

Novel Artificial Intelligence platform leads to the discovery of new protoporphyrinogen oxidase inhibitors

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Abstract

Weed control is essential in modern agriculture, though it has become more difficult with the emergence of resistance to most current herbicides and a slow registration process of new compounds. A new approach to identify possible herbicide candidates using an artificial intelligence algorithm that takes into effect biological parameters with the goal of reducing R&D time on new herbicides. Herein we describe the discovery of 4-chloro-2-pentenamides as novel inhibitors of protoporphyrinogen oxidase, a known herbicide target site, by the Agrematch AI. The herbicidal activity is confirmed in greenhouse assays, with the highest performing AGR001 showing good activity pre-emergent at 150 g/ha and post emergent as low as 50 g/ha on the troublesome weed palmer amaranth (*Amaranthus palmeri*). A lack of activity is reported on PPO resistant palmer amaranth carrying the glycine 210 deletion (Δ G210) mutation. The mechanism of action is confirmed by the herbicide-dependent accumulation of protoporphyrin IX, subsequent light-dependent loss of membrane integrity, and direct in vitro inhibition of protoporphyrinogen oxidase. Modeling of the docking of these inhibitors in the active site of protoporphyrinogen oxidase illustrates that their flexible side chains can accommodate a number of poses in the catalytic domain.

Introduction

Current agriculture faces new struggles against unpredictable weather pattern changes associated with climate change and managing a myriad of pests that impact crop productivity. Weeds are the most problematic pests because these undesirable plants reduce crop yields by competing for space and resources (light, nutrients, water) and cause billions of dollars in annual loss.^{1,2} Consequently, modern agricultural practices rely heavily on the use of synthetic herbicides. While herbicides have been a major contributor to the success of agriculture, they have imposed a strong selection pressure over millions of farming acres, leading to the evolution of herbicide resistance in hundreds of weed species.³ Both target-site resistance (TSR) and nontarget-site resistance (NTSR) mechanisms have evolved to most herbicide classes.⁴

Nearly three decades of silence in respect to new herbicide mechanisms of action (MoA) have left farmers in great need of new, effective tools. New weed management methods are being developed to address the current weed resistance problems, including non-chemical approaches,^{5,6} but chemical tools remain the most cost effective and no-tillage friendly.⁷ Recently, there has been a flurry of reports of new modes of action to break this silence.⁸ Most of these reports are from research groups within the AgChem industry. However, several startup companies have also joined the fray with novel approaches to the discovery of interesting chemistry and new mechanisms of action.

Understanding the process by which companies discover new herbicides grants insight into the lack of new herbicide options. There are five main steps to the development of a crop protection agent, which take on average 11 years of early research:⁹ discovery when a molecule is identified through some pipeline and its MoA and toxicity are investigated; optimization, when the molecule is taken through

formulation improvements and greenhouse testing against crops and weeds; field trials, when this testing is taken to realistic conditions and large scale safety studies; development, which includes regulatory hurdles and registration of the commercial product; and finally launch, when the product is released and stewarded by the company. Due to tightening regulations on target site knowledge and lower toxicity acceptance companies are screening upwards of 160,000 molecules to find one possible target.⁹ Large AgChem companies differ from startup companies (e.g., Agrematch, MoA Technology and Enko). AgChem companies still rely mostly on large scale, automated screening processes utilizing available molecule libraries. On the other hand, startup companies have developed innovative approaches to screen extremely large numbers of compounds and methods to guide them to new MoA.

Agrematch is a unique data-science product discovery and development company catering to industries that require novel compounds for their products, initially focused on Agriculture and food industries. Agrematch's utilizes compound-based data analytics to predict early and downstream considerations which are necessary for successful product launch.

Agresense, the Agrematch artificial intelligence (AI) algorithm system has been developed for rational identification of molecules with desired compound-organism interaction. This multi-layered system utilizes advanced artificial intelligence (AI) and machine learning (ML)/Deep Learning (DL) algorithms and other data science concepts combined with biology, chemistry, and agriculture know-how to provide insights that help make informed decisions along the product development journey. A key use case of Agresense is the discovery and characterization of biologically active (bioactive) compounds, such as crop protection and crop enhancement products by screening the vast chemical universe for the desired functionality.

We report in this paper a new chemical class of herbicide that was discovered using the artificial intelligence algorithm developed by Agrematch. These new 4-chloro-2-pentenamides are herein confirmed to act by inhibiting protoporphyrinogen oxidase (PPO), a key enzyme in porphyrin biosynthesis.¹⁰

Results And Discussion

Discovery of novel structures

The iterative process of Agresense resulted in a family of compounds, AGR001-AGR014 with herbicidal activity described in this paper (Supplemental Table 1). The log P values calculated by SwissADME¹¹ are on the higher end of suggested values for biological relevance in regard to herbicide uptake, as the "Briggs rule" suggests ideal values are less than 3. Surprisingly, the classifier algorithm predicted these compounds to belong to Herbicide Resistance Action Committee (HRAC) group 14 (inhibitors of PPO), even though classical similarity methods showed very low similarity scores to the structure of known group 14 herbicide (Fig. 1a). PPO inhibitors that have been registered to date share some structural similarities to the substrate, protoporphyrinogen (protogen). If the registered classes of PPO inhibitors, the diphenyl ethers, *n*-phenyl-oxadiazolones, pyraflufen-ethyl and some members of the *n*-phenyl imides and

n-phenyl-triazolinones have a two-ring structure which mimics one half of protogen. The others, pyraclonil and the rest of the *n*-phenyl imides and *n*-phenyl-triazolinones have three ring structures that still roughly resemble one half to three fourths of a protogen molecule (HRAC). Half of the structures reported in this paper do contain a single cyclic group. The usefulness of PPO inhibitors is evident in the extensive research using computational approaches to discover new chemical classes targeting this enzyme.^{12–14}

Herbicidal activity

Pre-emergent applications were tested for all 14 potential compounds, post-emergent applications were tested for the top 8 compounds. Pre-emergent activity was investigated in Palmer amaranth (*Amaranthus palmeri*), post emergent activity was investigated in *Xanthium strumarium*, *Datura ferox*, *Solanum nigrum*, *Amaranthus blitoides*, and Palmer amaranth. Post-emergent activity of AGR001 in net-house conditions is illustrated in Fig. 1b. Despite borderline log P values, these compounds were herbicidal. Most tested compounds, other than AGR012 and AGR014 had some pre-emergent activity, with AGR001, AGR002 and AGR009 providing good control at lower rates (Table 1). Each of the 8 compounds tested post-emergent had good activity, though AGR001, AGR004 and AGR005 were the most active (Table 2). Some of the compounds caused photobleaching, which was consistent with predicted activity as PPO inhibitors. AGR001 and AGR002 were selected as representative compounds of the structures with herbicidal activity to determine MoA.

Table 1
Herbicide activity at pre-emergence application on Palmer
amaranth in percent control

Compounds	Pre-emergence application (g/ha)		
	30	150	300
AGR001	40	90	100
AGR002	20	90	100
AGR003	-	-	100
AGR004	-	-	80
AGR005	-	-	70
AGR006	-	-	100
AGR007	-	-	80
AGR008	-	-	80
AGR009	30	80	85
AGR010	30	30	90
AGR011	0	20	90
AGR012	10	10	0
AGR013	-	-	40
AGR014	-	-	0

Table 2
Herbicide activity at post-emergence application on
Palmer amaranth expressed in percent control

Compounds	Post-emergence application (g/ha)				
	25	50	75	100	250
AGR001	90	95	-	100	-
AGR002	-	30	-	30	50
AGR003	-	-	-	100	100
AGR004	-	100	100	-	-
AGR005	-	90	100	-	-
AGR007	-	-	-	-	80
AGR008	-	-	-	-	95
AGR010	-	-	-	-	50

Mechanism of action studies

PPO inhibitors target the last common enzyme in the synthesis of heme and chlorophyll, a branch point in the tetrapyrrole pathway.¹⁵ This causes a buildup of the substrate, protoporphyrinogen, which leaks from the plastids to the cytoplasm and is oxidized into the product of the protein, protoporphyrin IX (proto). Proto is a photodynamic red pigment which generates reactive oxygen species (ROS) upon exposure to light. These ROS cause lipid peroxidation of the cell membranes leading to cell, and thereby plant death.¹⁵ The activity of PPO inhibitors is quantifiable in plant tissue through light-dependent bleaching of tissue, light-dependent electrolyte leakage from membrane disruption, and accumulation of proto after application. Activity can also be observed *in vitro* through an enzyme assay.

Initial electrolyte leakage assays were performed in cucumber cotyledons. Both AGR001 and AGR002 treatments at 100 μM showed the same increase in conductivity due to cell damage after exposure to light as has been reported previous with PPO inhibitors (Fig. 2a).¹⁶ The second electrolyte leakage assay in cucumber cotyledons was to determine the efficacy of lower doses, showing that AGR001 is approximately sixteen times more active than AGR002, with I_{50} values of $1.8 \pm 0.1 \mu\text{M}$ and $30.0 \pm 1.1 \mu\text{M}$, respectively (Fig. 2b). The bleaching observed in the leaf disks are typical of herbicides inhibiting PPO (Fig. 2c).

Additional experiments measuring proto levels in plant tissues exposed to 300 μM of selected compounds were performed. The accumulation of proto in treated tissue and not the control further supports inhibition of PPO as the MoA (Fig. 3).

A third electrolyte leakage assay was performed on leaf disks from PPO-resistant¹⁷ and wildtype Palmer populations (Fig. 4). The PPO-resistant line was not sensitive to either compound, whereas the susceptible line responded to the treatments with a marked increase in electrolyte leakage.

Finally, the MoA of these compounds was confirmed by directly testing on purified PPO enzyme extracted from recombinant wild-type *Amaranthus tuberculatus* PPO2 heterologously expressed in *E. coli* (Fig. 5). Dose-response curves were obtained for AGR001 and AGR002 at concentrations ranging from 0.3 to 333 μM . Inhibition of PPO activity by AGR001 and AGR002 was lower than most commercial PPO inhibitors, requiring micromolar concentrations to inhibit 50% of PPO enzyme activity, with I_{50} values of 1.84 ± 0.11 and 9.42 ± 1.37 μM , respectively. Most commercial PPO-inhibiting herbicides have I_{50} values in the submicromolar range.^{18,19} Nonetheless, the *in vitro* activity of AGR001 and AGR002 (Fig. 5) paralleled their ability to induce lipid peroxidation and loss of membrane integrity (Figs. 2b and c)

The predictive ability of the Agresense artificial intelligence algorithm system for rational identification of the mechanism of action of novel molecules was confirmed *post facto* following extensive literature searches that identified earlier papers describing the herbicidal activity of related alkenamide compounds.^{20,21} The activity of these compounds was light-dependent and was later on associated with inhibition of PPO.^{22,23}

Docking study

Docking studies were performed to further understand the interaction between AGR001 and AGR002 with the catalytic domain of PPO. The published binding energy of protogen and proto are -10.6 and -7.3 kcal mol^{-1} , respectively.²⁴ The average docking energies for AGR001 and AGR002 were -4.7 and -3.1 kcal mol^{-1} , respectively. These values are lower than the binding energies of other common PPO inhibitors, which range from -8.8 to -5.3 kcal mol^{-1} , indicating a less effective binding to the active site which is reflected in the higher I_{50} values reported above. AGR001 and AGR002 are smaller and structurally different from all commercial PPO-inhibiting herbicides. 4-Chloro-2-pentenamides have very flexible side chains and can hold several different conformations in the active site (Supplemental Fig. 2), whereas most PPO inhibitors are more rigid multicyclic molecules that occupy a more limited number of poses and are stabilized by interactions with highly conserved residues (e.g., Arg98, Phe353 and Leu356) known to interact with the substrate in the catalytic domain (Fig. 6). Most of the interactions involved hydrophobic interactions between the ligand and the surrounding residues. A few poses also involved hydrogen bonding. This ability of 4-chloro-2-pentenamides to assume different poses within the catalytic domain indicates an increase in entropic energy compared to other PPO inhibitors and may account for the low binding energies of this chemical class.

In conclusion, the discovery of new herbicides is critical to sustain crop productivity around the globe. Agrematch has introduced a transformative approach to explore chemical spaces for bioactive compound discovery. Their artificial intelligence system utilizes large data sets and advanced machine and deep learning algorithms to identify and then biologically validate leads with new modes of action.

This innovative approach vastly expands the reach into the chemical space that will lead to the discovery of much needed new products. The 4-chloro-2-pentenamides identified as putative PPO inhibitors by the Agresense AI algorithm have both pre- and post-emergent herbicidal activity on multiple weeds. Despite the lack of structural similarity with known PPO inhibitors, these compounds indeed acted by inhibiting PPO, causing the expected light-dependent loss of membrane integrity, photobleaching, accumulation of proto and inhibition of PPO. While some weeds have evolved resistance to certain PPO-inhibiting chemistry, group 14 herbicides remain an important group of chemicals to manage weeds,^{26–28} and many major agrochemical companies are developing new molecules with this mode of action.^{29,30} The Agresense algorithm continues to identify novel chemical spaces with potentially new MoAs.

Materials And Methods

Discovery of novel structures (Agrematch innovative approach)

The Agresense model generation platform enables specific functional modules to be developed by training with relevant positive and negative datasets. In most cases, it takes multiple training iterations that sometimes require new data generation to create a robust predictive module. Once trained, the predictive module can be used for the desired application where every use contributes to the accuracy of the prediction. Each model has different characteristics which depend on the complexity of the question and the domain it searches; each model run may take from milliseconds with a single computer to days with thousands of CPUs activated in the cloud.

A database of ~ 1.2B compounds was screened to create a library of 50 compounds, predicted by Agresense (Agrematch Artificial Intelligence system) to have herbicidal activity, based on a unique proprietary molecular representation of the screened compounds. The iterative process included testing the compounds identified by Agresense for their herbicidal activity in the lab and feeding back the results into the Agresense system to optimize it and to create additional iterations of compound libraries. These compounds were then analyzed by Agrematch's MoA classifier algorithm to predict whether these compounds have a new MoA or belong to an existing HRAC class.

Plant material and growth

For herbicidal activity assays, Palmer amaranth (*Amaranthus palmeri*), *Xanthium strumarium*, *Datura ferox*, *Solanum nigrum* and *Amaranthus blitoides* were planted in pots with a potting mix and grown in either growth-chamber or Net-house. All plants were watered as needed.

Cucumber seedlings (cultivar Straight eight) were planted in trays with promix and grown in the greenhouse for up to 3 weeks to collect fresh cotyledons. These plants were discarded once the 2 true-leaf started to emerge. Palmer amaranth plants that were either sensitive (S) or resistant (R) to PPO inhibitors due to a glycine210 deletion^{31–33} were grown in the greenhouse for 4 weeks under similar

conditions. All plants were watered as needed. The use of plants in the present study complied with international, national and/or institutional guidelines.

Herbicidal activity

For pre-emergence assays, compounds were dissolved in DMSO and diluted in water to a final concentration of 10–100 mg/L with 2% DMSO. Compounds were sprayed using a VL-SET Paasche Airbrush at a 3,000 L/ha application volume.

For post-emergence assays, compounds were dissolved in DMSO and diluted in water to a final concentration of 25–250 mg/L with 2% DMSO. Break-Thru® S-240 (EVONIK) at a final concentration of 0.05% was added. Compounds were sprayed using a VL-SET Paasche Airbrush at a 1,000 L/ha application volume. Weeds were sprayed at the 4–6 leaf stage.

For post-emergence net-house assay, 300 mg/L of compound AGR001 was dissolved with 900 mg/L xylenes, 73 mg/L ethoxylated castor oil (Kolliphor® RH 40) and 48 mg/L calcium dodecylbenzene sulfonate (Rhodacal® 60be) in water. Break-Thru® S-240 (EVONIK) at a final concentration of 0.05% was added. Weeds were sprayed using a VL-SET Paasche Airbrush at a 1,000 L/ha application volume at the 4–6 leaf stage (2-leaf stage for *D. ferox*).

Herbicidal activity was assessed and scored 7 days after application by visual inspection of the weeds in comparison to untreated controls. Activity score was in the range of 0 to 100, where 0 represents no herbicidal activity like control weeds and 100 represents the maximal herbicidal activity (i.e., total death of the weed).

Electrolyte leakage

First, time-course experiments were conducted over 40 h to measure the effect of AGR001 or AGR002 on electrolyte leakage from cucumber cotyledons using a modified method of Dayan and Watson.³⁴ For each compound, 36 discs (6 mm diam.) were cut from 7 to 15 day-old cucumber cotyledons and placed in a petri dish. The discs were floating over 5 mL of MES buffer (pH 6.5) with 2% sucrose with 100 µg/mL of either AGR001 or AGR002. This was done in low light intensity to prevent photodynamic damage.

Once the plates were prepared, the initial conductivity (a measure of electrolyte leakage) was measured using a FiveEasy Plus FP30 conductivity meter connected to an InLab 751-4mm microprobe (Mettler Toledo, Columbus, OH 43240). The plates were then kept in the dark at room temp for 16 h. Conductivity was measured after the dark incubation period and then the plates were moved into an LED-30L1 LED high intensity growth chamber (Percival, Perry Iowa 50220). Conductivity was measured 1, 5, 10, 24 h after exposure to light intensity (approx. 1050 µmol/m/s).

Second, dose-response curve experiments were conducted with AGR001 and AGR002 at 1, 3, 10, 30, 100 and 300 µM. Control treatment consisted of DMSO alone to determine the relative potency of these molecules. Conductivity was measured as described above after 16 h dark incubation followed by 24 h exposure to high light intensity.

Third, the effect of AGR001 and AGR002 was tested on biotypes of S and R Palmer amaranth plants. For this experiment, 60 leaf discs from 4-week old Palmer amaranth were used. Concentrations used were 10 μ M AGR001 and 100 μ M AGR002, conductivity was compared to controls with DMSO alone.

Protoporphyrin IX accumulation

The effects of AGR001 and AGR002 on protoporphyrin IX (proto) levels were measured in cucumber cotyledons exposed to 300 μ M of either compound and compared to DMSO control after 16 h dark incubation. Proto extraction and analysis followed a protocol described by Dayan et al.³⁵ Approximately 0.2 g of cotyledonary tissue was ground to a powder in liquid nitrogen and homogenized in 2 mL of extraction solvent (methanol:0.1 M NH_4OH , 9:1) and centrifuged at $10,000 \times g$ for 15 min. The supernatant was saved and the pellet rehomogenized in 1 mL of extraction solvent, then centrifuged again at $10,000 \times g$ for 15 min. Supernatants were pooled and then filtered through a 0.2- μ m nylon syringe membrane filter before quantification with the LC-MS/MS system. Proto was separated in a biphenyl column (100 by 4.6 mm, 2.6 μ m, 40 C) at a flow rate of 0.4 mL min^{-1} using a linear gradient of methanol (B) and 10 mM ammonium acetate (A): 0 min, 50% B; 8min, 70% B; 11 min, 90% B; 13 min, 90% B; 13.5 min, 50% B; 17 min, 50% B. The MRM was optimized to 340.10 > 227.95.³⁶ A standard curve generated with serial dilutions of authentic protoporphyrin IX (MilliporeSigma, St. Louis, MO) was used for quantification. Limit of detection (LOD) and limit of quantification (LOQ) for proto were 0.05 ng/ μ L and 0.15 ng/ μ L.

Protoporphyrinogen oxidase activity

Protoporphyrinogen oxidase was obtained by expression and purification of the *Amaranthus tuberculatus* wild type isoform as described by Dayan et al.³² Briefly, the cell line was cultured overnight at 37 C in 250 mL of LB with ampicillin, which was diluted into 1 L of LB with antibiotic and grown for 1 h before induction with 1 mM IPTG. After induction the culture was grown at 25 C for 5 h. Cells were harvested by centrifugation at 2,000 $\times g$ and washed with 0.1% NaCl. Cells were lysed by sonication (Model 120 Sonic Dismembrator with a Model CL-18 1/8 inch probe, Thermo Fisher Scientific, Waltham, MA, USA) in 3 x 30 s bursts with 60 s on ice in between in 50 mM sodium phosphate pH 7.5, 500 mM NaCl, 5 mM imidazole, 5% glycerol and 1 μ g/mL leupeptin. After lysis 1U of benzonase (Millipore Sigma, Burlington, MA, USA) and 1 mM PMSF were added. Debris were removed by centrifugation for 30 min at 2000 $\times g$. Proteins were purified on a HisPur Ni-NTA Spin Column (Thermo Fisher Scientific, Waltham, MA, USA) as per the instructions with elution at 20 mM sodium phosphate, 300 mM sodium chloride 250 mM imidazole, pH 7.4. Protein was desalted on a PD-10 column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) equilibrated with 20 mM sodium phosphate, pH 7.5, 5 mM MgCl_2 , 1 mM EDTA and 17% glycerol. Pure PPO which was stored at -80 C until use.

Protoporphyrinogen was prepared by reducing proto with sodium amalgam as described by Jacobs and Jacobs.³⁷ Assays were conducted with 20 μ g of protein per replicate as described by Dayan et al.³² with specific modifications for our spectrophotometer as follows: enzymatic activity was measured on an spectrofluorometer (Synergy H1, Agilent Technologies, Wilmington, DE USA). Excitation and emission

wavelengths were set to 395 and 633 nm, respectively. The assay was carried in black microplates (Costar 3915) out under kinetics conditions over 10 min. Enzyme activity was measured based on the linear portion of the curve. Proto amounts were calculated based on a calibration curve of proto standard (Sigma-Aldrich, Inc., St. Louis, MO 68178) at concentrations ranging from 8 nM to 2 μ M (Supplemental Fig. 1). AGR001 and AGR002 were tested at concentrations ranging from 0.3 to 333 μ M and compared to untreated control that received the same volume of solvent. Statistical analysis was performed using the R software (v 4.1.0). Dose responses were fit using the DRC package.³⁸ Regression curves were imported into Prism 9.1.1 (GraphPad, San Diego, CA 92103).

Docking study

All herbicide structures were downloaded as 3-D.sdf files from PubChem³⁹. The two experimental compounds were build using a molecular modelling and computational chemistry application (Spartan18, Wavefunction, Inc. Irvine, CA 92612). The bond angles and length were corrected, and the atom energies were calculated by submitting all the molecules to geometric minimization using density function theory calculations (wb97X-D 6–31*). The optimized structures were saved as mol2 files along with their electrostatic charges.

The crystal structure of spinach PPO was obtained from 1sez.⁴⁰ Prior to use for docking studies, the pdb file was modified to replace the seleniomethionine residues with methionine residues. Also, the atom types of the FAD cofactor were corrected, and the ligand was converted to its oxidized form using Spartan18.

All the herbicides were docked into the catalytic domain of PPO using a Autodock (AutoDock version 4.2, Scripps Institute, San Diego CA, USA).^{41,42} Additionally, the guanidino group of arginine 98 (atom id = 3782) was designated as important in the interaction between one of the propionate groups (coordinates of this proton are x = -43.390, y = -1.054 and z = 31.750). A grid box was used to delimitate the region of the catalytic domain according to the software. The gridbox dimensions were set to 38 $\times$ 34 $\times$ 34 points with a spacing to 0.375. The box was centered on the following coordinates: x = -40, y = -6, and z = 29. PPO was set as a rigid structure. The algorithm was set to generate 100 docking poses and the top clustered was selected as optimal conformation for the docking of each ligand. Interactions between the ligand and the catalytic domain of PPO were identified using the protein–ligand interaction profiler (PLIP).²⁵

Declarations

Author Contributions: All authors contributed substantially to the writing of this article and have reviewed it. ALB and FED carried out all the experiments related to the mode of action. YG, ES, NS, YP and IS conducted the AI discovery work as well as the initial greenhouse studies.

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Figures

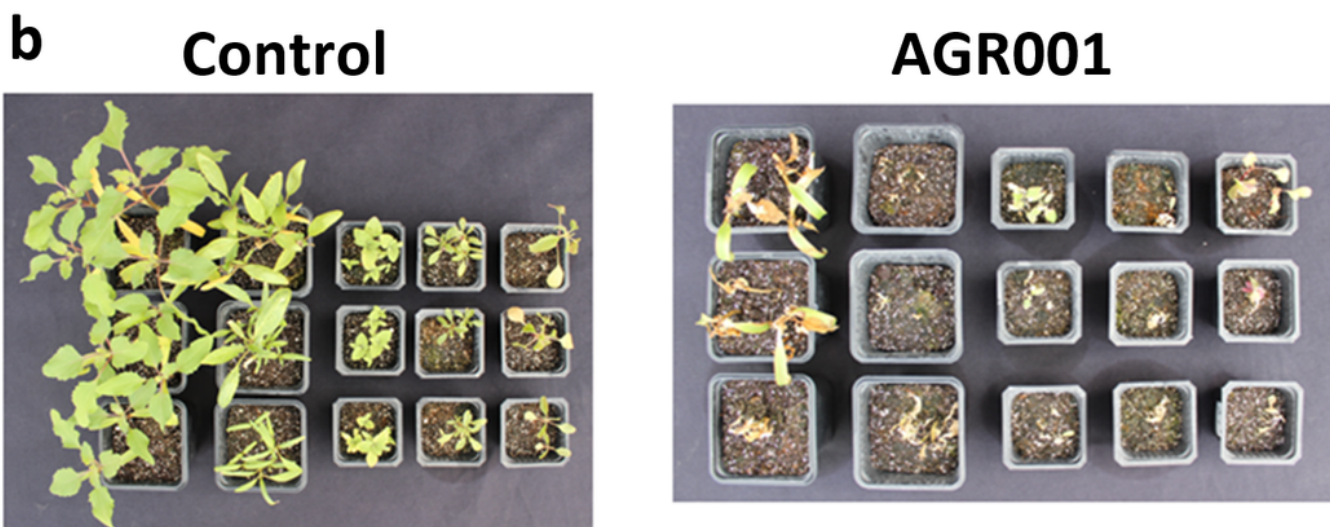
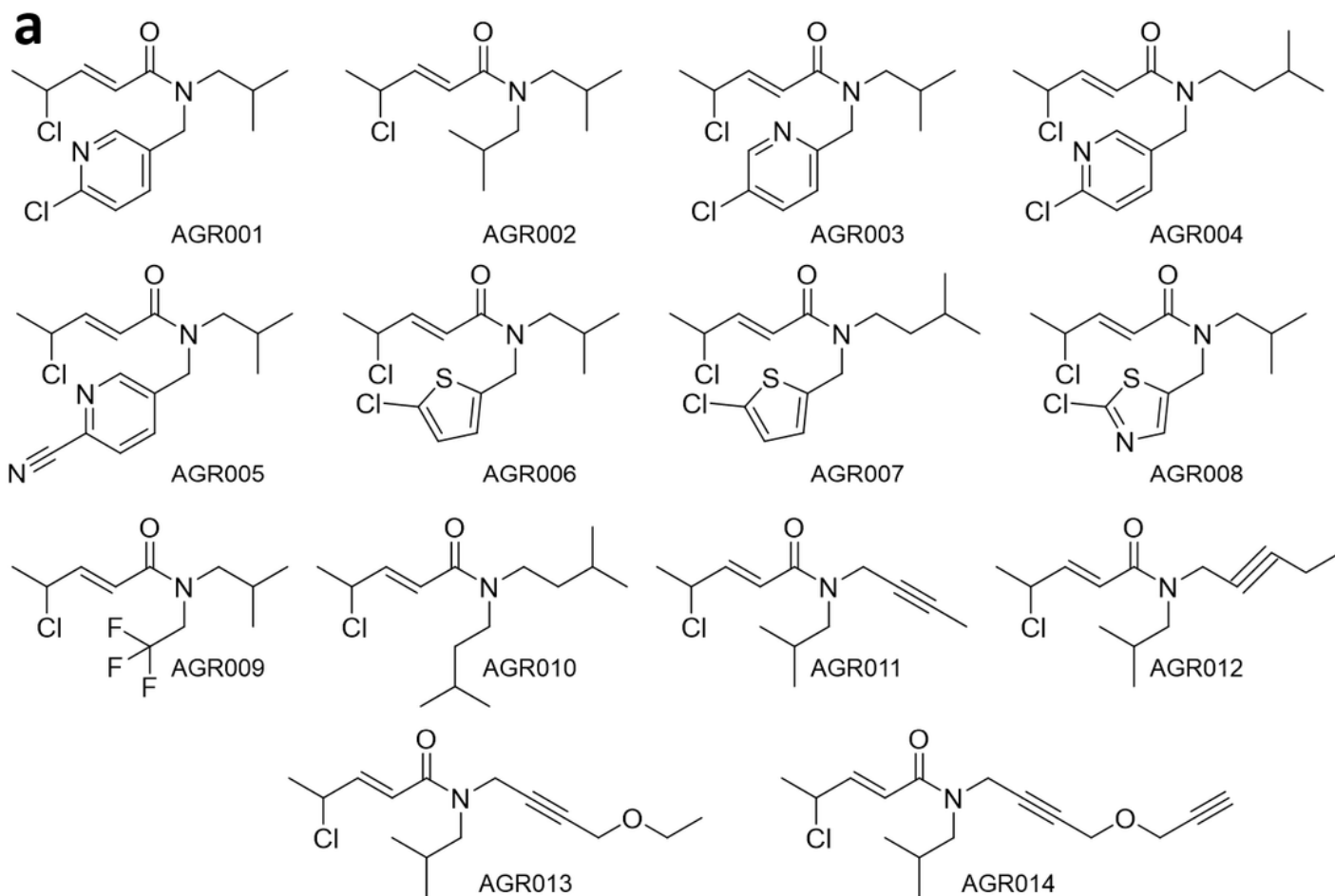


Figure 1

a) Structure of the novel 4-halo-2-pentenamides described in this paper; **b)** Herbicidal activity of AGR001 in post-emergence Net-house assay, 7 days after application of 300 g/ha. From left to right: *Xanthium strumarium*, *Datura ferox*, *Solanum nigrum*, *Amaranthus blitoides*, *Amaranthus palmeri*.

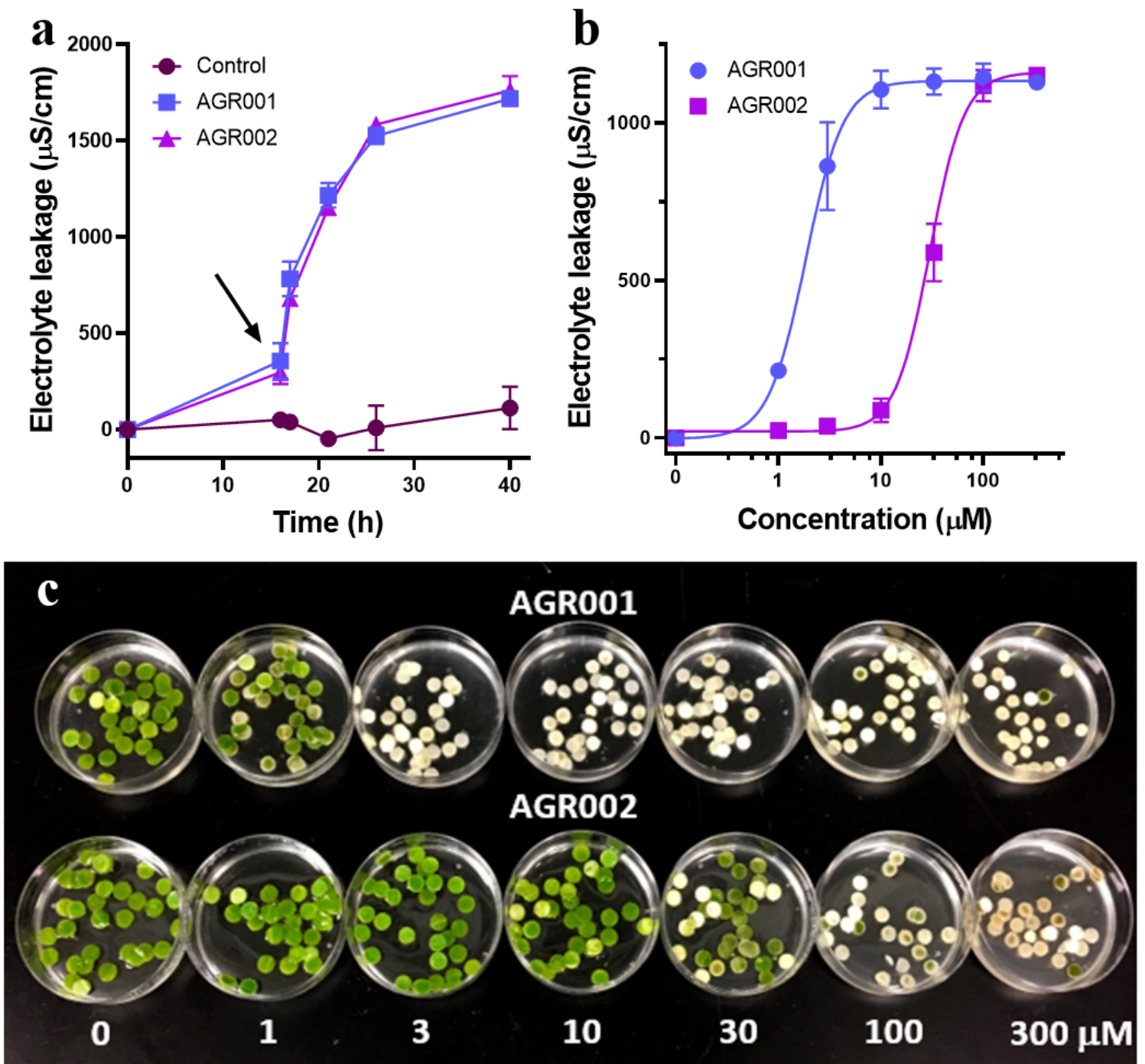


Figure 2

Electrolyte leakage experiments in cucumber cotyledons. **a)** Electrolyte leakage caused by 100 $\mu\text{g}/\text{mL}$ AGR001 (light blue) or AGR002 (pink) over 40 h. The arrow indicates when the plates started exposure to high light intensity (approx. 1400 $\mu\text{mol}/\text{m}^2/\text{s}$); **b)** Dose-response curves with AGR001 (light blue) and AGR002 (pink) following 15 h dark incubation and 11 h of exposure to high light intensity; **c)** Bleaching of tissues at the end of the dose-response curve experiments.

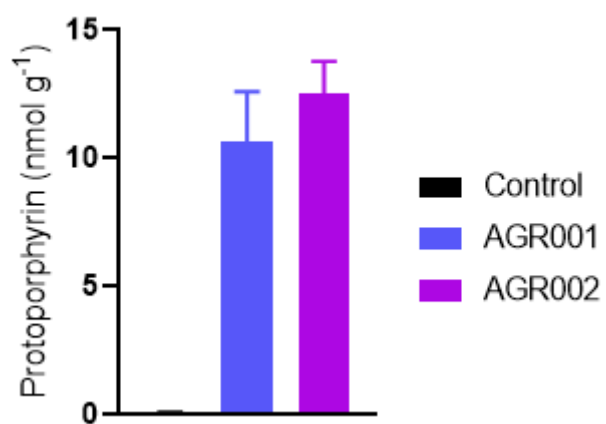


Figure 3

Accumulation of proto in cucumber cotyledons after 24 h exposure to 300 μ M AGR001 (light blue) or AGR002 (purple) in darkness, relative to DMSO control.

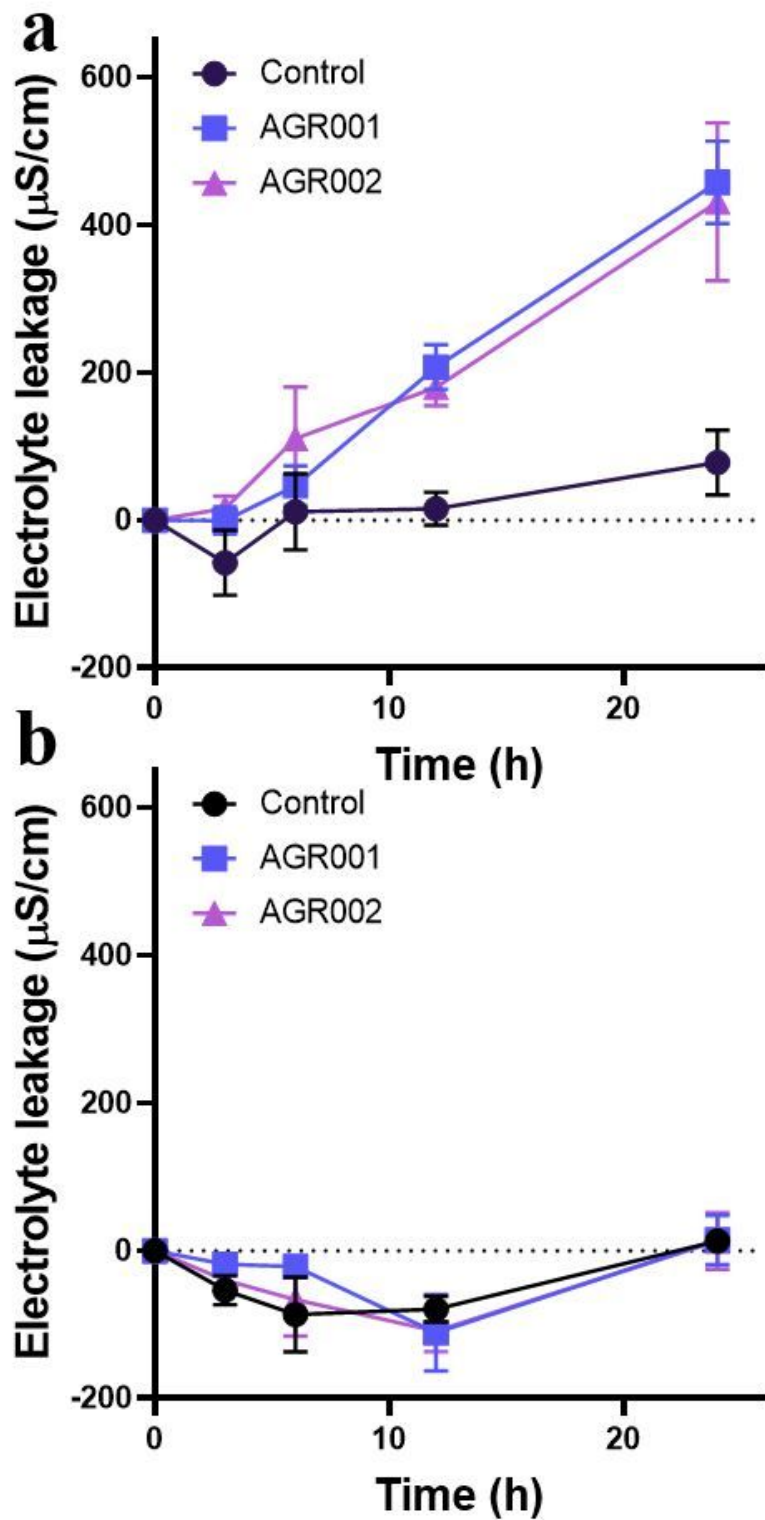


Figure 4

Electrolyte leakage caused by 10 μM AGR001 (light blue) and 100 μM AGR002 (purple) after 15 h dark incubation and subsequent exposure to high light intensity over 24 h on **a)** susceptible and **b)** PPO-resistant Palmer amaranth.

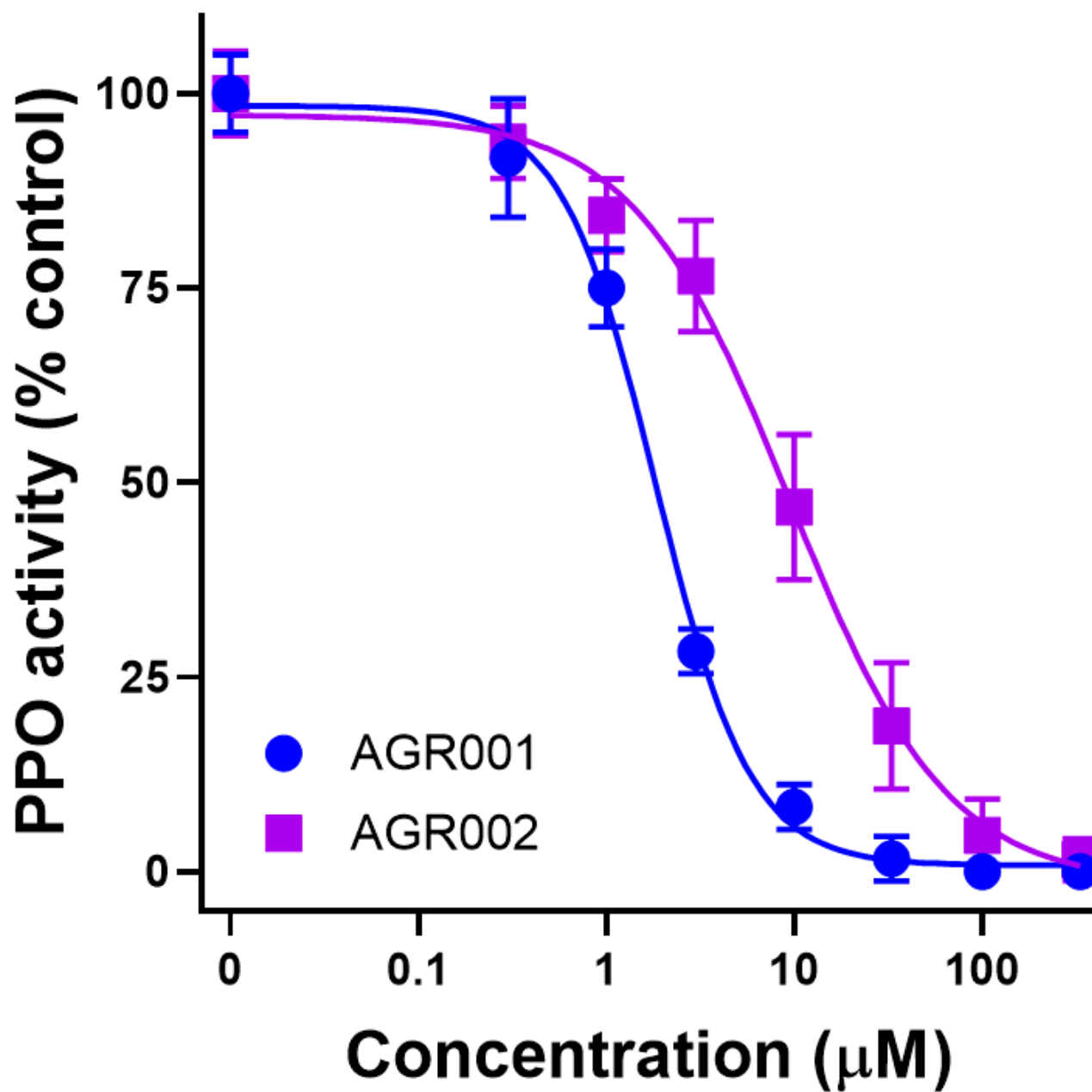


Figure 5

Inhibition of PPO activity by novel 4-chloro-2-pentenamides in in vitro assays using heterologously expressed enzyme. IC_{50} values for AGR001 and AGR002 were 1.84 ± 0.11 and 9.42 ± 1.37 μ M, respectively.

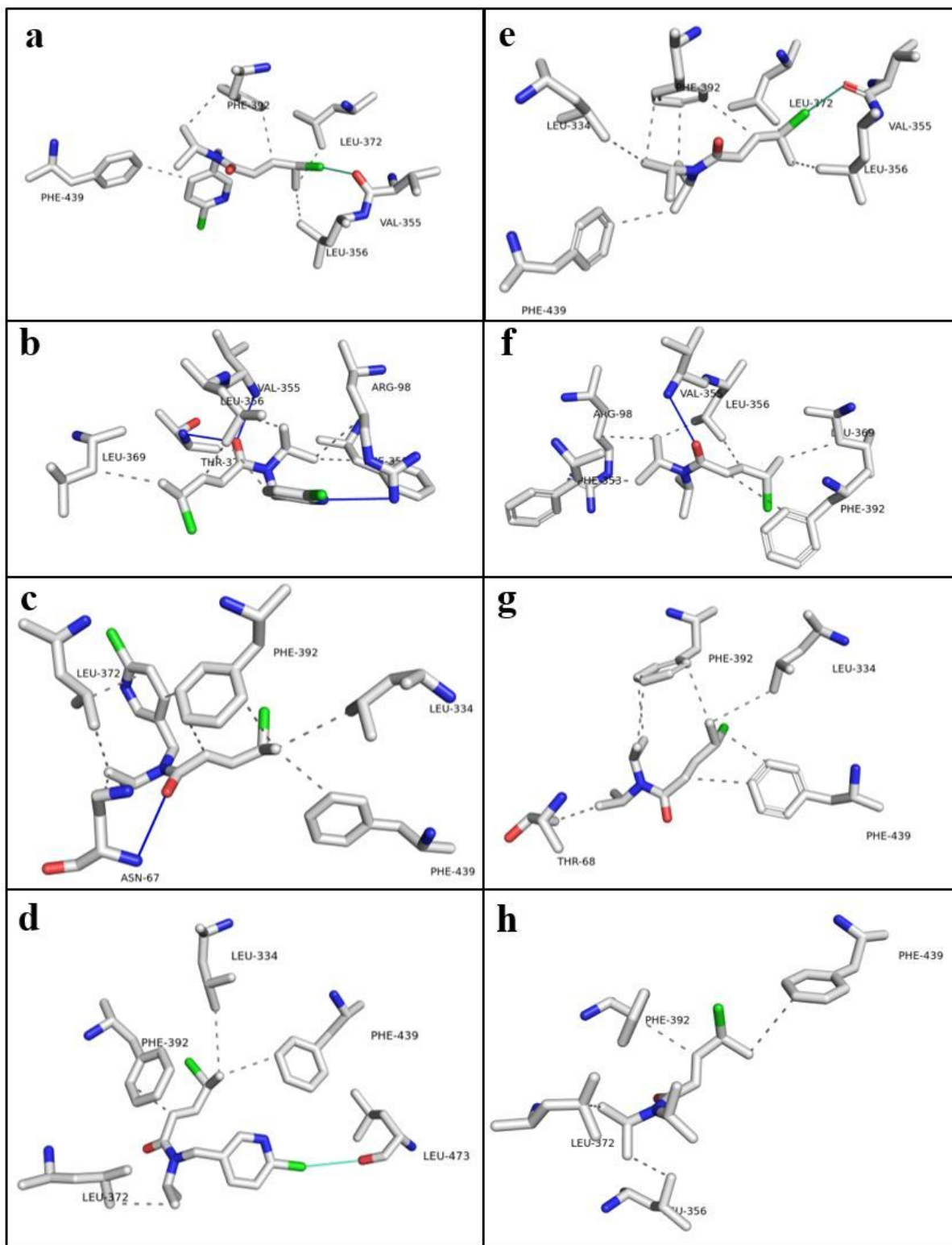


Figure 6

Representative docking of AGR001 poses **a)** group 1, **b)** group 2, **c)** group 3, and **d)** group 4 of and AGR002 poses **e)** group 1, **f)** group 2, **g)** group 3, and **h)** group 4 of AGR002 on PPO. Refer to Table 1 for number of poses in each group and average docking energies. Interactions were obtained using the protein–ligand interaction profiler (PLIP).²⁵ Gray dotted line = hydrophobic interaction; blue solid line = hydrogen bond; green solid line = halogen interaction.

Supplementary Files

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