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The discovery of fused pyrrole carboxylic acids as novel, potent D-amino acid oxidase (DAO) inhibitors

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Abstract—The 'NMDA hypofunction hypothesis of schizophrenia' can be tested in a number of ways. DAO is the enzyme primarily responsible for the metabolism of D-serine, a co-agonist for the NMDA receptor. We identified novel DAO inhibitors, in particular, acid **1**, which demonstrated moderate potency for DAO in vitro and ex vivo, and raised plasma D-serine levels after dosing ip to rats. In parallel, analogues were prepared to survey the SARs of **1**.

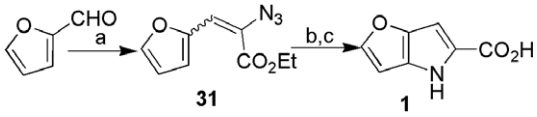
Schizophrenia is a condition that affects up to 1% of the population. Current therapies are inadequate and one new approach is to address the 'NMDA hypofunction' hypothesis[1] by developing drugs to enhance NMDA receptor function, for example, elevation of brain glycine through inhibition of the glycine transporter (GLYT1).[2]

An alternative approach is to increase the brain concentration of D-serine, a co-agonist of the NMDA receptor, and would therefore be expected to increase the activation of the NMDA receptor.

Clinical evidence to support this hypothesis is mixed, but some benefit was seen in patients when D-serine was used as an add-on to atypical anti-psychotics.[3] One approach to increase brain D-serine levels is to inhibit D-amino acid oxidase (DAO), the enzyme primarily responsible for metabolising D-serine.[4]

A number of reports profile compounds including indoles,[5a] benzisoaxoles[5b,c] and pyrrole/pyrazole derivatives[5c,d] with potent in vitro activity for DAO inhibition and benefits in animal models of cognitive and positive symptoms of schizophrenia. There are striking similarities between these classes of compounds; they are all low molecular weight, carboxylic acids.

Described herein is our approach to the identification of potent DAO inhibitors and follow-up studies utilising X-ray crystallography to design analogues suitable for in vivo profiling.



Scheme 1. Reagents and condition: (a) $EtO_2CCH_2N_3$, NaOEt, EtOH (67%); (b) Toluene, reflux (77%); (c) NaOH (97%).

Keywords: Schizophrenia; D-Amino acid oxidase (DAO) inhibitor.
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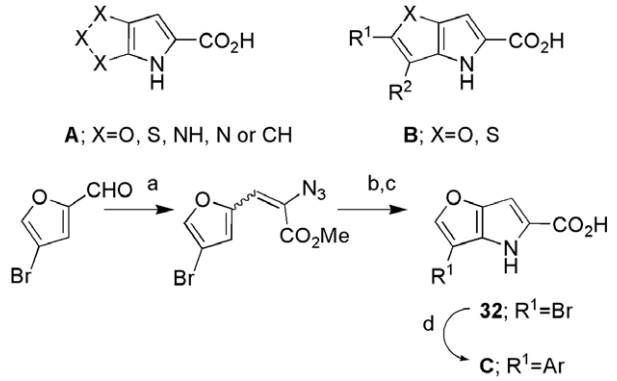
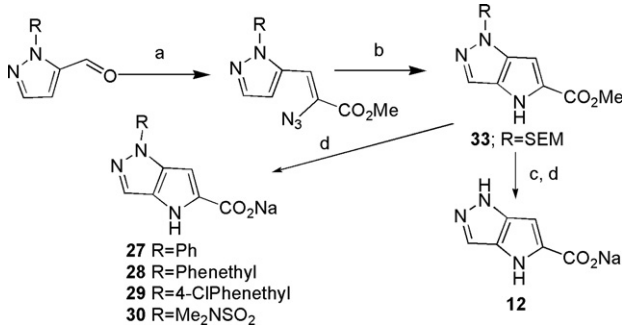


Scheme 2. Reagents and conditions: (a) $MeO_2CCH_2N_3$, NaOMe/MeOH (70%); (b) Xylene, reflux (65%); (c) NaOH (95%); (d) $Pd(PPh_3)_4$, Na_2CO_3, MeCN, H_2O, reflux (43–78%).



Scheme 3. Reagents and conditions: (a) $MeO_2CCH_2N_3$, NaOMe/MeOH (60%); (b) Xylene (51%); (c) 2 N HCl in EtOH, reflux; (d) NaOH (50–74%).

A HTS campaign using a subset of the Merck compound collection identified a small number of DAO inhibitors. Key hits were resynthesised for repeat testing, IC_{50} measurement and counter-screening assays. For example, compound **1** was synthesised by a method described previously,[6] whereby condensation of furfuraldehyde with ethyl azidoacetate followed by thermal cyclisation of **31** furnished **1** in multi-gram quantities (Scheme 1).

This methodology was adapted by using the appropriate readily available heteroaromatic aldehydes to generate a range of pyrrole carboxylic acid analogues to investigate core changes represented by **A** (compounds **7**–**12**) and substituted derivatives **B** (compounds **13**–**18**). To explore the SARs of other analogues represented by **C** (compounds **19**–**25**), aryl groups were introduced via Suzuki coupling of bromide **32** with the appropriate boronic acid (Scheme 2).

For the preparation of fused heterocyclic pyrrole **12**, using SEM-protected pyrazole-2-carboxaldehyde in the condensation and thermal cyclisation sequence afforded **33** which, after deprotection with HCl then saponification, furnished **12**. The utility of this method was enhanced through the availability of many N-substituted pyrazole aldehydes which allowed the standard route to be used to generate N-substituted acids **27**–**30** (Scheme 3).

Hits from the initial DAO inhibition assay[7] were followed up by performing further screening within the Merck compound collection. An additional set of hits was identified including a number of compounds previously reported as DAO inhibitors (e.g., compounds **3**,[5a] **4**[5b] and **5**[5c]). A counter-screen assay confirmed selectivity over D-aspartate oxidase (DDO) the nearest homologue of DAO. A selection of validated hits is shown in Table 1.

Table 1. In vitro potencies of acids **1**–**5**[7]

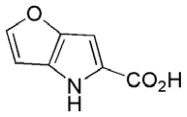
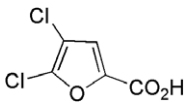
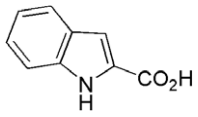
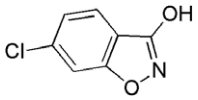
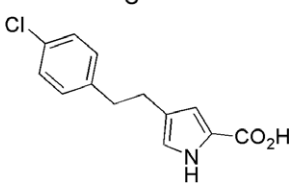
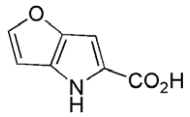
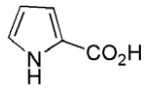
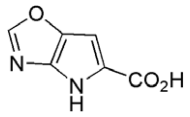
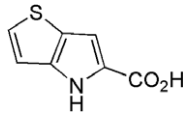
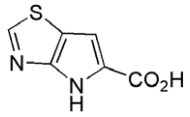
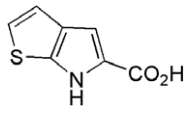
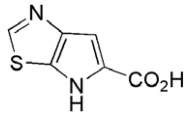
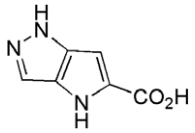
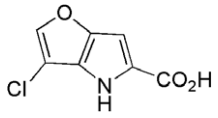
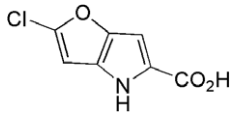
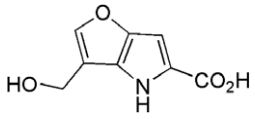
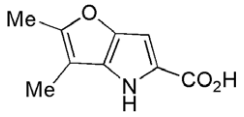
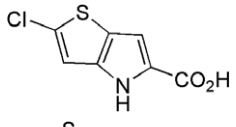
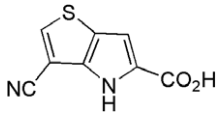
	Compound	h-DAO IC_{50} (nM)	h-DDO IC_{50} (nM)
	**1**	141 ± 73	>5000
	**2**	343 ± 84	>5000
	**3**	745 ± 377	>5000
	**4**	507 ± 236	>5000
	**5**	238 ± 99	>5000

Table 2. In vitro potencies of compounds **1** and **6**–**18**[7]

	Compound	h-DAO IC_{50} (nM)
	**1**	141 ± 73
	**6**	>5000
	**7**	>5000
	**8**	245 ± 170
	**9**	516 ± 174
	**10**	269 ± 77
	**11**	4204 ± 436
	**12**	>5000
	**13**	1426 ± 161
	**14**	>5000
	**15**	>5000
	**16**	>5000
	**17**	>5000
	**18**	1153 ± 236

From this subset of validated hits, compound **1**[8] was chosen to form the basis of a limited lead optimisation campaign. Initially, heterocyclic variations and substituted analogues were synthesised to explore the SARs of hit **1** with the aim of improving the potency for inhibiting DAO (Table 2).

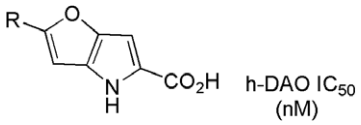
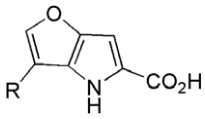
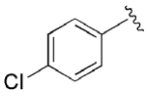
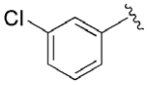
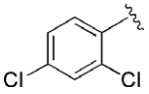
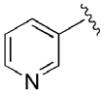
The SAR of analogues **6**–**18** is intriguing. The removal of the furan ring in **1** to pyrrole-2-carboxylic acid **6** resulted in complete loss of potency. Replacing the furan ring with other heterocycles such as thiophene analogues **8** and **10**, and thiazole **9** resulted in compounds with similar potencies. However, other heterocyclic replacements such as oxazole **7**, thiazole **11** and pyrazole **12** showed significant or complete losses in potency. Locating substituents on the furan or thiophene ring resulted in two compounds (**13** and **18**) with reduced potency, both with substituents on the 3-position of the fused heterocyclic core, but otherwise substituents on the 2-, 3- or both positions lost potency (compounds **14**–**17**). These data suggest limited scope for the replacement of the furan portion of the core with other heterocycles or substituted heterocycles.

The crystal structure of human DAO bound to compound **1** was determined by replacing the bound benzoate from the crystals of the ternary complex with FAD.[9] Compound **1** occupies the pocket adjacent to bound FAD, where benzoate is known to bind.[10] The carboxylate group of **1** hydrogen bonds with guanidyl group of Arg283 and the hydroxyl of Tyr228 (see Fig. 2 in Ref. 10). In addition, the N8 atom of **1** interacts with the main-chain NH of Gly313. The volume of the binding pocket occupied by **1** is not significantly different from that occupied by benzoate, despite some conformational differences in side chains observed around the binding pocket.

The information from X-ray studies and the low average molecular weight of hits identified from the HTS campaign hinted at a small binding site consistent with an enzyme capable of metabolising small amino acids such as D-serine. Despite the losses in potency found when small substituents were located off the core of acids **1** and **8**, an attempt was made to explore space further from the heterocyclic cores. The X-ray structure of **1**:DAO suggested space behind Tyr228 where hydrophobic groups could be accommodated if appended a suitable distance away from the core. Firstly, simple derivatives of **1** were synthesised where a small range of aromatic rings were appended from the two available positions on the furan ring. All compounds synthesised lost potency (Table 3).

When consideration was given to the potency of **5**, where the position of the aryl group and linker conferred increased potency compared with the parent (**5** vs **6**), it seemed plausible that potency could be gained by accessing the same space in the simple acids presented here. Thus, a simple selection of compounds was prepared with groups appended from the nitrogen atom adjacent to the pyrrole ring of compound **12** (compounds **27**–**30**). These compounds resulted in no gain

Table 3. In vitro potencies of acids **19**–**25**[7]

 h-DAO IC_{50} (nM)		R	 h-DAO IC_{50} (nM)	
1	141 ± 73	H	**1**	141 ± 73
19	>5000		—	—
20	>5000		**23**	>5000
21	>5000		**24**	>5000
22	>5000		—	—
—	—		**25**	>5000

in potency relative to the NH parent (IC_{50}s > 5 μM). These data along with the results for compounds **19**–**25** (Table 3) suggested that groups appended from the core of acids such as **1** and **12** were ineffective at improving or even maintaining potency. This could be due to the incorrect positioning of the appendages in **19**–**30** relative to the side chain of **5**.

Compounds **1** and **8** were further profiled to assess suitability for in vivo studies to establish if a correlation existed between the compound exposure in plasma, DAO enzyme inhibition and changes in D-serine levels. Firstly, no off-target activities were found for **1** and **8** for the inhibition of P450 enzymes (IC_{50}s > 10 μM for CYP3A4, CYP2D6 and CYP3C9) or binding to ion channels (IK_R or sodium channels; data not shown). In addition, compound **1** was found not to be an antagonist at the glycine site of NMDA receptors (data not shown).

To correlate D-serine levels with ex vivo tissue DAO enzyme inhibition, compound **1** was dosed at 25, 75 and 150 mg/kg ip to rats, and D-serine levels were measured out to 24 h in the plasma whilst enzyme activity was determined in kidney and cerebellum at 1, 2 and 4 h.[11] A dose dependent increase in the D-serine levels versus the vehicle was not observed in plasma; however, at 150 mg/kg of **1**, a significant increase in D-serine levels was observed versus the vehicle at 4, 8, 16 and 24 h (Fig. 1a). Ex vivo measurement of DAO enzyme inhibition revealed significant inhibition versus the vehicle at all doses and time points of **1** in kidney (Fig. 1b). In the cerebellum, significant inhibition was observed except for the 25 mg/kg ip dose at 1 h (Fig. 1c). After 24 h, enzyme activity had returned to baseline levels in both cerebellum and kidney (data not shown). Plasma levels of **1** after ip dosing at 25, 75, or 150 mg/kg revealed dose proportionality (Fig. 1d).

Compound **1** was identified from an HTS campaign and found to significantly inhibit DAO activity in vitro. Proof of principle in animals was demonstrated because **1** significantly inhibited ex vivo DAO enzyme activity in kidney and cerebellum. Consequently, D-serine levels in the plasma were increased. The work done more than 30 years ago showed that systemic administration of D- but not L-serine to rats induced nephrotoxicity, which manifested as acute necrosis of the proximal tubules and consequent aminoaciduria, proteinurea and glucosuria.[12] These effects were most noticeable 2 h after administration and were not apparent by 4–5 days when the tubules were re-lined by new epithelium indicating that the toxicity was reversible. Interestingly, others showed that D-serine nephrotoxicity was attenuated by sodium benzoate, an inhibitor of DAO.[13] This study indicated that the mechanism of D-serine nephrotoxicity may be related to the by-products of D-amino acid metabolism (e.g., H_2O_2). These effects will require further monitoring with the development of such inhibitors. This compound therefore represents a useful tool to further explore the role of D-serine in modulating central NMDA receptor function. Limited lead optimisation efforts identified equipotent analogues to **1** including compound **8**. The results of the in vivo pharmacological characterisation of **8**, including the effects on central D-serine levels, will be reported shortly (Fig. 2).[14]

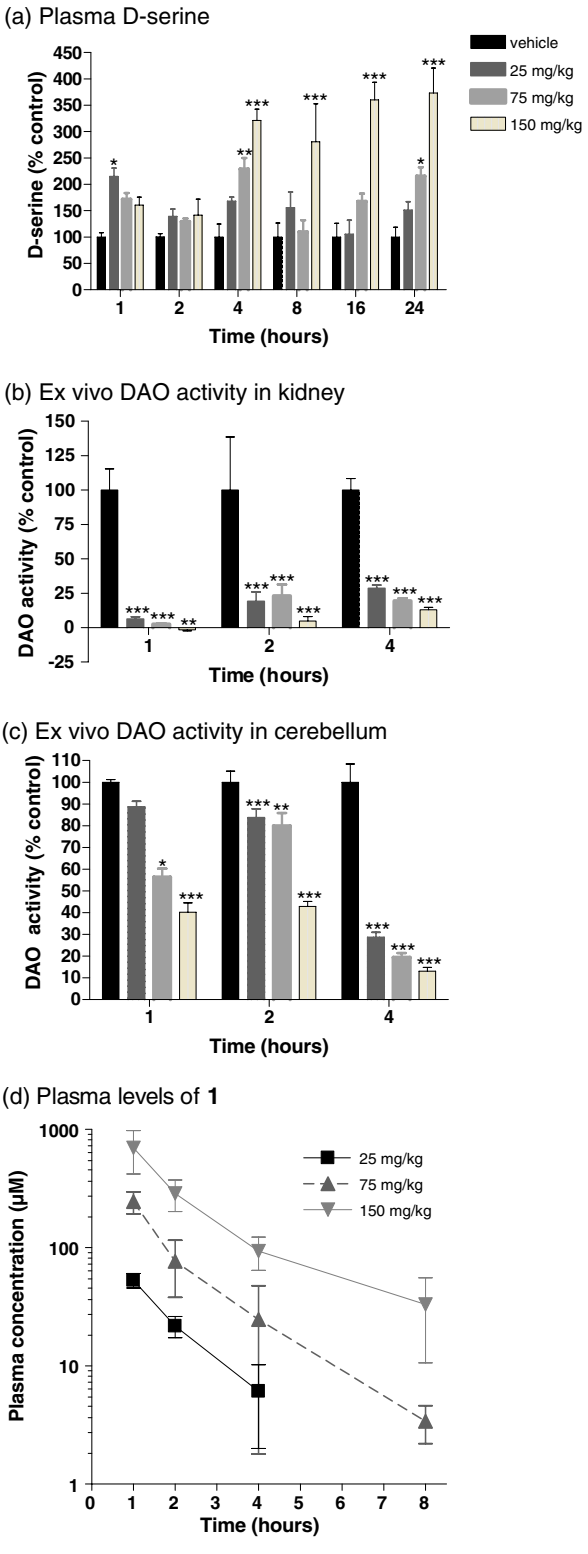


Figure 1. Plasma D-serine, DAO enzyme activity and plasma concentration over time following administration of 25, 75 or 150 mg/kg of **1** ip to rats. (a) D-serine levels in plasma; (b) Ex vivo DAO enzyme activity in kidney; (c) Ex vivo DAO enzyme activity in cerebellum (the effects of **1** on plasma D-serine and DAO enzyme activity were analysed using a two-way ANOVA to analyse time and treatment effect followed by a Bonferroni post test for individual group comparisons. $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$ dose vs vehicle). (d) Plasma levels of **1** out to 8 h.

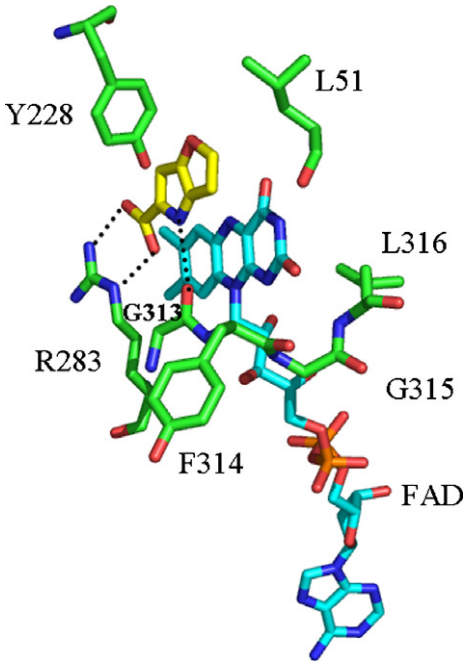


Figure 2. Interactions of compound **1** with hDAO. Part of the bound FAD is shown in cyan. The dotted lines represent hydrogen bonding interactions. The coordinates of the structure of hDAO bound to compound **1** have been deposited in Protein Data Bank (accession number 3CUK).

Acknowledgments

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7. Assayed in CHO cells transfected with DAO as described in Brandish, P. E.; Chiu, C. S.; Schneeweis, J.; Brandon, N. J.; Leech, C. L.; Kornienko, O.; Scolnick, E. M.; Strulovici, B.; Zheng, W. *J. Biomol. Screen.* **2006**, *115*, 481, All compounds were screened a minimum of 4 times.

8. For compound **1**: (400 MHz ^{1}H NMR DMSO-d_6) 6.58 (1H, s), 6.69 (1H, s), 7.76 (1H, s), 11.51 (1H, s), 12.36 (1H, s); (^{13}C NMR, DMSO-d_6) 94.3, 98.1, 123.1, 127.4, 145.6, 147.5, 161.3; *m/z* (AP^-) 150 $[M-H]^+$.
9. Prasad, S.; Munshi, S. Human DAO was crystallized as a ternary complex with bound FAD and benzoate. DAO (aa 1–347) at 2 mg/ml in buffer containing 50 mM sodium phosphate, pH 6.6, 200 mM FAD and 200 mM benzoate, was mixed with equal volume of crystallisation buffer containing 100 mM Tris-HCl pH 7.7, 150 mM Tri-Potassium Citrate and 15% (w/v) PEG 3350. The crystals obtained at room temperature diffracted X-rays to 2.5 Å resolution and belonged to space group C2 with cell dimensions a = 187.80 Å, b = 51.2 Å, c = 153.3 Å, $\alpha = 90.0°$, $\beta = 110.4°$, $\gamma = 90.0°$. The asymmetric unit contains four molecules associated as 2 dimers. Crystals were soaked in solution containing compound **1** for 48 hours prior to data collection.
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