Synthesis and Biological Evaluation of D-Amino Acid Oxidase Inhibitors

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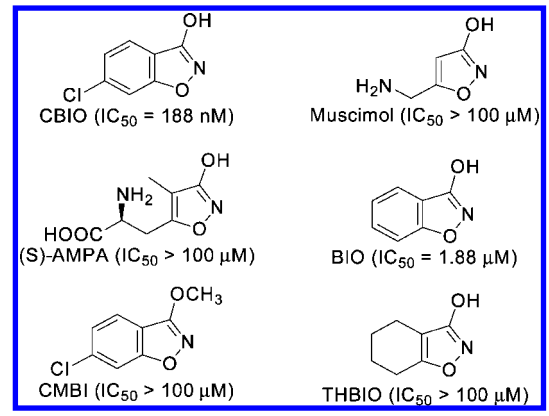
Abstract: D-Amino acid oxidase (DAAO) catalyzes the oxidation of D-amino acids including D-serine, a full agonist at the glycine site of the NMDA receptor. A series of benzo[*d*]isoxazol-3-ol derivatives were synthesized and evaluated as DAAO inhibitors. Among them, 5-chlorobenzo[*d*]isoxazol-3-ol (CBIO) potently inhibited DAAO with an IC_{50} in the submicromolar range. Oral administration of CBIO in conjunction with D-serine enhanced the plasma and brain levels of D-serine in rats compared to the oral administration of D-serine alone.

The preclinical and clinical evidence supporting the role of NMDA[a] receptor hypofunction in schizophrenia has prompted clinical trials of agents that enhance NMDA receptor function.[1] For example, schizophrenic patients receiving D-serine, a full agonist at the glycine site of the NMDA receptor, with concomitant neuroleptic therapy have shown significant improvements in their positive, negative, and cognitive symptoms.[2] Furthermore, reduced D-serine levels were reported in the serum and cerebrospinal fluid of schizophrenic patients.[3,4]

In animals, however, D-serine is believed to be metabolized substantially by D-amino acid oxidase (DAAO), diminishing its oral bioavailability.[5] In addition, at high doses, D-serine is reported to cause selective necrosis to the pars recta region of the renal proximal tubules in the rat.[6] The mechanism of D-serine-induced nephrotoxicity is believed to be associated with oxidative stress caused by hydrogen peroxide, a byproduct of DAAO-mediated metabolism of D-serine.[7]

These findings prompted us to identify small molecule DAAO inhibitors that can be coadministered with D-serine to minimize its metabolism by DAAO. DAAO inhibition should not only improve bioavailability of D-serine but also reduce its nephrotoxic effects. This paper describes the synthesis of a series of small molecule DAAO inhibitors based on a benzo[*d*]isoxazol-3-ol core structure and the in vivo effects of DAAO inhibition on D-serine pharmacokinetics.

Using a fluorescence-based DAAO assay,[8] we screened a large number of compounds for their ability to inhibit DAAO and found that 6-chlorobenzo[*d*]isoxazol-3-ol (CBIO) potently inhibits DAAO with an IC_{50} of 188 nM.[9] Subsequent kinetic studies showed that CBIO is a competitive inhibitor with respect to D-serine with a K_i of 100 nM.



We tested other structurally related compounds, muscimol, (*S*)-AMPA, benzo[*d*]isoxazol-3-ol (BIO), 6-chloro-3-methoxybenzo[*d*]isoxazole (CMBI), and 4,5,6,7-tetrahydrobenzo[*d*]isoxazol-3-ol (THBIO). All of these compounds except BIO exhibited negligible DAAO inhibition.

These findings led us to speculate that CBIO inhibits DAAO in a mode similar to that of benzoic acid, a prototype competitive DAAO inhibitor ($K_i \approx 16\ \mu M$).[10] Figure 1A illustrates a crystal structure of DAAO complexed with benzoic acid.[11] As illustrated in Figure 1B, we propose that the isoxazole group of CBIO plays a role similar to the carboxylate group of benzoic acid, forming a critical hydrogen bond network with Arg283 and Tyr228. This explains the loss of DAAO inhibitory potency observed in CMBI. The benzene ring of CBIO is believed to lay parallel to the flavin ring with Tyr224 on the opposite side of the benzene ring, leading to strong $\pi-\pi$ stacking interactions. This may explain the negligible DAAO inhibition achieved by a saturated analogue, THBIO, due to its inability to form these interactions. These preliminary biological results prompted the synthesis of a variety of benzo[*d*]isoxazol-3-ols to evaluate structure−activity relationships within this class of compounds.

Our initial attempt to synthesize benzo[*d*]isoxazol-3-ol derivatives **3** involved the formation of *N*-hydroxysalicylamides **2** from salicylic acid methyl esters **1** (Scheme 1). Cylization of **2** by the treatment with thionyl chloride (step b)[12] or carbonyl diimidazole (step c),[13] however, afforded undesired product **4** or **5**, respectively, as major products, providing **3** in low yields.

To circumvent the formation of these byproducts, we developed an alternative method that utilizes 2-fluorobenzoyl chlorides **6** and *N*-(2,4,6-trimethoxybenzyl)hyroxylamine **7** (Scheme 2). Coupling of **6** and **7** afforded *N*-hydroxybenzamide derivatives **8**. Subsequent treatment with potassium carbonate followed by deprotection yielded benzo[*d*]isoxazol-3-ol derivatives **3**.

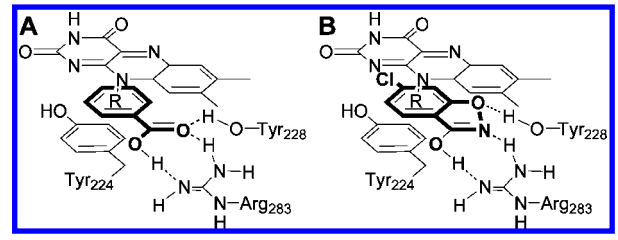


Figure 1. (A) Schematic illustration of the active site of DAAO in complex with benzoic acid. (B) Proposed model of CBIO bound to DAAO.

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[a] Abbreviations: NMDA, *N*-methyl-D-aspartate; DAAO, D-amino acid oxidase; CBIO, 6-chlorobenzo[d]isoxazol-3-ol; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BIO, benzo[*d*]isoxazol-3-ol; CMBI, 6-chloro-3-methoxybenzo[*d*]isoxazole; THBIO, 4,5,6,7-tetrahydrobenzo[*d*-]isoxazol-3-ol.

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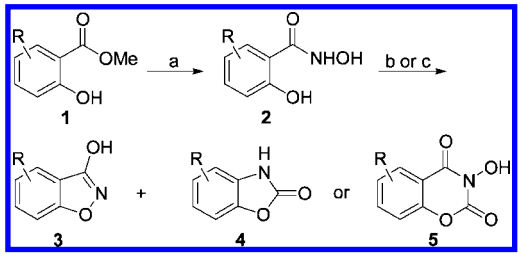
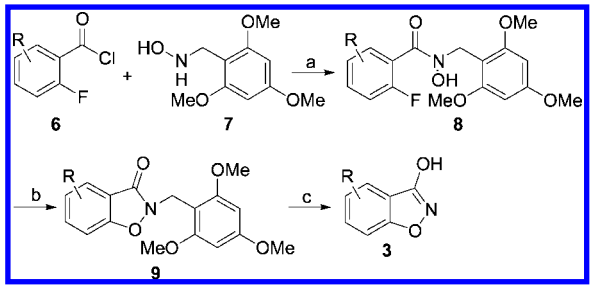
Scheme 1. Synthesis of Benzo[*d*]isoxazol-3-ols[a]



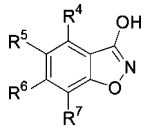
[a] Reagents and conditions: (a) hydroxylamine hydrochloride, KOH, methanol, room temp, 20−95%; (b) pyridine, thionyl chloride, THF, room temp, 27−37%; (c) carbonyl diimidazole, reflux, 1−33%.

Scheme 2. Synthesis of Benzo[*d*]isoxazol-3-ols[a]



[a] Reagents and conditions: (a) triethylamine, dichloromethane, room temp, 45−60%; (b) potassium carbonate, DMF, 120 °C, 47−99%; (c) triisopropylsilane, TFA, dichloromethane, room temp, 12−61%.

Table 1. Inhibition of DAAO by Benzo[d]isoxazol-3-ol Derivatives



compd	R^4	R^5	R^6	R^7	IC_{50} (μM)[a]
BIO	H	H	H	H	1.88 ± 0.00
3a	CF_3	H	H	H	>100
3b	F	H	H	H	7.82 ± 0.96
3c	H	Br	H	H	0.599 ± 0.006
3d	H	I	H	H	1.51 ± 0.12
3e	H	NO_2	H	H	2.95 ± 0.15
3f	H	H	F	H	0.444 ± 0.032
CBIO	H	H	Cl	H	0.188 ± 0.001[b]
3g	H	H	CH_3	H	0.269 ± 0.013
3h	H	H	OMe	H	2.57 ± 0.27
3i	H	H	OEt	H	>100
3j	H	H	NO_2	H	0.722 ± 0.024
3k	H	H	CF_3	H	5.00 ± 0.24
3l	H	H	CF_3	F	>100
3m	H	H	H	F	23.0 ± 0.3
3n	H	H	H	CH_3	>100

[a] Values are the mean ± SE of duplicate experiments. [b] $n = 4$.

A large number of benzo[*d*]isoxazol-3-ols were synthesized using one of the three methods described above and tested for their ability to inhibit DAAO. Table 1 summarizes in vitro DAAO inhibitory data of these compounds.

In general, larger substituents on the benzene ring caused a significant increase in IC_{50} values. Smaller groups are better tolerated especially at positions 5 and 6. Compounds with similar potency to CBIO have small substituents at one of these positions as represented by **3c**, **3f**, **3g**, and **3j**.

These observations are in a good agreement with DAAO's preferred substrate specificity for D-amino acids bearing hydrophobic side chains up to four carbon atoms long or compact ring systems.[14] Our SAR analysis coupled with previous findings suggests that the active site pocket of DAAO is of limited space and only accommodates substrates or inhibitors of moderate size. This may pose a challenge to further optimization of benzo[*d*]isoxazol-3-ol-based DAAO inhibitors because minor structural modifications are unlikely to generate an additional hydrophobic interaction at the active site that could contribute to a higher affinity for DAAO.

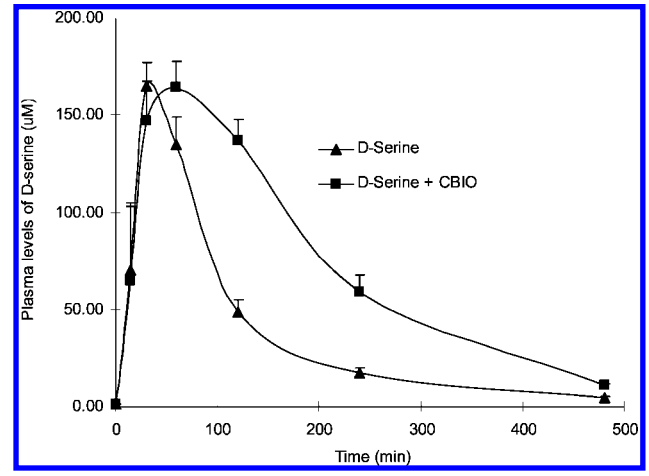


Figure 2. Effects of CBIO on rat plasma levels of D-serine following oral administration of D-serine. Male SD rats were administered D-serine (30 mg/kg, po) or D-serine (30 mg/kg, po) and CBIO (30 mg/kg, po). Each point shown is the mean ± SD ($n = 4$).

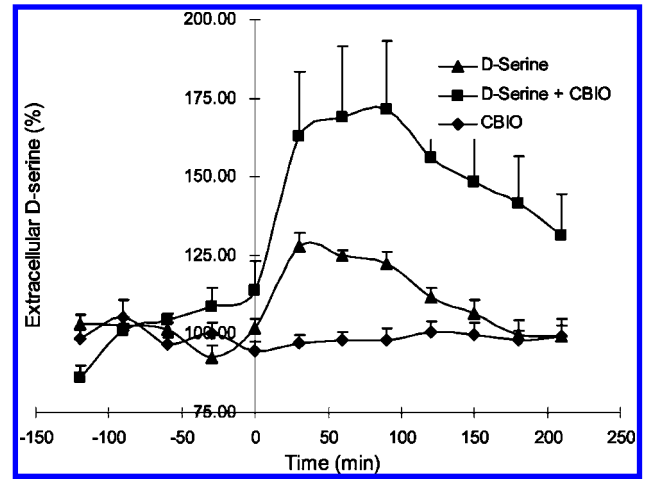


Figure 3. Effects of CBIO on rat brain levels of D-serine following oral administration of D-serine. Male SD rats were administered D-serine (30 mg/kg, po), CBIO (30 mg/kg, po), or D-serine (30 mg/kg, po) and CBIO (30 mg/kg, po) at $t = 0$ min. D-Serine levels are expressed as percent of the basal level. Each point shown is the mean ± SD ($n = 4$).

To assess the effects of DAAO inhibition on plasma levels of D-serine in rats, D-serine was given orally with or without CBIO, and plasma samples were analyzed for D-serine levels using an enantiospecific bioanalytical method.[15]

As shown in Figure 2, coadministration of CBIO (30 mg/kg) markedly enhanced the plasma levels of D-serine in rats compared to D-serine alone treatment. The results demonstrate that an orally administered DAAO inhibitor can enhance the oral bioavailability of coadministered D-serine.

To assess whether the increased plasma concentrations of D-serine would reflect the extracellular D-serine levels, we treated rats with oral D-serine +/− CBIO and analyzed microdialysis samples from the prefrontal cortex for D-serine concentrations.[15] As shown in Figure 3, oral D-serine increased the extracellular D-serine levels by 25% compared to the basal levels. At some

points, the extracellular levels of D-serine following coadministration of CBIO (30 mg/kg) increased by more than 60% compared to the basal levels.

Figure 3 also shows that oral administration of CBIO alone did not affect the extracellular levels of D-serine. Therefore, the enhancement of D-serine levels in the prefrontal cortex following coadministration of D-serine and CBIO is likely due to increased systemic D-serine levels because D-serine is capable of penetrating the blood−brain barrier.[17] One could attribute CBIO's inability to autonomously enhance D-serine levels in the brain to its poor blood−brain barrier (BBB) permeability.

Interestingly, a recent report from Dr. Adage's group showed a slight increase in D-serine levels in rat cortex and midbrain following intravenous administration of a pyrazole-3-carboxylate based DAAO inhibitor alone.[16] This effect may be due to its superior ability to penetrate the blood−brain barrier. It has been reported, however, that there is little overlap in distribution of DAAO and NMDA receptors in the brain.[18] Therefore, even a brain-penetrable DAAO inhibitor may not be able to significantly enhance NMDA receptor-mediated neurotransmission by itself.

Our study indicates that coadministration of D-serine and a DAAO inhibitor represents a more effective approach for delivering D-serine to the site of its action. Inhibition of DAAO should also reduce oxidative stress and reduce nephrotoxicity.[7] Pharmacological evaluation of D-serine/CBIO coadministration is currently underway using animal models of schizophrenia.

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Supporting Information Available: Synthetic procedures for CMBI and **3a**−**n**, elemental analysis data, in vitro D-amino acid oxidase assay method, and in vivo experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Millan, M. J. *N*-Methyl-D-aspartate receptors as a target for improved antipsychotic agents: novel insights and clinical perspectives. *Psychopharmacology* **2005**, *179*, 30–53.

(2) Tsai, G.; Yang, P.; Chung, L. C.; Lange, N.; Coyle, J. T. D-Serine added to antipsychotics for the treatment of schizophrenia. *Biol. Psychiatry* **1998**, *44*, 1081–1089.

(3) Hashimoto, K.; Fukushima, T.; Shimizu, E.; Komatsu, N.; Watanabe, H.; Shinoda, N.; Nakazato, M.; Kumakiri, C.; Okada, S.; Hasegawa, H.; Imai, K.; Iyo, M. Decreased serum levels of D-serine in patients with schizophrenia: evidence in support of the *N*-methyl-D-aspartate receptor hypofunction hypothesis of schizophrenia. *Arch. Gen. Psychiatry* **2003**, *60*, 572–576.

(4) Hashimoto, K.; Engberg, G.; Shimizu, E.; Nordin, C.; Lindstrom, L. H.; Iyo, M. Reduced D-serine to total serine ratio in the cerebrospinal fluid of drug naive schizophrenic patients. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **2005**, *29*, 767–769.

(5) Curti, B.; Ronchi, S.; Simonetta, P. M. D- and L-Amino Acid Oxidases. In *Chemistry and Biochemistry of Flavoenzyme*; Müller, F., Ed.; CRC Press: Boca Raton, FL, 1992; Vol. 3, pp 69−94.

(6) Ganote, C. E.; Peterson, D. R.; Carone, F. A. The nature of D-serine-induced nephrotoxicity. *Am. J. Pathol.* **1974**, *77*, 269–282.

(7) Williams, R. E.; Lock, E. A. Sodium benzoate attenuates D-serine induced nephrotoxicity in the rat. *Toxicology* **2005**, *207*, 35–48.

(8) Cook, S. P.; Galve-Roperh, I.; Martinez del Pozo, A.; Rodriguez-Crespo, I. Direct calcium binding results in activation of brain serine racemase. *J. Biol. Chem.* **2002**, *277*, 27782–27792.

(9) Simultaneous to our work on this series, two patent applications were published disclosing the same series of DAAO inhibitors. (a) Kennis, L. E. J.; Vanhoof, G. C. P.; Bongartz, J.-P. A. M.; Luyckx, M. G. M.; Minke, W. E. Benzisoxazoles. PCT Int. Appl. WO 2005/089753, 2005. (b) Fang, Q. K.; Hopkins, S.; Jones, S. Benzo[*d*]isoxazol-3-ol DAAO Inhibitors. U.S. Pat. Appl. 20050143434, 2005.

(10) Vanoni, M. A.; Cosma, A.; Mazzeo, D.; Mattevi, A.; Todone, F.; Curti, B. Limited proteolysis and X-ray crystallography reveal the origin of substrate specificity and of the rate-limiting product release during oxidation of D-amino acids catalyzed by mammalian D-amino acid oxidase. *Biochemistry* **1997**, *36*, 5624–5632.

(11) Mattevi, A.; Vanoni, M. A.; Todone, F.; Rizzi, M.; Teplyakov, A.; Coda, A.; Bolognesi, M.; Curti, B. Crystal structure of D-amino acid oxidase: a case of active site mirror-image convergent evolution with flavocytochrome b2. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 7496–7501.

(12) Kalkote, U. R.; Goswami, D. D. New synthesis of 1,2-benzisoxazole derivatives. *Aust. J. Chem.* **1977**, *30*, 1847–1850.

(13) Geffken, D. The cyclisation of salicylamides and salicylohydroxamic acids with 1,1′-carbonyldiimidazole. *Liebigs Ann. Chem.* **1981**, 1513–1514.

(14) Dixon, M.; Kleppe, K. D-Amino acid oxidase II. Specificity, competitive inhibition and reaction sequence. *Biochim. Biophys. Acta* **1965**, *96*, 368–382.

(15) Fukushima, T.; Kawai, J.; Imai, K.; Toyo'oka, T. Simultaneous determination of D- and L-serine in rat brain microdialysis sample using a column-switching HPLC with fluorimetric detection. *Biomed. Chromatogr.* **2004**, *18*, 813–819.

(16) Adage, T.; Trillat, A. C.; Quattropani, A.; Perrin, D.; Cavarec, L.; Shaw, J.; Guerassimenko, O.; Giachetti, C.; Greco, B.; Chumakov, I.; Halazy, S.; Roach, A.; Zaratin, P. In vitro and in vivo pharmacological profile of AS057278, a selective D-amino acid oxidase inhibitor with potential anti-psychotic properties. *Eur. Neuropsychopharmacol.* **2008**, *18*, 200–214.

(17) Bauer, D.; Hamacher, K.; Broer, S.; Pauleit, D.; Palm, C.; Zilles, K.; Coenen, H. H.; Langen, K. J. Preferred stereoselective brain uptake of D-serine, a modulator of glutamatergic neurotransmission. *Nucl. Med. Biol.* **2005**, *32*, 793–797.

(18) Horiike, K.; Tojo, H.; Arai, R.; Nozaki, M.; Maeda, T. D-Amino-acid oxidase is confined to the lower brain stem and cerebellum in rat brain: regional differentiation of astrocytes. *Brain Res.* **1994**, *652*, 297–303.

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