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Identification of some novel AHAS inhibitors via molecular docking and virtual screening approach

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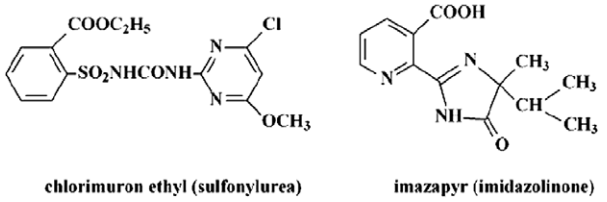
Abstract—Acetohydroxyacid synthase (AHAS; EC 2.2.1.6) catalyzes the first common step in branched-chain amino acid biosynthesis. This enzyme is an important target for the design of environmental-benign herbicides. Based on the crystal structure of AHAS/sulfonylurea complex, we have carried out computational screening of the ACD-3D database in order to look for novel non-sulfonylurea inhibitors of AHAS for the first time. Three novel compounds were found to inhibit plant AHAS in vitro among 14 procured compounds. One compound showed promising activity in vivo for rape root growth inhibition bioassay. This research provided useful clues for further design and discovery of AHAS inhibitors.

1. Introduction

Most animals are not able to survive without vegetable diet because they lack the biosynthetic ability of many essential compounds and precursors. The biosynthesis pathway of branched-chain amino acids valine, leucine and isoleucine belongs to such a case that exists in microbes and plants while absent in mammal bodies.[1] As a result, the enzymes involved in this pathway thus become good targets for the design of inhibitors which could prevent synthesis of these branched-chain amino acids, with no or little mammalian toxicity. Acetohydroxyacid synthase (AHAS; EC2.2.1.6)[1] catalyzes the first common step in the biosynthesis of these amino acids and as a result, inhibitors of AHAS are specific to non-animals. Indeed, several classes of widely and safely used commercial herbicides, including sulfonylureas[2] and imidazolinones[3] (Fig. 1), are potent inhibitors of AHAS.

Although AHAS was recognized about two decades ago[2,3] as the target of sulfonylurea and imidazolinone herbicides, it was not until very recently that the mechanism of inhibition of AHAS started to become clear by determining the crystal structure of *Saccharomyces cerevisiae* AHAS (yeast AHAS) alone[4] and with ligand bound,[5,6] and later AHAS from *Arabidopsis thaliana* (plant AHAS),[7,8] in complex with either a sulfonylurea or an imidazolinone herbicide. The active sites for both yeast AHAS and plant AHAS are located at the interface of the two subunits, forming a deep hydrophobic tunnel and the herbicide binds in the pocket with multiple residues.[9] Mutations of most surrounding residues result in resistance for herbicides. A previous detailed investigation of structure–activity relationships for a new family of sulfonylurea herbicides[10] revealed that better electrostatic, van der Waals, hydrophobic and H-bond interaction with surrounding residues in the binding site could enhance the binding affinity. These developments have opened a new view for design of novel AHAS inhibitors from a more rational way based on the herbicide binding mode other than the traditional me-too synthetic chemistry.



chlorimuron ethyl (sulfonylurea) imazapyr (imidazolinone)

Figure 1. Typical structures of sulfonylurea and imidazolinone herbicides.

Keywords: Acetohydroxyacid synthase; Docking; Virtual screening; Inhibitor; Design of herbicides.
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Structure based drug design (SBDD) is an approach for designing inhibitors using the information of the receptor geometry.[11,12] Molecular docking programs are usually used to virtual screen a three-dimensional database of small molecules to evaluate potential binding energy to predict how well a novel ligand might fit in with the binding site. The calculated binding affinities together with other scoring functions help to rank the potential ligand candidates. Several softwares, such as DOCK,[13] Gold,[14] FlexX[15] and AutoDock[16] have been developed to undertake such computational tasks. Compared to the conventional high throughout put screening (HTS) technique or random screening method, virtual screening comparatively has some advantages in costing less input.[17] With the protein databank expanding rapidly and the computational techniques developing continuously, SBDD in combination with virtual screening plays a more and more important role to discover novel compounds to overcome the structural limitation of available inhibitors.[18–24]

The binding pockets of yeast AHAS and plant AHAS share significant similarity,[8] in which both crystal structures are nearly the same for sulfonylurea herbicide chlorimuron ethyl (CE). This indicates that an inhibitor of yeast AHAS might be also active towards plant AHAS. In the present study, we have tried to look for novel compounds target to AHAS, by virtual screening small molecule database against both these AHAS apoenzymes. Hydrophobic parameters were also taken into consideration as a necessary filter for the resulting database. An investigation revealed that some compounds possess same structures in either resulting database. After carefully analyzing their unique structures and predicted binding modes in this cross intersection, 14 compounds were found to have favourable shape complementarity and potentiality in forming H-bonds. They were procured and the inhibition of plant AHAS in vitro and their herbicidal activity in vivo were examined. This is the first report to design AHAS inhibitors based on the binding sites. This virtual screening of AHAS has provided some promising information in discovering new lead compounds.

2. Results and discussion

2.1. Identification of molecules by virtual screening

Against both yeast AHAS and plant AHAS structural binding models, MDL ACD-3D database[25] with approximately 164,000 small compounds was searched by using the program DOCK 4.0.[13] This database had been truncated to filter all the metal-containing compounds in order that the remaining compounds could be recognized properly by the program. For each virtual screening, the top 2000 candidate molecules with the best energy scores were considered as potential AHAS inhibitors for further study. The calculated interaction energy of these molecules covered a range from −53.27 kcal/mol to −36.71 kcal/mol for yeast AHAS, and for plant AHAS in the range of −53.42 kcal/mol to −36.34 kcal/mol. The two resulting databases were compared and nearly 30 percent molecules were found to be the same as an intersection. This intersection was then subjected to further analyses.

For pharmaceutical compound, hydrophobic nature is an important factor to judge if a certain compound could effectively reach its site of action. It is the same for agrochemicals that this aspect is also a key factor. Lipinski et al.[26] have concluded that for most commercial herbicides, their $\log P$ values are in the range of −0.5 to 3.0. We calculated their $\log P$ values of the compounds in the resulting database by using XLOGP[27] program and picked out those compounds with predicted $\log P$ between −0.5 and 3.0.

For docking programs and scoring functions, usually a number of false positives would appear in the top ranking list. It is necessary to check and analyze the binding mode of each compound to determine if it has reasonable interaction and geometry fitting. We have investigated all their binding mode and binding sites of the compounds for their shape complementarity and potentiality in forming hydrogen bonds in this intersection. The molecules that did not fit either yeast AHAS or plant AHAS binding site well were excluded from the list after this inspection. Together with the $\log P$ criterion, a final database containing 296 compounds was obtained and considered as possible potential AHAS inhibitors.

On the basis of previous virtual searching and $\log P$ prediction, we selected 14 compounds as candidates to test their biological activity. The binding free energy and binding modes of these candidates were further investigated by the advanced docking program AutoDock3.0.[16] This program used Lamarckian Genetic Algorithm to obtain more precise results than DOCK but it consumed more calculation. To test the biological activities against plant AHAS in vitro, only plant AHAS was docked with these compounds by AutoDock3.0. Table 1 shows the structures of these compounds, their binding energy and calculated $\log P$ values.

2.2. In vitro and in vivo biological activity

The 14 compounds have been obtained from different commercial suppliers. All these compounds were subjected to both in vitro plant AHAS assay and in vivo herbicidal activity measurement. Out of the 14 compounds, 3 compounds were found to possess different rates of inhibition against plant AHAS. Their inhibition constants are 15.2 ± 1.3 μM (for compound **2**), 42.1 ± 10.4 μM (for compound **11**) and 90.7 ± 9.2 μM (for compound **13**), respectively (Fig. 2). These compounds were weaker than commercial sulfonylurea and imidazolinone herbicides, whose K_i values are usually between nanomolar and micromolar level. However, this preliminary result has significance because these structures are completely different from any known AHAS inhibitors. Further modification of these novel structures might help to design and discover new structures with enhanced binding affinity towards AHAS enzyme.

Herbicidal activity ultimately depends upon inhibition of AHAS, however there might exist difference between

Table 1. Structures of the investigated compounds and their docked energy from Dock and AutoDock calculations

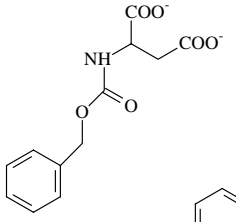
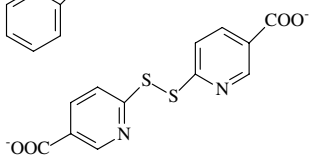
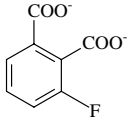
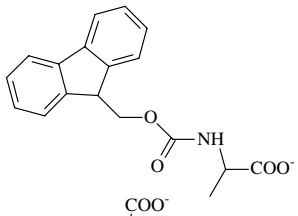
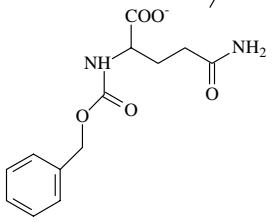
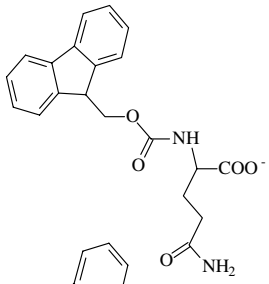
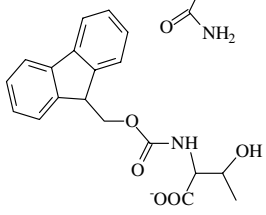
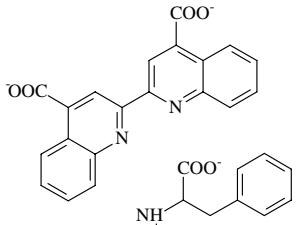
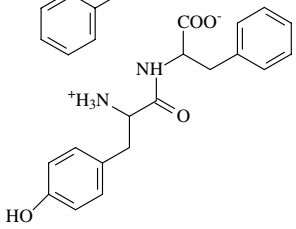
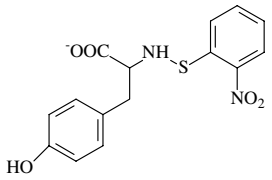
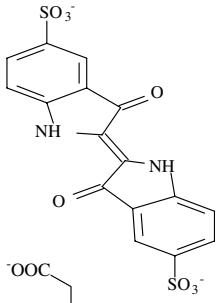
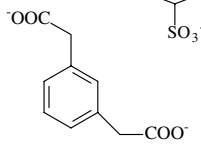
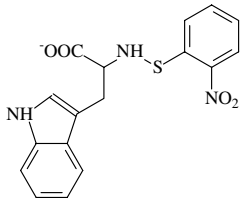
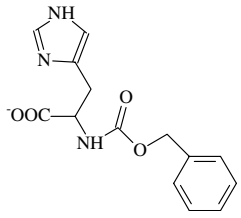
Compound	Structure	BE1[a]	BE2[b]	log *P*
1		−43.00	−9.53	−0.28
2		−42.39	−11.56	−0.16
3		−42.34	−7.94	0.52
4		−42.30	−11.93	2.62
5		−42.20	−10.56	−0.2
6		−41.77	−13.88	0.68
7		−41.56	−11.91	1.37
8		−39.71	−4.80	2.04
9		−39.43	−14.02	1.08

Table 1 (*continued*)

Compound	Structure	BE1[a]	BE2[b]	log *P*
10		−39.41	−11.89	2.48
11		−39.26	−8.88	0.44
12		−38.92	−8.22	−0.02
13		−38.35	−12.52	2.96
14		−37.56	−11.86	0.76

[a] BE1: binding energy (kcal/mol) from DOCK.
[b] BE2: binding energy (kcal/mol) from AutoDock.

in vivo and in vitro activity due to barriers during the different phase of transfer, degradation and detoxification within the plant.[9] Here the growth inhibition greenhouse tests of rape root were undertaken to evaluate their in vivo herbicidal activity (Table 2). Two concentrations of 100 μg/ml and 10 μg/ml were measured for all compounds. Compound **13** exhibited 60.5 percent inhibition of rape root length at 10 μg/ml and a weak inhibition of 6 percent was observed for compound **2**. All the other compounds did not inhibit rape root at this concentration. This is in general parallel with the in vitro assay results. At 100 μg/ml concentration, compound **4**, compound **5**, compound **6** and compound **7** had very low activity.

2.3. Binding modes of the new inhibitors

The binding modes of all the inhibitors with *A. thaliana* AHAS are shown in Figure 3. In order to compare the binding mode of CE with the inhibitors, we plotted both CE (red one) and each inhibitor (blue one) together at all the pictures. For compound **2** (Fig. 3A), it occupied similar space with CE. For compound **13** (Fig. 3B) and compound **11** (Fig. 3C), they have overlaps with CE in binding site, however they also interact with some different residues. It is possible that, some new binding sites might be considered when designing new inhibitors. For compound **11**, it is a symmetric molecule, which indicates that half moiety of the molecule might be also active towards AHAS. We have ordered some new compounds to validate this idea.

3. Conclusion

Herbicidal sulfonylureas and imidazolinones are most widely used AHAS inhibitors. However, these herbicides might not originally be designed based on the approach o target enzyme binding site. Now that the crystal structures of AHAS have been elucidated,[4–6,8] it becomes

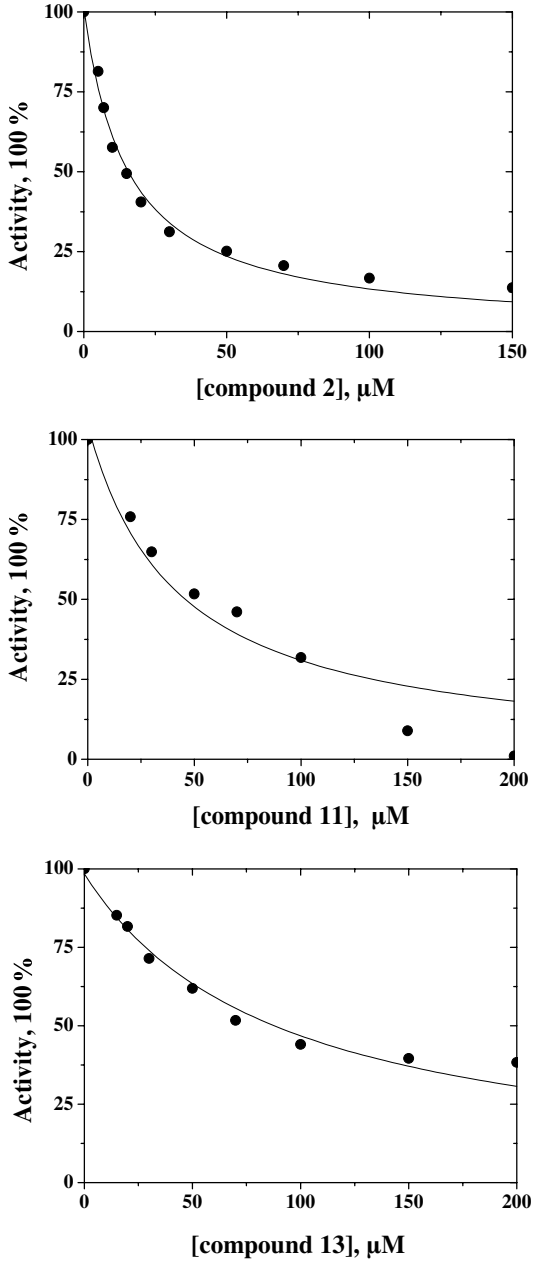


Figure 2. Inhibition curves of compound **2**, compound **11** and compound **13** against plant AHAS.

Table 2. In vivo herbicidal activity of the compounds (% inhibition)

Compound	100 μg/ml	10 μg/ml
1	0	0
2	12.1	6.0
3	0	0
4	12.9	0
5	6.3	0
6	13.9	0
7	25.7	0
8	1.4	0
9	0	0
10	0	0
11	0	0
12	2.6	0
13	74.8	60.5
14	0	0

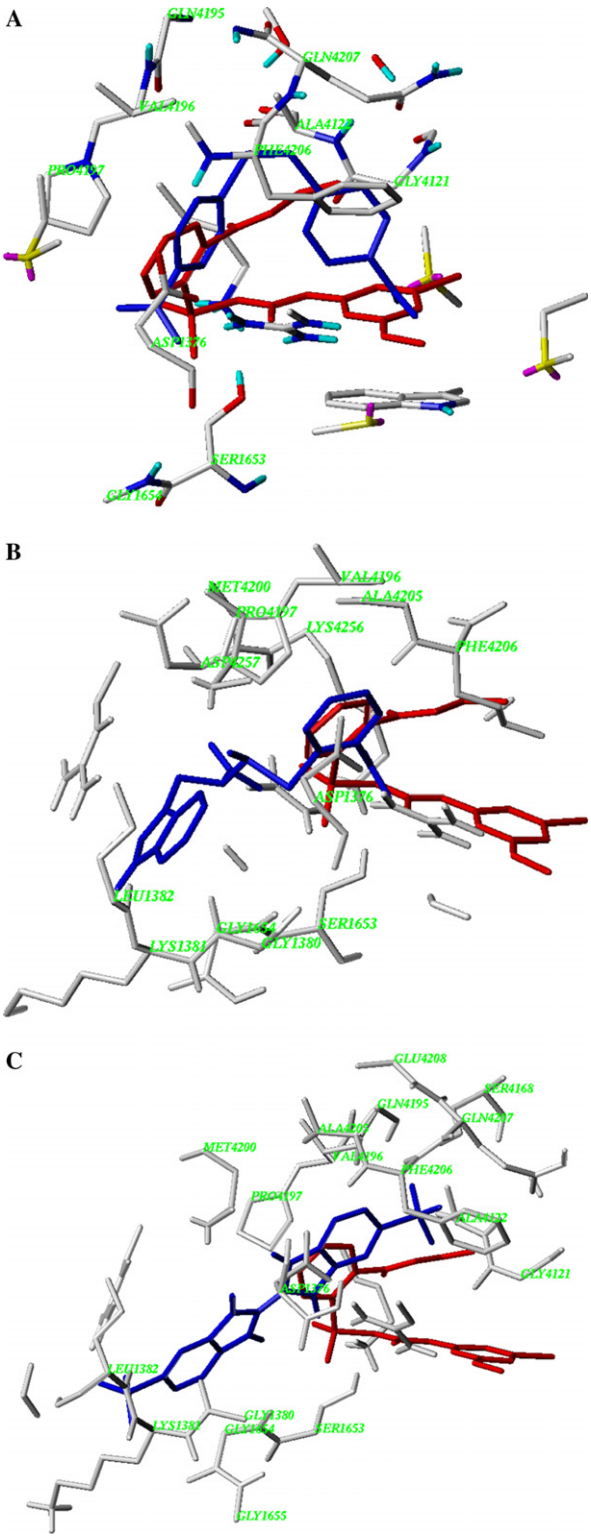


Figure 3. Binding modes of compound **2** (A), compound **13** (B) and compound **11** (C).

possible to find original lead compounds via virtual screening based on the binding site. Within this paper, we tried for the first time with computational docking to verify this possibility. We found 3 novel inhibitors of AHAS, among 14 different compounds. The in vivo herbicidal activity of these 3 inhibitors was in general

accordance with their in vitro activity. This result again indicated that in herbicide discovery research, interaction with the binding site is an important factor to consider; however, other factors that influence the in vivo behaviour should also be taken into consideration. Further research based on the novel AHAS inhibitors might assist in the design of new compounds with favourable herbicidal activity.

4. Experimental

4.1. Preparation of the macromolecules, ligand database and computational screening

The crystal structures of yeast AHAS in complex with CE (pdb code 1N0H, 2.8 Å in resolution) and plant AHAS in complex with the same herbicide (pdb code 1YBH, 2.5 Å in resolution) were used as the basis of the docking experiments. Yeast AHAS is a dimer and plant AHAS is a tetramer.[4–6,8] Their herbicide binding modes share significant similarities and we only maintained half of the plant AHAS structure to carry out molecular dockings. Usually if there does not exist any particular reason for preserving any of the water molecules or ions in the crystal, it is probably best to remove all of them.[13] And we did not find suitable parameters for FAD and ThDP for the docking program. Thus all waters and cofactors were removed, leaving only the free enzymes, as suggested by Dock 4.0.[13] Hydrogen atoms were added to the proteins and kollman-all-atom charges were assigned. For the ACD-3D database, we first eliminated the compounds that contain metals with a program written by ourselves. The database was then converted from isis SD format to sybyl mol2 format with the sdf2mol2 and sybdb modules, available in the DOCK suite. Gasteiger-Marsili charges were assigned to the small molecules. With an aim to reproduce the crystal structure conformation, CE was extracted from either complex structure and prepared individually. The procedures were finished in SYBYL6.9 (Tripos Associates, St. Louis, MO)[28] on an SGI Origin 350 server (R16000) and workstation (R4000).

All docking procedures were done in 'NanKai-Star' supercomputer. DOCK 4.0 was employed for the primary screening. For both structures, the computer program SPHGEN was used to characterize the binding site with negative image within 5.5 Å of binding site, creating clusters of overlapping spheres of the binding center. GRID was used to precompute score grids for rapid dock evaluation. DOCK matched each compound in turn to the site's negative image to search for favourable binding orientations and best binding energy. The virtual screening was performed using an anchor-first search, with important parameters setting as follows: anchor size of 10, configuration per cycle of 100, maximum orientation of 5000 and maximum iteration of 1000. The top 2000 molecules with lowest binding energy were kept for investigation.

The binding modes of the purchased compounds were further modelled by AutoDock 3.0. For AHAS, essential-only hydrogens were added and kollman-uni-atom charges were assigned. AutoTors was used to define which bonds in the ligand are rotatable. AutoGrid pre-calculated a 3D grid of interaction energy based on the macromolecular target using the AMBER force field. During AutoDock process, the Lamarckian genetic algorithm (LGA) was applied to search the binding orientation and conformation of each candidate molecule. The number of generation, energy evaluation and docking trials was set to 27,000, 25,000 and 10, respectively. For all the other parameters, their default values were used.

4.2. log *P* value prediction

XLOGP 2.0, developed by Lai et al.,[27] is an atom-additive program for calculating log *P* values. The octanol/water partition coefficient of the top 2000 molecules from each initial virtual screening was calculated by this method. It gives the log *P* value for a given compound by adding the contributions from component atoms and correction factors as described in the following equation:

$$\log P = \sum_i a_i A_i + \sum_j b_j A_j, \tag{1}$$

where a_i and b_j are regression coefficients, A_i is the number of occurrences of the ith atom type and B_j is the number of occurrences of the jth correction factor.

4.3. Measurement of in vitro and in vivo biological activity

4.3.1. In vitro AHAS inhibition. The plant AHAS was expressed and purified as described previously.[29] AHAS activity was measured using the colorimetric assay in 50 mM potassium phosphate (pH 7.0) containing 50 mM pyruvate, 1 mM thiamine diphosphate, 10 mM $MgCl_2$ and 10 μM FAD. The compounds to be tested were made into emulsions to aid dissolution. The reaction mixture was incubated at 37°C for 30 min and the reaction stopped with 25 μL of 10% H_2SO_4 and heated at 60 °C for 15 min to convert acetolactate into acetoin. The acetoin formed was quantified by incubation with 0.5% creatine and α-naphthol (5%, w/v) for 15 min at 60 °C and A_{525} was measured. The data were analyzed by nonlinear regression using the following equation to estimate the values and standard errors for the apparent inhibition constant (K_i^{app}) and the uninhibited rate (v_o).

$$v = v_o/(1 + [I]/K_i^{app}) \tag{2}$$

4.3.2. In vivo inhibition of the root growth of rape (*Brassica campestris* L). The in vivo experimental sections were similar to our previous publication.[10] Rape seeds were soaked in distilled water for 4 h before being placed on a filter paper in a 6-cm Petri plate, to which 2 ml of inhibitor solution had been added in advance. Usually, 10 seeds were used on each plate. Duplicate was tested for each trial. The plate was placed in a dark room and allowed to germinate for 72 h at 28 (±1) °C. The lengths of 10 rape roots were measured and the means were calculated. The percentage inhibition was calculated relative to controls using distilled water instead of the inhibitor solution.

Acknowledgments

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