



**BUDAPESTI MŰSZAKI ÉS GAZDASÁGTUDOMÁNYI EGYETEM
VEGYÉSZMÉRNÖKI ÉS BIOMÉRNÖKI KAR OLÁH GYÖRGY DOKTORI
ISKOLA**

Design and synthesis of D-amino acid oxidase inhibitors

Thesis booklet

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1. Introduction

Psychotic symptoms can be divided into 3 categories: positive, negative and cognitive¹. Positive symptoms include delusions, confused thoughts and hallucinations, while negative symptoms include a lack of normal human emotional reactions, and in addition to these symptoms, patients also struggle with learning and memory. Positive symptoms can be treated well with current medications, but negative and cognitive factors are less responsive to medication. Studies have shown that inhibition of D-amino acid oxidase enzyme and administration of D-serine may indirectly alleviate negative and cognitive symptoms. During my PhD work I aimed to identify, synthesize and test heterocyclic molecules with D-amino acid oxidase inhibitory properties.

2. Background

2.1. Biology and structure of D-amino acid oxidase

D-amino acid oxidase (DAAO) is a flavoprotein that catalyzes the oxidative deamination of neutral D-amino acids to the corresponding imino acids, which is hydrolyzed to α -keto acids and ammonia in the presence of water, while the reduced FAD cofactor is reoxidized by molecular oxygen while hydrogen peroxide is formed.

In mammals DAAO is expressed in three different organs: brain, kidney and liver. The main function of DAAO in brain is the regulation of the level of D-serine acting as a neurotransmitter by functioning as co-agonists of the *N*-methyl-D-aspartate (NMDA) receptor. DAAO is mainly localized in peroxisomes, however in neuronal cells, the enzyme does not show peroxisomal localization exclusively. It can be found both in neurons and glial cells.

Despite the fact that human DAAO (hDAAO) was already found in the human brain in 1966, its role in the central nervous system remained unexplored until 1992. Due to the development of analytical methods, the presence of free D-serine was confirmed in rat brain.

The first crystal structure was determined from porcine DAAO isolated from kidney in 1996, and later, the three-dimensional structure of the hDAAO was successfully determined. After the human enzyme was identified as a potential schizophrenia target, the structural investigation received increased attention and over a dozen experimental structures of the wild-type enzyme in complex with different ligands and in free form have been published and deposited in the RCSB Protein Data Bank.

The tertiary structure of hDAAO consists of two interconnected domains, a FAD-binding domain formed by residues 1-87, 140-195 and 286-347; and a substrate-binding domain formed by residues 88-139 and 196-285. A superposition of the available 3D structures of the hDAAO indicates that the conformational flexibility of the protein is generally low. However, there are three regions with increased mobility and they include residues of the active site loop covering the entrance of the active site.

¹ Sims A (2002). *Symptoms in the mind: an introduction to descriptive psychopathology*. Philadelphia: W. B. Saunders.

The active site of hDAAO is delineated by the positively charged Arg283, the hydrogen bond donor Tyr228 and hydrogen-bond acceptor Gly313 backbone carbonyl, the flexible Tyr224 and several hydrophobic residues including Leu51, Leu215 and Ile230 and the isoalloxazine ring of FAD.

2.2. Relevance of DAAO in schizophrenia

Amino acids are peptide and protein building blocks but they also play important role as biochemical regulators, such as autophagy regulators and neurotransmitters.^{2,3}

The development of the sensitivity of analytical methods has greatly contributed to the detection of D-amino acids such as D-serine, D-alanine and D-aspartate in mammals. At the same time, the enzymes that synthesize or metabolize D-amino acids have been localized that allowed identifying the functions of the amino acids in the endocrine and nervous systems.

D-serine can be found in the nervous, and at a lesser extent, in the endocrine system. D-serine rich regions in the central nervous system are the striatum, cerebral cortex and the hippocampus. D-serine is also detectable in the cerebellum, midbrain and spinal cord of rodents and humans.

D-serine concentration is regulated by serine racemase (SR) and DAAO. It is synthesized by SR from L-serine, and degraded by both SR itself and DAAO. The expression of the latter in the human brain is both age and brain region dependent.

The distribution of D-serine in the central nervous system is highly similar to that of the NMDA receptor. The activation of NMDA receptor requires both glutamate and D-serine or glycine. Influencing the NMDA receptor has a profound effect on synaptic activity, learning, and memory.⁴ The dysfunction of NMDA receptors is involved in the positive (psychotic), negative and cognitive symptoms in schizophrenia.⁵ Genetic studies have found association between schizophrenia and single nucleotide polymorphisms in DAAO and its regulator (G72).

Moreover, DAAO expression and enzyme activity have been reported to be increased in post mortem brain tissue samples from patients with schizophrenia compared to healthy controls.⁶ In patients with schizophrenia, it was found that the SR / DAAO ratio was reduced, resulting in decreased SR levels and / or increased DAAO levels.

The above findings suggest that increasing the body's D-serine levels is beneficial for treating schizophrenia, but to achieve optimum effect, high doses of D-serine are needed, which, in turn, is poorly tolerated due to nephrotoxic effects. Studies have shown that the cause of nephrotoxicity is the hydrogen peroxide produced by the degradation of D-serine metabolized by DAAO. Another potential way to increase D-serine level is to inhibit the enzyme activity

² Efeyan, A.; Comb, W.C.; Sabatini, D.M. Nutrient-sensing mechanisms and pathways. *Nature* **2015**, *517*, 302–310, doi:10.1038/nature14190

³ McDonald, J.W.; Johnston, M. V Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. *Brain Res. Brain Res. Rev.* **1990**, *15*, 41–70, doi:10.1016/0165-0173(90)90011-C

⁴ Mothet, J.-P.; Le Bail, M.; Billard, J.-M. Time and space profiling of NMDA receptor co-agonist functions. *J. Neurochem.* **2015**, *135*, 210–25, doi:10.1111/jnc.13204

⁵ Newcomer, J.W.; Krystal, J.H. NMDA receptor regulation of memory and behavior in humans. *Hippocampus* **2001**, *11*, 529–542, doi:10.1002/hipo.1069

⁶ Burnet, P.W.J.; Eastwood, S.L.; Bristow, G.C.; Godlewska, B.R.; Sikka, P.; Walker, M.; Harrison, P.J. D-amino acid oxidase activity and expression are increased in schizophrenia. *Mol. Psychiatry* **2008**, *13*, 658–60, doi:10.1038/mp.2008.47

that is responsible for its metabolism, so increase in D-serine level and recovery of the NMDA signal can be achieved through the inhibition of DAAO.

2.3. DAAO inhibitors, their *in vitro* properties and *in vivo* effects

Human DAAO is active preferentially on bulky aromatic D-amino acids like D-Tyr, D-Phe, and D-Trp, followed by small uncharged D-amino acids like D-Ser, D-Ala and D-Pro. Many of the known inhibitors are similar to the endogenous ligands being small and polar compounds.

The important binding motifs of DAAO inhibitors include a negatively charged moiety where two heteroatoms of the ligand form a salt bridge with Arg283, and a planar, electron rich moiety positioned parallel with the isoalloxazine ring of the FAD cofactor forming π - π interaction. Further interactions contributing to the binding of some of the ligands are hydrogen bonds to the backbone of Gly313 and to the sidechain of Tyr228 and stacking interactions with Tyr224. It is worth noting that the binding pocket is highly polar at the Arg283 side while it is predominantly hydrophobic at the opposite side.

Because of the properties of the active site of DAAO (relatively small and highly polar), examination of fragment size molecules as DAAO inhibitors seemed relevant. Fragment libraries are constructed from compounds with small size and low complexity. The compounds must meet the following criteria⁷:

- molecular weight less than 300 Da (or number of heavy atoms $8 \leq N_{\text{heavy}} \leq 22$),
- number of hydrogen bond donors and acceptors ≤ 3
- number of rotatable bonds ≤ 3
- $\log P_{\text{calculated}} \leq 3$
- Polar surface area (PSA) is often restricted to $\text{PSA} \leq 60 \text{ \AA}^2$.

A significant portion of DAAO inhibitors known in the literature meet these criteria, while based on the type of the negatively charged moiety and the size of the planar, electron rich moiety these inhibitors can be distributed in three different generations.

⁷ Congreve, M.; Carr, R.; Murray, C.; Jhoti, H. A “rule of three” for fragment-based lead discovery? *Drug Discov. Today* **2003**, *8*, 876–7

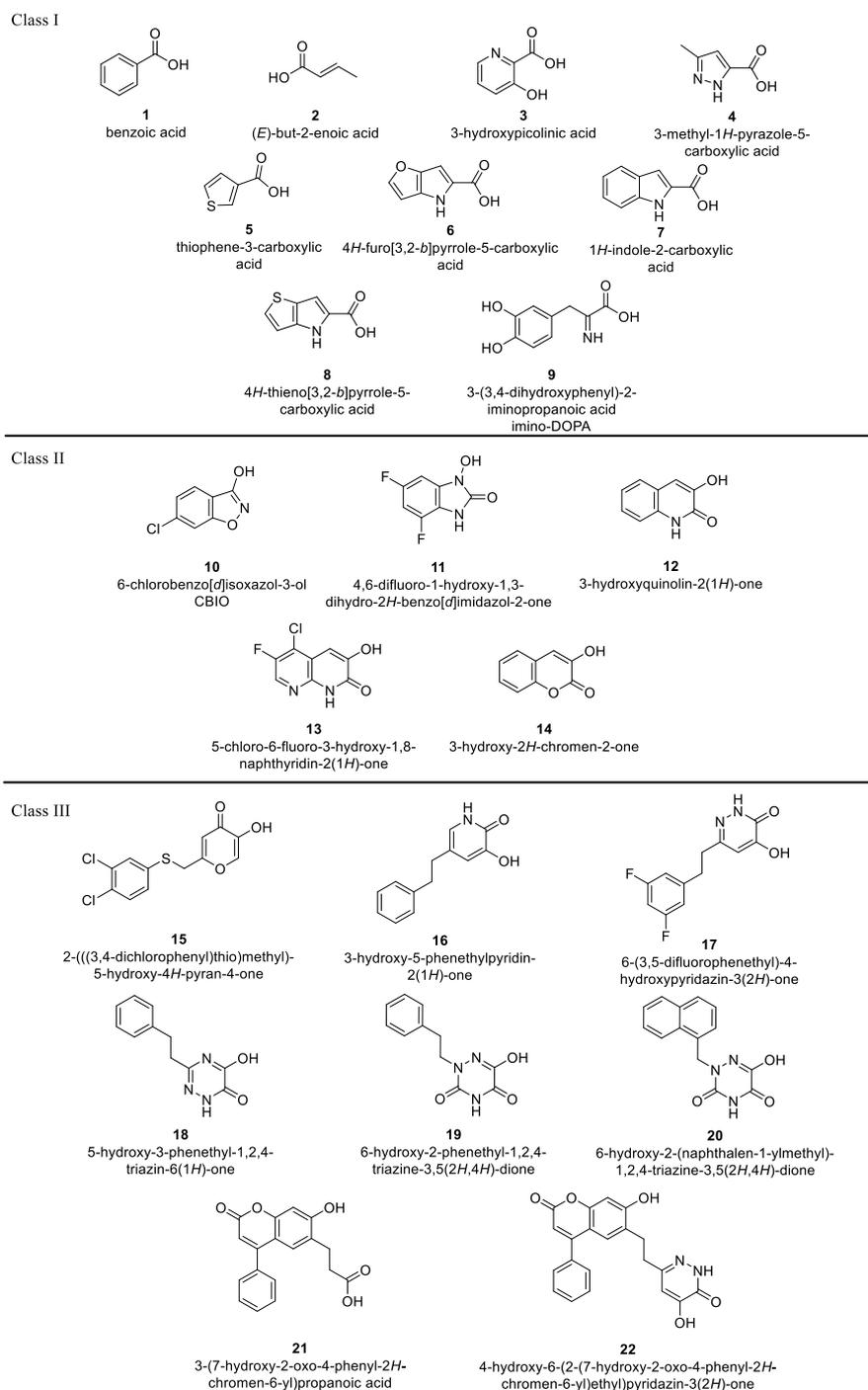


Figure 1. Examples of D-amino acid oxidase inhibitors

The first generation DAAO inhibitors are carboxylic acids (Figure 1, compounds **1-9**). The carboxylic group of these compounds interacts with Arg283 in the catalytic site. These molecules also contain a small aliphatic or aromatic group that is either a single ring or a fused double ring. Aromatic rings are advantageous in this position, as they form π - π stacking interactions with the isoalloxazine ring of the FAD cofactor and the side chain of Tyr224. An

appropriately positioned hydrogen bond donor (**4**, **6-9**) is able to form hydrogen-bond with the backbone carbonyl of Gly313.⁸

In second generation inhibitors (Figure 1, **10-14**), the carboxylic group is replaced by a bioisosteric moiety, while maintaining the fused double ring structure. Various cyclic headgroups anellated with benzene or 6-member heteroaromatic ring were found to be potent DAAO inhibitors. The headgroups are 5- or 6-member rings with endo and exo heteroatoms. It is a general observation that the substitution of the core structures with small groups (F, Cl, Me) is potentially beneficial, while larger groups diminish affinity.⁸

The third generation inhibitors (Figure 1, **15-22**) are based on cyclic carboxylic acid bioisosters connected to bulky nonpolar moieties with a flexible linker. This type of inhibitors keeps the essential interactions of former inhibitors, namely those with Arg283, Tyr228 and the isoalloxazine ring of FAD and they extend in the direction perpendicular to the plane of the isoalloxazine ring. This extension is possible owing to the flexibility of the Tyr224 residue first observed in the X-ray structure of pDAAO complexed with imino tryptophan. Flexibility of Tyr224 was detected in complexes of hDAAO with imino-DOPA (**9**) and also with inhibitors containing phenethyl substituents.⁸

3. Experimental methods

3.1. General information

Melting points were determined on an OptiMelt SRS (Sunnyvale, CA, USA). NMR measurements were performed on Varian NMR System 500 spectrometer (Palo Alto, CA, USA) or Varian NMR System 300 spectrometer (Palo Alto, CA, USA), ¹H and ¹³C-NMR spectra were measured at 30 °C in an appropriate solvent. ¹H and ¹³C chemical shifts are expressed in parts per million (δ) referenced to TMS or residual solvent signals. Reactions were monitored with Merck silica gel 60 F₂₅₄ TLC plates (Darmstadt, Germany). All chemicals and solvents were used as purchased. HPLC-MS measurements were performed using a Shimadzu LC-MS-2020 device (Kyoto, Japan) equipped with a Reprospher 100 C18 (5 μm, 100 × 3mm) column and positive-negative double ion source (DUIS±) with a quadrupole mass spectrometer in a range of 50-1000 m/z. Sample was eluted with gradient elution using eluent A (0.1% formic acid in water) and eluent B (0.1% formic acid in acetonitrile). Flow rate was set to 1.5 mL/min. The initial condition was 0% B eluent, followed by a linear gradient to 100% B eluent by 2 min, from 2 to 3.75 min 100% B eluent was retained, and from 3.75 to 4.5 min back to initial condition and retained to 5 min. The column temperature was kept at 30 °C and the injection volume was 1 μl. High resolution mass spectrometric measurements were performed using a Q-TOF Premier mass spectrometer (Milford, MA, USA) in positive electrospray ionization mode.

3.2. Biological measurements

D-2-Amino-4-(2-aminophenyl)-4-oxobutanoic acid (D-KYN) was used to measure D-amino acid oxidase activity based on the protocol described in ref.. Human DAAO (purchased

⁸ Szilágyi B, Ferenczy GG, Keserű GM. Drug discovery strategies and the preclinical development of D-amino-acid oxidase inhibitors as antipsychotic therapies. *Expert Opin. Drug Discov.* 2018;13:973–982.

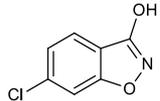
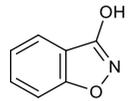
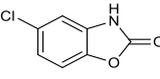
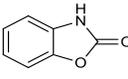
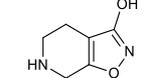
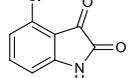
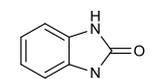
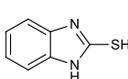
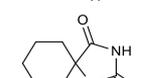
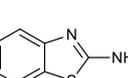
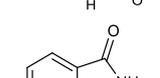
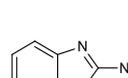
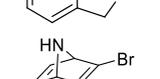
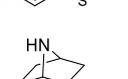
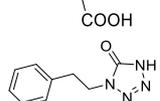
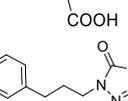
from TargetEx Ltd. (Dunakeszi, Hungary)) was used for the measurements. The buffer contained 20 mM TRIS-HCl and 100 mM NaCl. Flavin adenine dinucleotide (FAD – obtained from Sigma (St. Louise, MO. USA)) was also included in the assay. Compounds were dissolved originally in DMSO and the measured samples were diluted with the buffer solution (the final DMSO concentration was always below 5%). The mixed solution was incubated at 37°C for 1 hour. After the enzymatic reaction ZnCl₂ dissolved in H₂O was added and vortex-mixed. Single point measurements were performed at 20 μM inhibitor concentration, for the IC₅₀ measurements the inhibitors were used at 5 nM, 50 nM, 500 nM, 2.5 μM, 5 μM, 10 μM, 50 μM. Measurements were carried out on a Citation3 cell imaging multi-mode microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) with 364 well plates. The applied wavelengths were 340 nm and 396 nm.

4. Results

4.1. Fragments fit to the catalytic pocket

As mentioned earlier, testing fragments as DAAO inhibitors seemed advantageous, so in order to identify new DAAO inhibitors, the first step was to filter the fragment size [6+5] type fused and spiro heterocyclic compounds as well as some alkyl chain-linked benzene and heterocyclic compound through a virtual screening. Compounds with high docking score were purchased or synthesized and tested *in vitro*.

Table 1. Heterocyclic compounds tested for DAAO inhibition

Compound	Structure	Inhibition % at 20μM ^a	Compound	Structure	Inhibition % at 20μM ^a
10 (CBIO)		114 (3)	23 (BIO)		101 (2)
24		0 (2)	25		26 (1)
26		0 (1)	27		46 (6)
28		0 (2)	29		1 (0)
30		-1 (1)	31		0(0)
32		26 (1)	33		-4 (4)
34		7 (2)	35		0 (1)
36		9 (2)	37		12 (3)

^a all measurements were performed in duplicates; standard deviation in percentage is shown in parenthesis

Among scaffolds, compound **27** with the isatin backbone showed the highest activity so in the next step we examined various derivatives. These isatin derivatives were commercially available.

Table 2. DAAO inhibitory activity of isatin analogues

Compound	Structure	IC_{50} [μM] or % inhibition at 20 μM^a	Compound	Structure	IC_{50} [μM] or % inhibition at 20 μM^a
46		19% (1)	50		7.87(7)
47		0% (1)	51		49% (7)
27		46% (6)	52		12% (2)
48		13% (2)	53		8% (3)
49		-1% (1)	54		13.1(20)

^a all measurements were performed in duplicates; standard deviation in percentage is shown in parenthesis

Among these compounds **50** and **54** show noticeable activity, but their low micromolar potency did not validate further efforts for optimisation.

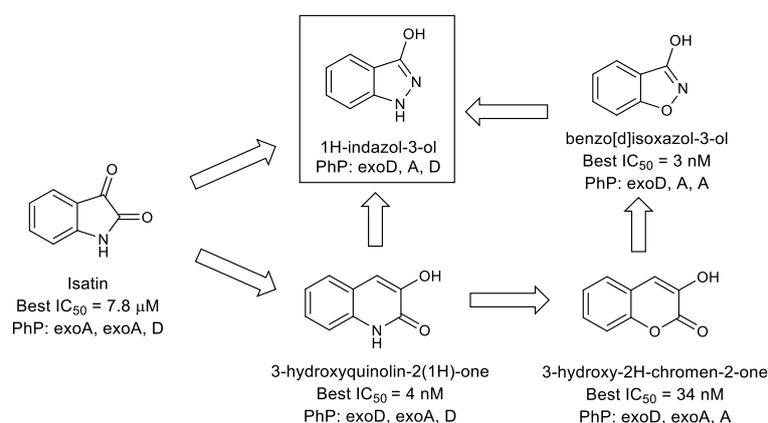


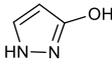
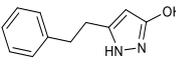
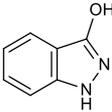
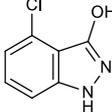
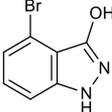
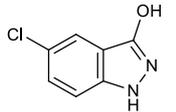
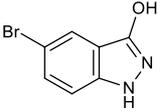
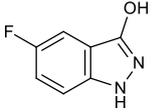
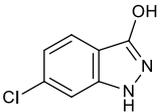
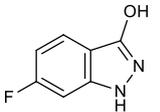
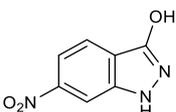
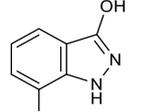
Figure 2. Design paradigm leading to the 1H-indazol-3-ol scaffold

The analysis of pharmacophore groups of these inhibitors lead us to a promising scaffold, 1H-indazol-3-ol. In the case of 1H-indazol-3-ol, the pharmacophore contains an endo H-bond donor (NH), an endo H-bond acceptor (N) and an exo H-bond donor (OH). This scaffold is very

similar to the benzo[*d*]isoxazol-3-ol, but has a difference in the role of the heteroatom at position 1 in the heterocyclic ring. This change in that position seemed favorable because in case of [6+6] membered inhibitors with similar difference the one with H-donor had one-fold lower IC₅₀ value because of the extra H-bond formed between the NH group and Gly313.

Consequently, the aim of our chemical program was to explore the 1*H*-indazol-3-ol scaffold. Several 1*H*-indazol-3-ol fragments were prepared and tested as DAAO inhibitors for this purpose (Table 3).

Table 3. DAAO inhibitory activity of 1*H*-indazol-3-ol fragments.

Compound	Structure	IC ₅₀ [μM] or % inhibition at 20μM ^a	Compound	Structure	IC ₅₀ [μM] or % inhibition at 20μM ^a
57		0% (0)	60		66% (2)
61		not measurable ^b	62		19% (8)
63		34% (0)	64		not measurable ^b
65		not measurable ^b	66		not measurable ^b
67		0.15 (13)	68		0.12 (42)
69		1.20 (25)	70		10.1 (13)

^a all measurements were performed in duplicates; standard deviation in percentage is shown in parenthesis

^b Strong autofluorescence

Although the heterocycle alone (**57**) did not show activity, both the phenethyl derivative (**60**) and the benzo-condensed derivatives proved to be active. Among the substituted derivatives of 1*H*-indazol-3-ol, compounds modified at positions 6 and 7 had significant activity, 6-chloro and 6-fluoro showed low nanomolar inhibition values.

The pK_a values of the test compounds are typically between 7 and 8, suggesting at least partial deprotonation at the pH of the assay, while some compounds had strong autofluorescence properties, so they could not be measured using the available enzyme assay (interference or sensitivity problems in other assays used in the literature), but it was also possible to test compounds for enzyme binding by isothermal titration calorimetry.

According to the PAMPA test the 6-Cl derivative (**67**) shows high and the 6-F derivative (**68**) shows medium permeability. These data demonstrate that these compounds, and more

generally, appropriately substituted derivatives in the 1*H*-indazol-3-ol series have potentially advantageous intestinal absorption.

Based on the beneficial *in vitro* DAAO inhibitory activity, good predicted absorption and metabolic stability we selected compound **68** for measuring its *in vivo* effect on mice D-serine level. It was found that the co-administration of **68** and D-serine results in a significant ($p < 0.05$) increase of the D-serine level compared to the administration of D-serine alone (33% increase after 10 minutes and 27% increase after 60 minutes with corresponding blood levels of 13.1 μM and 0.77 μM of **68**, respectively).

4.2. Inhibitors against the lid-open enzyme conformation

4.2.1. Linear shaped compounds

To find novel structural classes in-house compound library^{9,10,11} of over 2000 unique compounds at the Faculty of Pharmacy, University of Ljubljana was used for virtual screening. Compounds were docked into DAAO structures.

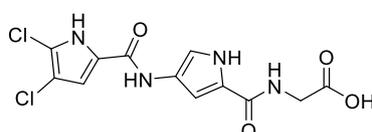


Figure 3. Structure of compound **71**

Hits were selected using Glide's docking score. Docked poses of top scored compounds were visually inspected and the best 16 compounds were subject to *in vitro* testing with D-kynurenine assay. This procedure led to the identification of compound **71** (Figure 3) as a micromolar DAAO inhibitor.

We performed an SAR study based on the newly identified DAAO inhibitor, compound **71**, with two objectives; first, to understand the structural requirements of lid opening, and second, to explore the interaction network around the inhibitor scaffold.

Compounds **83-91** (Table 4) were synthesized to explore how the interaction with the lid contributes to the activity. These compounds either have reduced size presumably not interacting with the lid, or they have varied substitution pattern on the terminal pyrrole ring.

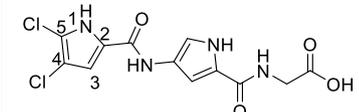
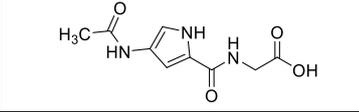
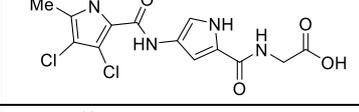
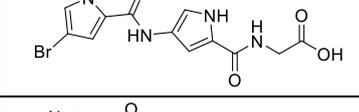
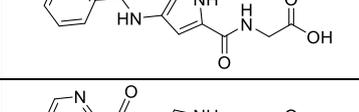
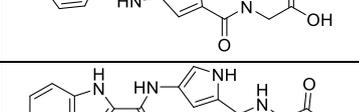
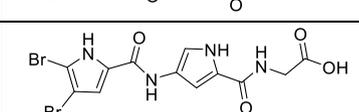
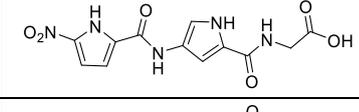
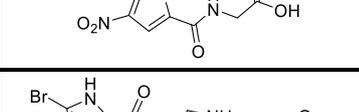
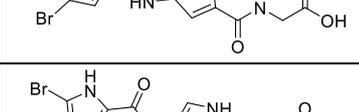
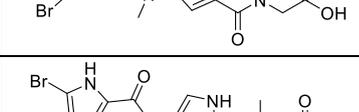
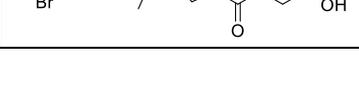
In order to explore the impact of the polar interaction network, specifically the role of the NH groups, methyl groups were added to the two amide N-atoms and also to the middle pyrrole ring N-atom (**99,102,103,109**). *N*-methylation affects the H-bonding ability of the compounds, the space they fill and also their preferred conformation.

⁹ Cotman, A.E.; Trampuž, M.; Brvar, M.; Kikelj, D.; Ilaš, J.; Peterlin-Mašič, L.; Montalvão, S.; Tammela, P.; Frlan, R. Design, Synthesis, and Evaluation of Novel Tyrosine-Based DNA Gyrase B Inhibitors. *Arch. Pharm. (Weinheim)*. **2017**, *350*, 1700087, doi:10.1002/ardp.201700087

¹⁰ Ilaš, J.; Pecar, S.; Hockemeyer, J.; Euler, H.; Kirfel, A.; Müller, C.E. Development of spin-labeled probes for adenosine receptors. *J. Med. Chem.* **2005**, *48*, 2108–14, doi:10.1021/jm049513x

¹¹ Brus, B.; Košak, U.; Turk, S.; Pišlar, A.; Coquelle, N.; Kos, J.; Stojan, J.; Colletier, J.-P.; Gobec, S. Discovery, Biological Evaluation, and Crystal Structure of a Novel Nanomolar Selective Butyrylcholinesterase Inhibitor. *J. Med. Chem.* **2014**, *57*, 8167–8179, doi:10.1021/jm501195e

Table 4. DAAO inhibitory activity of designed linear open-lid compounds

Compound	Structure	% INH at 20 μM^a	IC ₅₀ [μM] ^a
71		100 (0) %	5.2 (11)
83		39 (0) %	-
84		100 (0) %	8.8 (26)
85		100 (0) %	6.7 (27)
86		45 (2) %	-
87		18 (2) %	-
88		8 (1) %	-
89		100 (0) %	0.39 (5.1)
90		33 (8) %	-
91		35 (4) %	-
99		100 (0) %	0.49 (12)
102		0 (8) %	-
103		0 (3) %	-

109		32 (1) %	-
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^a all measurements were performed in duplicates; standard deviation in percentage is shown in parenthesis

Data in Table 4 show that all active compounds include a terminal pyrrole ring with large substituents. Compounds with unsubstituted pyridine moiety (**86**, **87**) or without the terminal substituted pyrrole (**83**, **88**, **91**) exhibit low inhibitory activity. Investigating their docked poses, we observe the presence of a salt bridge between the carboxylate group and Arg283, but the compounds fail to form other interactions characteristic to high affinity DAAO inhibitors. No stacking with the isoalloxazine ring of the FAD and limited favorable interactions by the central part of the ligands are detected.

Best activity in the series is obtained for compounds **89** and **99**, both containing Br-substituents on the terminal pyrrole ring. It is notable that compound **85** having a single Br-substituent at the 4th position and unsubstituted at the 5th has an order of magnitude lower activity than the 4,5 disubstituted compound **89**. The importance of the substituent at the 5th position is underlined by the activity measured for compounds **71** (IC₅₀=5.2 μM) and **84** (IC₅₀=8.7 μM) containing 5-Cl and 5-methyl substituents, respectively. The 10-fold increase in activity of the two latter compounds can be attributed to the presence of Br that is larger than either Cl or Me on one hand, and is able to form a halogen bond with Ser223 backbone carbonyl, on the other hand.

The methylation of the central pyrrole ring leads to a significant loss of activity (compare compounds **89** and **109**). This methyl group prevents the H-bond formation with Gly313 backbone and, according to the docked poses, it causes steric clash with the protein backbone. Methylation of the amide nitrogen close to the carboxyl end of the molecules does not affect activity according to the comparison of compounds **89** and **99**. Docking studies suggest that this amide can adopt both cis and trans conformation without significantly affecting the position of the terminal substituted pyrrole ring in either **89** or **99**. The trans amide conformation is proposed to be preferred for amide (**89**) and the cis conformation is proposed to be preferred for methylamide (**99**).

The amide group between the pyrrole rings is predicted by docking to adopt cis conformation. This results in a bend molecule that is accommodated in the binding pocket extended by the movement of loop 216-228 (lid opening). The presence of a secondary cis amide is notable, since amides have high preference for the trans conformation both in proteins¹² and in small molecules¹³. Nevertheless, there are examples where ligands bind to proteins with cis amide conformation (PDB: 1HTG, 1AJ6, 1YBH) showing that intermolecular interactions are able to compensate for the energy increase due to amide trans-cis transformation. Converting secondary amides to tertiary amides increases the propensity for cis conformation. Therefore, the methylation of the amide that is assumed to adopt cis-

¹² Weiss, M.S.; Jabs, A.; Hilgenfeld, R. Peptide bonds revisited. *Nat. Struct. Biol.* **1998**, *5*, 676, doi:10.1038/1368

¹³ Brameld, K.A.; Kuhn, B.; Reuter, D.C.; Stahl, M. Small molecule conformational preferences derived from crystal structure data. A medicinal chemistry focused analysis. *J. Chem. Inf. Model.* **2008**, *48*, 1–24, doi:10.1021/ci7002494

conformation when bound may be able to increase activity by facilitating the adoption of cis conformation. Another effect of methylation, however, is the increase of bulkiness. Docking of compounds **102** and **103** shows that this methyl group is close to loop 48-63. The effect of this steric clash observed for **102** and **103** was not possible to assess *a priori* since the conformation of this loop either varies (see e.g. PDB codes 3ZNQ and 4QFC) or is partially missing (3CUK and 4QFD) in available crystal structures that indicate structural flexibility. The complete loss of activity observed with the methylation of the amide between the pyrrole rings (compare compounds **89** and **99** with **102** and **103**) suggests that this loop is unable to adopt a conformation that allows accommodation of the methylated amide group.

4.2.2. L shaped compounds

In our research group, previous studies have investigated how the substitution of the aromatic moiety of compound **22** affects activity. It was found that substitution of the phenyl group at positions 3 and 4 (Figure 4) results in a slight improvement in the activity, IC₅₀ values of these compounds (**22,110-114**) were between 52 and 474 nM.

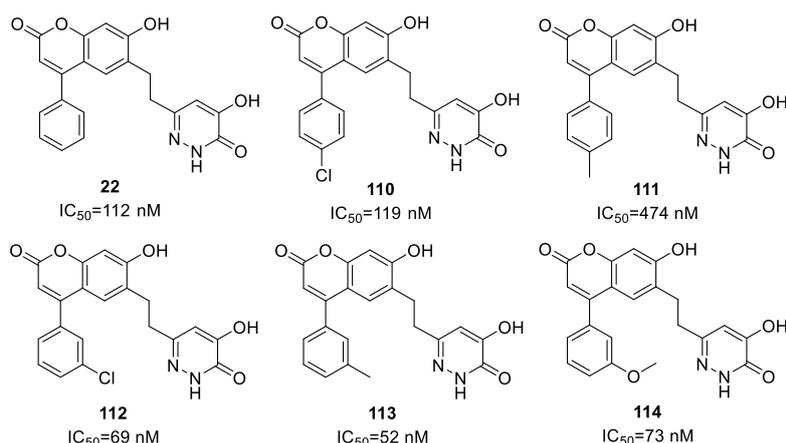


Figure 4. Derivatives of compound **22** mono-substituted at the aromatic part

In view of these results, new compounds have been designed that contain a phenyl group substituted at the same position as the previously produced **22** derivatives.

Here we focused on the linker region. Starting from the original linker used in compounds **21** and **22**, we selected 1,5-dihydro-2*H*-pyrrolo[3,2-*d*]pyrimidine-2,4(3*H*)-dione and 1,2,3,4-tetrahydroisoquinoline skeletons that might resemble in shape and possible substitution pattern.

As a warhead, the best option would have been the pyridazine ring used in the original compound, but we failed to produce a suitable derivative for the coupling reaction. Therefore, we decided to attach a known acidic headgroup (pyrrole-2-carboxylic acid) as warhead.

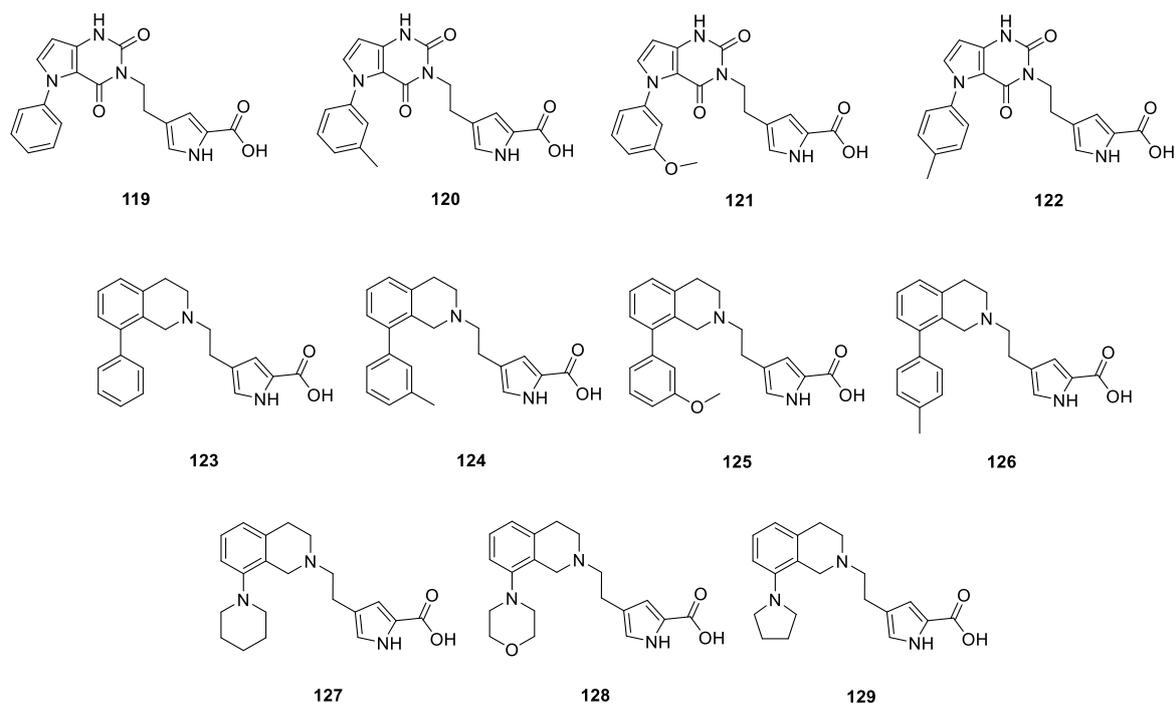


Figure 5. Designed compounds with new linker part.

The prepared new compounds (**119-122**; Figure 5) were tested in the KYNA enzyme inhibitory assay, but unfortunately they did not show any inhibitory activity at 20 μM concentration. Comparing these compounds with DAAO inhibitors **22** and **110-114** and with pyrrole-2-carboxylic acid containing inhibitors known from the literature strongly suggests that it is the linker part of molecules **119-122** that prevents DAAO activity. The replacement of the 7-hydroxy-2*H*-chromen-2-one linker of compound **22** changes the interactions of the linker, in addition it modifies the exit vectors of both the acidic headgroup and the pendant aromatic moiety in compounds **119-122**. While the presence of H-bond to the Gln53 backbone is highly possible in DAAO complexes of **119-122**, no H-bond acceptor to the water-mediated interaction with Tyr224 can be assumed. Perhaps more importantly, the different orientation of the pendant aromatic group in **119-122** compared to compounds **22** and **110-114** might be responsible for the decreased activity. This group in derivatives **110-114** points toward the flexible loop formed by residues 218–224 and may not form beneficial interactions with the loop. These observations suggest that the linker contributes significantly to DAAO activity both by its interactions with the enzyme and by properly orienting the pendant aromatic moiety.

5. Thesis points

1. I have identified low nanomolar DAAO inhibitors with 1*H*-indazol-3-ol structure, which, in addition to enzymatic kinetics and titration calorimetry, have been subjected to protonation and tautomeric mapping, and their *in vivo* efficacy has been demonstrated following metabolic stability and membrane permeability assays. [P1, P2]
2. I synthesized linear compounds with polar terminus that is typical to DAAO inhibitors which have nanomolar activity and analyzed their structure-activity relationship by studying the derivatives produced. [P3]
3. I have produced compounds derived from a DAAO inhibitor that, in contrast to most known inhibitors, interacts with loop 218-224. This information may be useful in designing new compounds having better physicochemical properties and ADME profile. [P4]

6. APPLICABILITY

By identifying 1H-indazol-3-ol as a novel DAAO inhibitor, a new pharmacophore moiety was discovered, that could function as a possible carboxylic acid bioisostere group for the newly prepared compounds.

In addition to the synthetic value of the newly identified inhibitors that bind to the lid-open enzyme conformation, the results obtained during structure-activity relationship can help to identify and design new compounds.

7. Publications

7.1. Publications related to the thesis:

P1. Szilágyi B, Kovács P, Ferenczy GG, et al. Discovery of isatin and 1*H*-indazol-3-ol derivatives as D-amino acid oxidase (DAAO) inhibitors. *Bioorganic Med. Chem.* 2018;26:1579–1587. DOI: 10.1016/j.bmc.2018.02.004; IF: 2.881 ; I(FI):2(1)

P2. Orgován Z, Ferenczy GG, Szilágyi B, et al. Validation of tautomeric and protomeric binding modes by free energy calculations. A case study for the structure based optimization of D-amino acid oxidase inhibitors. *J. Comput. Aided. Mol. Des.* 2018;32:331–345. DOI: 10.1007/s10822-018-0097-y; IF:2.356 ; I(FI):4(1)

P3. Szilágyi B, Skok Ž, Rácz A, et al. Discovery of D-amino acid oxidase inhibitors based on virtual screening against the lid-open enzyme conformation. *Bioorganic Med. Chem. Lett.* 2018;28:1693–1698. DOI: 10.1016/j.bmcl.2018.04.048; IF:2.442 ; I(FI):1(1)

P4. Szilágyi B, Hargitai C, Kelemen ÁA, et al. Synthesis and Biochemical Evaluation of Lid-Open D-Amino Acid Oxidase Inhibitors. *Molecules.* 2019;24:290. DOI: 10.3390/molecules24020290; IF: 3.060 (2018); I(FI):0(0)

P5. Szilágyi B, Ferenczy GG, Keserű GM. Drug discovery strategies and the preclinical development of D-amino-acid oxidase inhibitors as antipsychotic therapies. *Expert Opin. Drug Discov.* 2018;13:973–982. DOI: 10.1080/17460441.2018.1524459; IF:4.421 ; I(FI):1(1)

7.2. Further publications:

Polyák M, Varga G, Szilágyi B, et al. Synthesis, enzyme kinetics and computational evaluation of *N*-(β -D-glucopyranosyl) oxadiazolecarboxamides as glycogen phosphorylase inhibitors. *Bioorganic Med. Chem.* 2013;21:5738–5747. DOI: 10.1016/j.bmc.2013.07.024; IF: 2.951; I(FD):11(3)

7.3. Presentations:

1. MTA TTK Szerves Kémiai Szeminárium, 2016. június 17.; D-aminosav oxidáz inhibitorok szintézise - Szilágyi Bence, Ferenczy György, Keserű György
2. MTA Alkaloid- és Flavonoidkémiai Munkabizottsági ülés, 2018. április 13.; D-aminosav oxidáz enzimgátló molekulák szintézise és vizsgálata - Szilágyi Bence, Ferenczy György, Keserű György
3. MTA Heterociklusos és Elemorganikus Kémiai Munkabizottsági ülés, 2018. május 07.; D-aminosav oxidáz enzimgátló molekulák szintézise és vizsgálata - Szilágyi Bence, Ferenczy György, Keserű György, Janez Ilaš