensuring the biological activity observed was not the result of an active metabolite.

The intermediate for the preparation of the hydroxylated methylisoxazole **A** was readily obtained utilising a [3 + 2] dipolar cycloaddition of an in situ generated nitrile oxide and propargyl alcohol (Scheme 1). Conversion of the resulting ester **3** to the hydrazide **4** allowed subsequent cyclisation to the pyrazolo[1,5-*d*][1,2,4]triazine **6**, upon treatment of **4** with the pyrazoloaldehyde **5**[8] and cyclisation in Dowtherm A at 200 °C.[10] After unmasking the pyrazolone, alkylation with 5-chloromethyl-1-methyl-1*H*-1,2,4-triazole (**7**)[11] furnished compound **8**.

Introduction of a hydroxyl group onto the *tert*-butyl as in hypothetical metabolite **B** required an entirely differ-



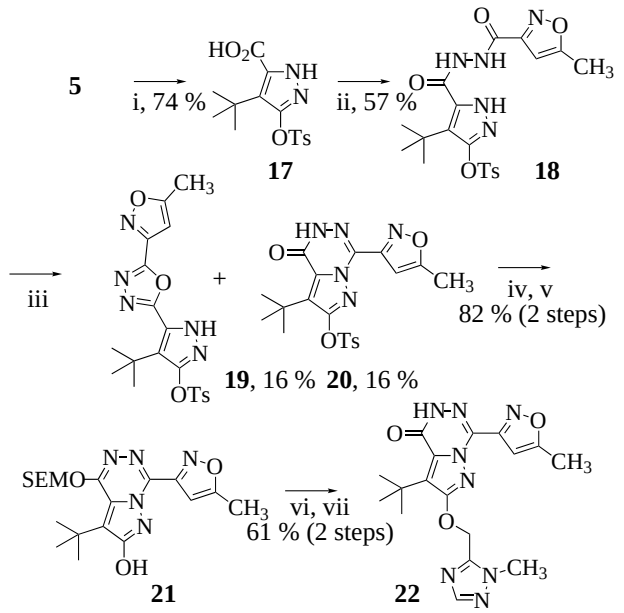
Scheme 1. Reagents and conditions: (i) Et_3N; (ii) $NH_2NH_2 \cdot H_2O$; (iii) **5**, xylene, reflux, then Dowtherm A, 200 °C; (iv) NaOH; (v) **7**, K_2CO_3.



Scheme 2. Reagents and conditions: (i) $Ph_3P{=}CHCO_2CH_3$; (ii) $NH_2NH_2 \cdot H_2O$, AcOH; (iii) TsCl, Et_3N; (iv) SEMCl, NaH; (v) NaOH; (vi) **7**, K_2CO_3, 50 °C; (vii) DIBAL, −78 °C; (viii) 40% TFA/DCE; (ix) **15**, xylene, 145 °C.

ent synthetic approach as shown in Scheme 2 and dihydro-4,4-dimethyl-2,3-furandione (**9**) was chosen as a suitable hydroxyl-*tert*-butyl synthon. Wittig olefination of **9** gave diester **10**, which upon treatment with $NH_2NH_2 \cdot H_2O$ in AcOH resulted in 1,4-addition to the least sterically encumbered end of the alkene, concurrent ring opening of the lactone, followed by cyclisation of the hydroxyl onto the ethyl ester to furnish the pyrazololactone **11** as described by Hackler.[12] Further protection group manipulation,[13] alkylation with **7** and controlled DIBAL reduction of the lactone gave the critical cyclisation precursor, lactol **14**. Addition of the isoxazole hydrazide **15**[14] triggered a cascade sequence: opening of the lactol; condensation with the hydrazide; and thermal cyclisation to yield compound **16**.

Formation of the putative aldehyde oxidase metabolite **C** necessitated an entirely different cyclisation protocol, Scheme 3. Oxidation of the pyrazoloaldehyde **5** to the



Scheme 3. Reagents and condition: (i) $AgNO_3$, KOH; (ii) **15**, BOPCl, Et_3N; (iii) mesitylene, 165 °C; (iv) SEMCl, NaH; (v) NaOH; (vi) **7**, K_2CO_3; (vii) TFA, then K_2CO_3.

corresponding carboxylic acid **17** was achieved with $AgNO_3$/KOH, and coupling to the isoxazole hydrazide **15** gave the required cyclisation precursor **18**. However, all attempts to prepare the pyrazolotriazinone skeleton **20** were accompanied with formation of the 1,3,4-oxadiazole **19** as the major product (4:1 upon acid catalysed cyclisation). Even the optimised procedure in refluxing mesitylene yielded only 16% of the required pyrazolo[1,5-*d*][1,2,4]triazin-4(5*H*)-one **20**, together with 16% of **19**.[15] Subsequent protecting group manipulation and alkylation with **7** gave compound **22**, which was identical to the material isolated from biological sources.[16]

Utilisation of combinations of the above synthetic approaches enabled the preparation of the three possible M + 32 metabolites, Figure 3. Compound **23** was prepared from **4** + **17**; **24** from **14** + **4**; whereas **25** was prepared using a modification of Scheme 2 using **13**, hydrazine and 5-methylisoxazole-3-carbonyl chloride.

With all the potential metabolites of **1** on hand, their biological profiles were determined (Table 1): in vitro

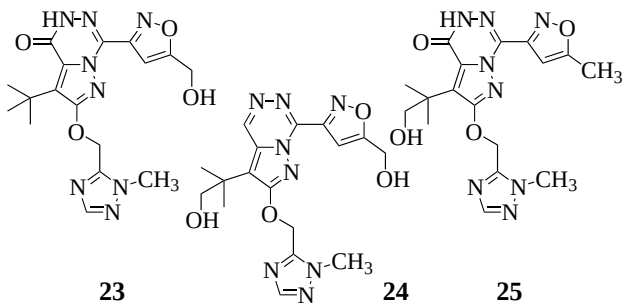


Figure 3. Other potential metabolites.

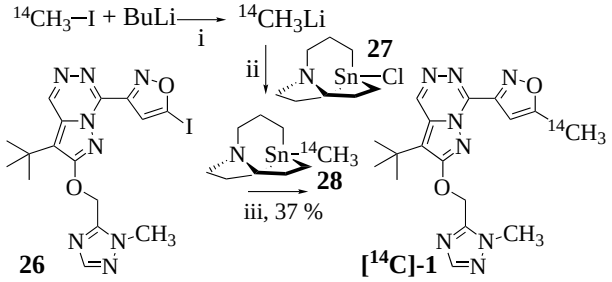
Table 1. Affinity and efficacy of metabolites

Compound	K_i (α5) nM	Efficacy in L(tk$^-$) cells (%)				Occ. (3 mpk ip)
		α1	α2	α3	α5	
1	1.4	−16	+6	−9	−55	> 95%
8	1.1	−11	−1	−15	−52	12%
16	9.4	−41	−23	−32	−40	21%
22	700	1	0	−8	−47	ND
23	370	ND	ND	ND	ND	ND
24	21	−45	−31	−39	−44	8%
25	>666	ND	ND	ND	ND	ND

binding affinity;[17] efficacy values were determined using whole cell patch-clamp recordings from mouse fibroblast L(tk$^-$) cells stably expressing the human $GABA_A$ receptor subtypes relative to the GABA-evoked current using a submaximal (EC_{20}) GABA concentration;[18] and receptor occupancy was measured for those compounds showing high affinity as determined by inhibition of in vivo binding of [^{3}H]Ro 15-1788, a non-selective BZ receptor ligand.[8] All six potential metabolites showed either weak affinity or poor receptor occupancy at 3 mpk ip, at which dose **1** gave complete occupancy, it was concluded that the activity of **1** was not due to circulating metabolites.

In order to assist ADME studies and to ensure that no metabolites had been missed radiolabelled **1** was prepared, introducing a ^{14}C-isotope at the C-5 position of the isoxazole, through a palladium-catalysed cross-coupling of the iodoisoxazole **26** with an appropriately radiolabelled methyl organometallic, Scheme 4.[19] Lithiation of [^{14}C]methyl iodide with *n*-BuLi gave $^{14}CH_3Li$, which was then reacted with chlorostannatrane **27** to give the required radiolabelled methyl organometallic **28**.[20] Coupling to the iodoisoxazole using catalytic $Pd(PPh_3)_4$ in DMF at 100 °C occurred successfully to give [^{14}**C**]-**1**.[21]

Excretion studies with [^{14}**C**]-**1** in rat showed that the dose was distributed equally between urine and faeces, 40 ± 8% and 45 ± 12%, respectively, the majority being cleared in 24 h. Analysis of urine following dosing with [^{14}**C**]-**1** showed one major component, the hydroxyl-*tert*-butyl metabolite **16**, accounting for 40% of the urine extract. Other minor metabolites included **8**, hydroxylation of the 5-methylisoxazole, and **24** a M + 32 metabolite (see Fig. 4).



Scheme 4. Reagents and conditions: (i) hexanes, −78 °C; (ii) **27**, THF, −20 °C; (iii) **28**, cat. $Pd(PPh_3)_4$, DMF, 100 °C.

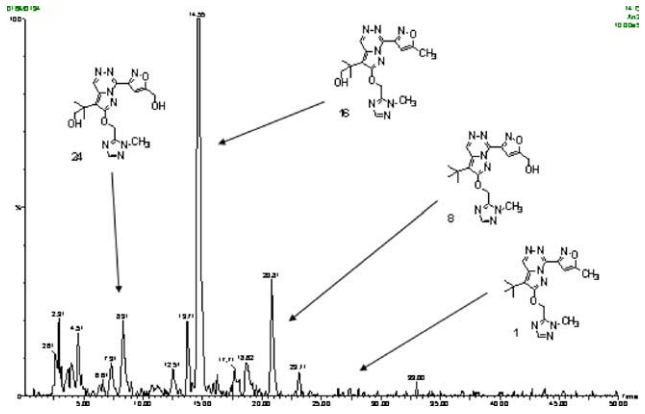


Figure 4. Radiochemical analysis of rat urine upon dosing of [^{14}C]-**1**.

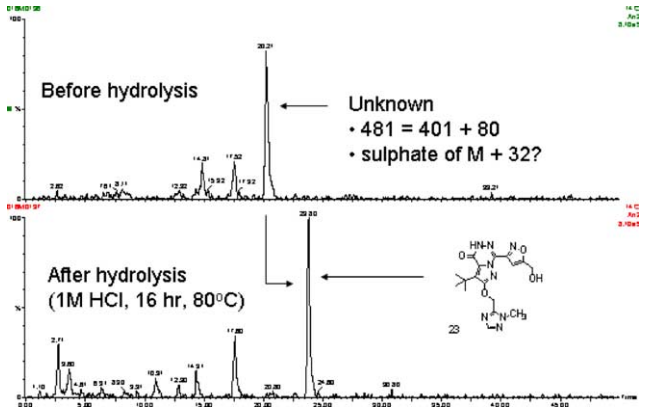


Figure 5. Radiochemical analysis of rat bile upon dosing of [^{14}C]-**1**.

Meanwhile, analysis of bile revealed that the major peak (40%) did not correspond to any of the synthetic metabolites. Mass spectroscopy showed that the component had a mass of 481, corresponding to a sulfate conjugate of a M + 32 metabolite. Hydrolysis of this conjugate liberated **23**, the product of oxidation of the methylisoxazole and also by aldehyde oxidase (see Fig. 5).

In summary, through synthesis of potential metabolites of **1** and radiolabel [^{14}C]-**1** the fate of the compound in vivo has been fully characterised and profiling of the authentic samples has revealed that they probably have little relevance to the pharmacological activity seen with **1**. There is no single dominant metabolic pathway, the two major routes being renal excretion of **16** and biliary excretion of a sulfate conjugate of **23**, each accounting for 15% of the dose. Direct excretion of **1** accounts for <2% dose. Although the metabolite **22** arising from oxidation of the pyrazolo[1,5-*d*][1,2,4]triazine core by aldehyde oxidase is not a significant excreted metabolite, oxidation by this enzyme contributes to the formation of **23**.

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