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The discovery of the novel lead compound of *N*-nitroureas target on acetohydroxyacid synthase

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ABSTRACT

The weeds are a primary reason causing the yield decreasing of agriculture. Acetohydroxyacid synthase (AHAS, EC: 2.2.1.6) is the key target enzyme of many kinds of herbicides, such as sulfonylureas and imidazolinones, the requirements toward the novel herbicide is urgent owing to the gradually emerging resistance with the long-term application. *N*-nitrourea compounds are a novel urea compounds owning various effects, such as growth regulation, weeding, bacteriostasis. In this study, α-[*N*-nitro-*N*-(2,4,6-triCl) phenyl] leucine ethyl ester urea (compound 3g), a novel *N*-nitrourea compound, was discovered as a potential lead compound for the AHAS target. Based on the molecular docking analysis compound 3g optimization work was performed, and then a new structure backbone of *N*-nitrourea compound was designed for further research.

1. Introduction

Throughout the history of agriculture, more time, energy and money have been devoted to weed control than to any other agricultural activity [1]. Weed management is a perennial challenge for growers, and continual innovation is essential to maintain the effectiveness of management technologies [2]. For the past many years, herbicides have played a vital role in crop production. Many new herbicides were discovered and developed in the 1960s and 1970s [3], and their use has allowed for significant diversification in crop types and has triggered a major shift towards more soil- and water-conserving and energy-efficient farming systems. In recent years, with the increasing numbers of herbicide-resistant weeds emergence [4,5], there is a dire need of new, more selective and even more potent herbicides to control the unwanted vegetables, so as to reduce crop loss and to avoid an epidemic.

There are many metabolic differences among plants, animals and microorganisms, which are the basis for the action of various selectively toxic compounds. For example, the branched-chain amino acids are synthesized by plants, algae, fungi, bacteria and archaea, but not by human and animals [6]. There are several enzymes in the biosynthetic pathway of the branched-chain amino acid, such as acetohydroxyacid synthase, ketol-acid reductoisomerase, threonine deaminase and dihydroxyacid dehydratase. Moreover, they are all unique in this biosynthetic pathway. So, these enzymes are potential targets of bioactive compounds, and several herbicides actually act in this way [7]. Especially, many herbicides focus on the first enzyme in this pathway, acetohydroxyacid synthase (AHAS; EC: 2.2.1.6). AHAS could catalyze two molecules of pyruvate to form 2-acetolactate for valine and leucine formation, or one molecule pyruvate and another molecule 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate [8]. It contains three cofactors: flavin adenine dinucleotide (FAD), thiamine diphosphate (ThDP) and Mg^{2+}, they are necessary for the activity of AHAS. ThDP involves in the decarboxylation reaction of pyruvate to form stabilized carbanion, and protonation to form hydroxyethyl-ThDP, which is an extremely unstable intermediate. Then the intermediate condense another molecule of pyruvate or 2-ketobutyrate. Finally, the products of 2-acetolactate or 2-aceto-2-hydroxybutyrate can be obtained, and release the ThDP [9]. Due to no pure redox reaction in this reaction, the function of FAD is still indistinct. But some researchers suppose that FAD is able to stabilize the enzyme, and properly increase activity of AHAS [10]. Meanwhile, the others infer that FAD acts as a protective role in the reaction. Mg^{2+} likes as an anchor and is coordinated to ThDP by two diphosphate oxygen atoms and two amino acid sidechains. By these connections, ThDP can be holded in proper position [6].

AHAS was target of many classes of herbicides, such as sulfonylureas, imidazolinones, sulfamides, triazolo pyrimidines, pyrimidine ether, pyrimidine salicylic acid, as well as its analogs. The catalytic subunit of *Saccharomyces cerevisiae* AHAS, the structure

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in apo form and in the presence of sulfonylurea herbicides have been solved [11]. More recently, six crystal structures of *Arabidopsis thaliana* AHAS (*At*AHAS) in complex with five different sulfonylurea and one imidazolinone herbicide have been reported [8]. Both the sulfonylurea and imidazolinone herbicides bind at the entrance of channel leading to the active site, preventing access of the substrate [8,9]. These structures have explained the molecular basis of herbicides and opened the way to the rational design of alternative inhibitors of AHAS.

The sulfonylurea herbicide was initially developed by Levitt. Sulfonylureas were more popular and extensive application in the worldwide. They have possessed a great share of herbicide market. The basic structure is as Fig. 1 [8]. Sulfonylureas own several prime advantages which are high selectivity and potency, broad-spectrum weed control, low mammalian toxicity [12]. Up to now, more than thirty herbicides which have registered come from this family [8].

As the widespread and long-term application of herbicides, the resistant weeds are emerging continuously as time goes on. It is urgent to develop novel compounds as lead inhibitors to combat resistant problem. *N*-nitrourea compounds are a novel urea compounds owning various effects, such as growth regulation, weeding, bacteriostasis. Our laboratory has made efforts to research *N*-nitrourea compounds for years. And we have synthesized hundreds of *N*-nitrourea compounds. Some of these compounds have acquired patents. The general feature of *N*-nitrourea compounds (Fig. 2) are a central urea bridge with an o-substituted aromatic ring which is attached to the nitrogen atom, and a nitro group links to the same nitrogen atom. It has extremely similar construction with the sulfonylureas.

Based on the similarity of structure between sulfonylureas and *N*-nitrourea compounds, we suppose the *N*-nitrourea compounds have the same targeted enzyme with the sulfonylureas. In order to develop more selective and potent *N*-nitrourea herbicides, in the present study we verified our hypothesis combining by molecular docking and biological activity assay. Subsequently, we analyzed the interaction mechanism between the *N*-nitrourea lead compounds and the active site of AHAS target by molecular docking analysis, and then *N*-nitrourea lead compounds optimization work was performed, and an optimized structure backbone with potential high inhibitory activity compounds was designed for further research.

2. Materials and methods

2.1. Preparation of N-nitrourea compounds

The *N*-nitrourea compounds selected for molecular docking and biological testing study were shown in Table 1, and they were different types of skeleton structure and all came from our laboratory reported previously in literature [13–15]. The *N*-nitrourea compounds were selected out by jointly using the structural diversity, chemical and physical character.

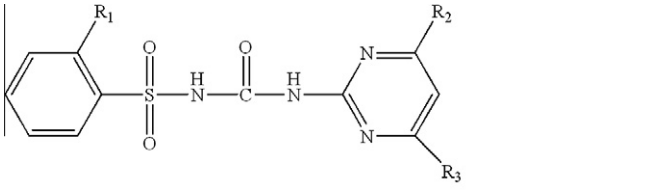

Fig. 1. The basic structure of sulfonylureas compound.

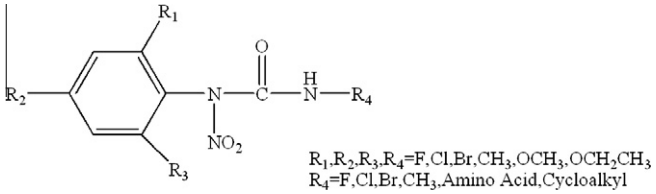

Fig. 2. The basic structure of *N*-nitrourea compound.

2.2. Molecular docking analysis

Based on the similarity of structure between sulfonylureas and *N*-nitroureas, we supposed the *N*-nitroureas have the same target enzyme with the sulfonylureas. In order to prove our hypothesis, we performed molecular modeling analysis. All the selected synthesis *N*-nitrourea compounds were docked into the active site of *At*AHAS to investigate the interaction mechanism between the ligand and target by FlexX docking methods [16–18]. The FlexX is a fast and automated docking program, which takes ligand's conformational flexibility into account during the docking process by an incremental fragment placing technique. The base fragment (the ligand core) is automatically selected and is placed into the active site using a new algorithmic approach based on pattern recognition technique called pose clustering [19–21]. Next the remainder of the ligand is built up incrementally form other fragments. The conformational flexibility of the ligand is included by generating multiple conformations for each fragment and including all in the ligand building steps. Placement of the ligand is scored on the basis of protein-ligand interactions (pairwise assignments of interaction geometries), as shape alone is a weak descriptor, especially for small or flexible ligands. Finally, the binding energy is estimated, and placements are ranked. The scoring function used to rank the solutions is an estimate of the free binding energy ΔG of the protein-ligand complex [22]. The equation used is basically the ΔG for an ideal hbond, ionic, aromatic, or lipophilic interaction, adjusted by a penalty that depends on deviation from the ideal interatomic radius for the two interacting elements.

The active site for molecular docking analysis was defined as follows: all atoms located within the range of 6.5 Å from any atom of the sulfonylureas herbicide methyl 2-{[(4-methylpyrimidin-2-yl)carbamoyl]sulfamoyl}benzoate (SM) in the X-ray crystallographic structure of *At*AHAS (PDB id: 3EA4) were selected into the so-called active site, and the amino acid residue was, therefore, involved into the active site if at least one of its atoms was selected. The docking process was performed as follow: The docking ligand was set as mol2 file type and assigned Gasteiger–Hückel charge, the input options for 3D coordinate generation of ligand was set as "If Necessary", formal charge assignment was set as "If Necessary", add hydrogens was set as "Use Existing", test atom typing was set as "No Testing (Assign Atom if Necessary)", assign delocalized types was set as "Use Existing". The output options for maximum number of poses per ligand was set as "30", output format was set as "SYBYL Database". Performing CScore calculations, the CScore execution mode was set as "Parallel" model.

Based on the binding mode of known ligands in the active site, and the validated molecular docking process for the specific *At*AHAS system, the representative candidate *N*-nitrourea compounds were screened out for further bioaffinity testing on enzyme level.

2.3. AHAS extraction and purity

The maize plant grows fast, and there is high abundance of ALS enzyme in the maize seedling. At the same time, the amino acid sequence analysis showed that there is high homology of ALS enzyme from maize and *A. thaliana*, respectively. So, to obtain AHAS enzymes, we extracted it from 3-d-old maize plant which was cultivated in constant light at 26 °C after germination. The