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Design, Synthesis, and Herbicidal Activity of *N*-Benzyl-5-cyclopropyl-isoxazole-4-carboxamides

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ABSTRACT: Based on the structures of isoxaflutole (IFT) and *N*-isobutyl-*N*-(4-chloro-benzyl)-4-chloro-2-pentenamide, a series of *N*-benzyl-5-cyclopropyl-isoxazole-4-carboxamides was designed by connecting their pharmacophores (*i.e.*, a multitarget drug design strategy). A total of 27 *N*-benzyl-5-cyclopropyl-isoxazole-4-carboxamides were prepared from 5-cyclopropylisoxazole-4-carboxylic acid and substituted benzylamines, and their structures were confirmed by NMR and MS. Laboratory bioassays indicated that **I-26** showed 100% inhibition against *Portulaca oleracea* and *Abutilon theophrasti* at a concentration of 10 mg/L, better than the positive control butachlor (50% inhibition for both weeds). A strong growth inhibition was observed, but a typical bleaching phenomenon of IFT could not be observed in the Petri dish assay. **I-05** displayed excellent postemergence herbicidal activity against *Echinochloa crusgalli* and *A. theophrasti* at a rate of 150 g/ha, and bleaching symptoms were observed in the leaves of treated weeds. The bleaching effect of *Chlamydomonas reinhardtii* treated by **I-05** could be reversed by adding homogentisate. Enzymatic bioassays indicated that **I-05** could not inhibit 4-hydroxyphenylpyruvate dioxygenase (HPPD) activity, but **II-05**, an isoxazole ring-opening product of **I-05**, could inhibit HPPD activity with an EC_{50} value of 1.05 μM, similar to that of mesotrione (with an EC_{50} value of 1.35 μM). Detailed discussion about observed herbicidal symptoms is provided in the Results and Discussion section. This investigation provided a proof-of-concept foundation that a multitarget drug design strategy could be applied in agrochemical research.

KEYWORDS: *4-hydroxyphenylpyruvate dioxygenase, herbicidal activity, isoxazole, benzylamine*

INTRODUCTION

4-Hydroxyphenylpyruvate dioxygenase (HPPD) is an important enzyme of the biosynthetic pathway to plastoquinone in plants, and inhibition of plastoquinone biosynthesis through HPPD blockade results in herbicidal activity with unique bleaching symptoms.[1] In 1997, HPPD was identified as the target site of triketones.[2] Great progress has been made in the discovery of HPPD inhibitors in recent years, and triketones, isoxazoles, and pyrazoles have been widely used in the management of weeds.[3] Because of their high activity, lack of resistance, and low mammalian toxicity, the discovery of novel HPPD inhibitors attracts much attention in the herbicide industry nowadays.[4−7]

Isoxaflutole (IFT) is a pre-emerging HPPD herbicide for the control of broadleaf and grass weeds in maize and sugarcane.[8] Although IFT is a highly effective herbicide, the weed spectrum and crop selectivity of IFT are not good enough in practical applications. To overcome these shortcomings, IFT is usually applied by mixing with flufenacet, calonifen, or atrazine to improve its herbicidal activity against grassy weeds, and cyprosulfamide is used as a safener in the formulation.[9−11] It is necessary to develop novel chemistry to overcome such shortcomings.

The multitarget drug design is a novel strategy in the medicinal field, and a variety of successful examples have been reported.[12−14] However, this strategy has seldom been reported in agrochemical research. Different approaches could be realized for multitarget drug designs. Among these approaches, merging pharmacophores with and without a linker is a well-recognized method.[15,16] This method requires the similarity of chemical structures and bioactivities of the two lead compounds. IFT does not inhibit HPPD in plants. Its activity comes from the product of a ring-opening conversion (*i.e.*, from isoxazole to diketonitrile or DKN). This ring-opening conversion is a rapid nonenzymatic hydrolyzation and occurs both in the plant and in soil.[17] The chelating 1,3-diketone of DKN is responsible for the binding to the active site of HPPD. Therefore, the isoxazole-4-carbonyl moiety of IFT is considered as its pharmacophore. Another lead herbicide adopted for this design is *N*-isobutyl-*N*-(4-chloro-benzyl)-4-chloro-2-pentenamide, which is a light-dependent amide herbicide reported by Kumai Chemical Co. Ltd. It can effectively control barnyard grass, throughout inhibiting cell division.[18] According to its structure, carbonyl benzylamine is considered to be more important than the flexible side chain. Therefore, this moiety was assumed as a pharmacophore for this lead herbicide.

The objective of the present study was to adopt a multitarget drug design strategy by merging the pharmacophore of IFT

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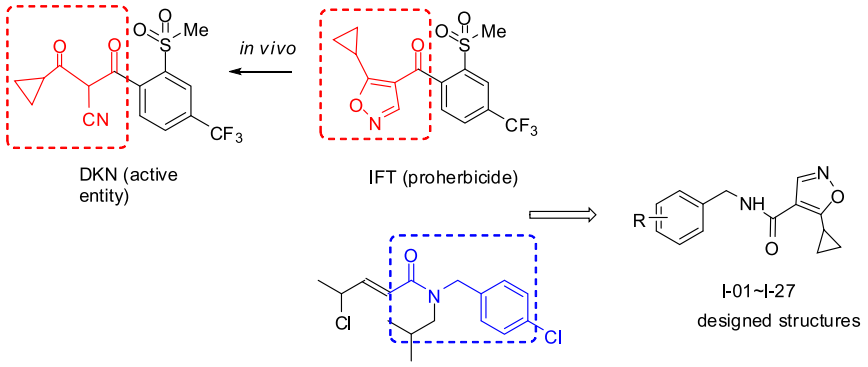

Figure 1. Design strategy of *N*-benzyl-5-cyclopropylisoxazole-4-carboxamides.

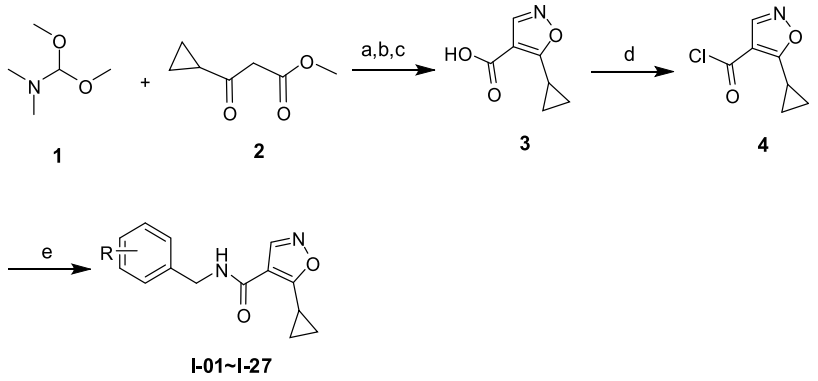

Figure 2. Synthesis of compounds **I-01**−**I-27**. Reagents and conditions: (a) 60 °C, 20 h; (b) H_2NOH−HCl, H_2O, MeOH, 90 min, 60 °C; (c) concentrated HCl, AcOH, 4 h, reflux; (d) oxalyl chloride, CH_2Cl_2, 30 min, room temperature; and (e) substituted benzylamines, CH_2Cl_2, 30 min, 0 °C.

(*i.e.*, isoxazole-4-carbonyl) and the pharmacophore of *N*-isobutyl-*N*-(4-chloro-benzyl)-4-chloro-2-pentenamide (*i.e.*, carbonyl benzylamine). Because of sharing a common carbonyl group of two pharmacophores, merging without a linker was adopted, forming novel carboxamides (Figure 1). A total of 27 *N*-benzyl-5-cyclopropylisoxazole-4-carboxamides were designed and synthesized, and their structures were confirmed by NMR and MS. Their herbicidal activities were evaluated by Petri dish assays and pot experiments in a glasshouse. Active compounds (*e.g.*, **I-05**, **I-24**, and **I-26**) were discovered, and typical bleaching phenomenon of HPPD inhibition could be observed with algae and postemergence pot assays for some compounds (*e.g.*, **I-05** and **I-24**). These active compounds could be used as herbicidal leads for further optimization.

MATERIALS AND METHODS

Reagents and Procedures. All chemical reagents were commercially available and used without further purification. Precoated silica gel plates (Si60 F_{254}, Merck Chemical Co. Ltd) were used to monitor the progress of reactions. Purification of target compounds was performed on silica gel column chromatography (200−300 mesh, Qingdao Marine Chemical Co. Ltd, China). ^1H and ^{13}C NMR spectra were recorded on a Bruker AVANCE III-500 NMR spectrometer, and the residual solvent signals were used as reference. Mass spectral analysis was carried out on a Finnegan LCQ Advantage MAX LC/MS spectrometer equipped with an ESI source. The melting points were conducted on a WRS-3 apparatus and uncorrected.

General Procedures for Preparation of 5-Cyclopropylisoxazole-4-carboxylic Acid (3). The key intermediate 5-cyclopropylisoxazole-4-carboxylic acid (**3**) was prepared by following a procedure described in a patent with minor modification (Figure 2).[19] 1,1-dimethoxy-*N*,*N*-dimethylmethanamine (**1**) (24 g, 0.2 mol) and methyl 3-cyclopropyl-3-oxo-propanoate (**2**) (28 g, 0.2 mol) were mixed and heated for 20 h at 60 °C. The obtained yellow oil was first dissolved in methanol (200 mL) and water (100 mL), and then, hydroxylamine hydrochloride (14 g, 0.2 mol) was added. The solvents were evaporated under vacuum after the mixture was heated for 90 min at 60 °C. The residue was dissolved in the mixture of acetic acid (100 mL) and concentrated HCl (100 mL) and refluxed for 4 h. The reaction mixture was diluted with water (500 mL) and extracted with ethyl acetate (200 mL × 3). The organic layer was combined and washed with brine and then dried with anhydrous sodium sulfate. Ethyl acetate was evaporated under vacuum. The residue was subjected to a silica gel column and eluted with the mixture of ethyl acetate and petroleum ether at the ratio of 1:3 (v/v) to afford **3**.

General Procedures for Preparation of 5-Cyclopropylisoxazole-4-carbonyl Chloride (4). Oxalyl chloride (38.1 g, 0.3 mol) in 50 mL of anhydrous dichloromethane was added dropwise to the solution of **3** (15.3 g, 0.1 mol) in 150 mL of anhydrous dichloromethane. The mixture was reacted for 30 min at room temperature. After completion of the reaction based on TLC monitoring, dichloromethane was evaporated under vacuum to afford **4**.

General Procedures for Preparation of *N*-Benzyl-5-cyclopropylisoxazole-4-carboxamides (I-01−I-27). The target compounds, **I-01**−**I-27**, were synthesized by following a reported procedure.[20] To a solution of substituted benzylamines (1 mmol) and **4** (1 mmol) in 20 mL of anhydrous dichloromethane, pyridine (3 mmol) in 5 mL of anhydrous dichloromethane was added dropwise at 0 °C. The mixture was reacted at 0 °C for 30 min. After completion of the reaction based on TLC monitoring, the solution was washed with water (30 mL) and saturated sodium chloride solution (30 mL) successively. The organic layer was dried over anhydrous sodium sulfate. After the solvent was removed under vacuum, the residue was

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purified on a silica gel column by using ethyl acetate/petroleum ether (1:5) as an eluent to afford **I-01**−**I-27**.

General Procedures for Preparation of *N*-Benzyl-2-cyano-3-cyclopropyl-3-oxopropanamides (II-03, II-04, II-24, and II-25). The DKNs of **I-03**, **I-04**, **I-24**, and **I-25** were synthesized following a procedure in literature (Figure 3).[21] To a solution of **I-03**, **I-04**, **I-24**,

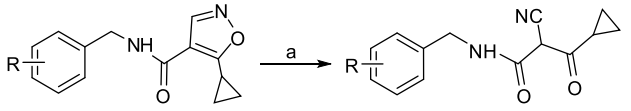

Figure 3. Preparation of **II-03**, **II-04**, **II-24**, and **II-25**. Reagents and conditions: (a) Et_3N, CH_2Cl_2, room temperature.

or **I-25** (1 mmol) in dichloromethane (10 mL), triethylamine (3 mmol) was added, and the mixture was stirred for 12 h at room temperature. After completion of the reaction based on TLC monitoring, dichloromethane was evaporated under vacuum. The residue was diluted with cold water and acidified with 1 M dilute hydrochloric acid and then extracted with ethyl acetate (40 mL). The organic layer was washed with brine and dried with anhydrous sodium sulfate. **II-03**, **II-04**, **II-24**, and **II-25** were obtained by using silica gel column chromatography eluted by ethyl acetate/petroleum ether (1:4).

Petri Dish Assay. Seeds of monocotyledon weeds, such as *Echinochloa crusgalli* and *Digitaria sanguinalis*, and dicotyledon weeds such as *Amaranthus retroflexus*, *Portulaca oleracea*, and *Abutilon theophrasti* were collected from the campus of Northwest A&F University in 2017. Herbicidal activity of the title compounds against these target weeds was first evaluated by Petri dish assay as described previously.[22] A total of 10 germinating seeds were placed in Petri dishes (90 mm diameter) containing two layers of filter paper impregnated with 5 mL of the solutions of tested compounds at 100 and 10 mg/L, respectively. Water was used as a blank control; IFT and butachlor were used as positive controls. Then, the Petri dishes were placed in a light incubator at 25 °C with a light intensity of 3000 lux. The growth inhibition rates of roots and stems were observed after 5 days. Each treatment was repeated three times, and average inhibition of stems and roots are listed in Tables 1 and 2.

Pre- and Postemergence Herbicidal Activity. Pre- and postemergence herbicidal activities of the title compounds against *E. crusgalli* and *A. theophrasti* were evaluated according to a procedure reported previously.[23] Each test compound was first dissolved in DMSO to prepare a concentration of 100 g/L solution. Each solution was then diluted with water containing 0.1% Tween 80 to the desired concentrations before applications. The soil used was a mixed soil (33.3% garden soil and 66.7% seedling substrate). Plastic pots with an inner diameter of 7.5 cm were filled with the soil to three-fourths of

Table 1. Herbicidal Activity of Title Compounds in Petri Dish Tests (100 mg/L)

		inhibition rate (%)									
		EC[a]		DS[a]		AR[a]		PO[a]		AT[a]	
no.	R^1	root	stem	root	stem	root	stem	root	stem	root	stem
I-01	H	50	30	60	30	80	80	100	100	90	80
I-02	2-CH_3	50	40	70	70	80	70	100	100	90	90
I-03	3-CH_3	50	40	60	30	80	60	100	100	100	100
I-04	4-CH_3	50	30	70	50	60	50	100	100	100	100
I-05	2-OCH_3	60	50	60	50	60	50	100	100	100	100
I-06	3-OCH_3	30	20	50	30	70	50	100	100	100	100
I-07	4-OCH_3	30	20	60	40	80	80	100	100	100	100
I-08	3,4-diOCH_3	30	30	20	20	40	30	100	100	100	100
I-09	3,5-diOCH_3	50	40	50	50	40	40	100	100	100	100
I-10	4-$CH(CH_3)_2$	60	50	50	50	70	60	70	80	100	100
I-11	4-$C(CH_3)_3$	50	50	50	40	70	60	70	70	60	60
I-12	2-F	80	80	100	100	100	100	100	100	100	100
I-13	3-F	80	90	100	100	100	100	100	100	100	100
I-14	4-F	60	60	100	100	100	100	100	100	100	100
I-15	2-Cl	60	60	40	50	40	40	80	80	100	100
I-16	3-Cl	100	100	100	100	100	100	100	100	100	100
I-17	4-Cl	100	100	100	100	100	100	100	100	100	100
I-18	3-Br	100	100	100	100	100	100	100	100	100	100
I-19	2,4-diF	80	70	80	80	100	100	100	100	100	100
I-20	2,4-diCl	80	80	60	60	90	90	80	70	60	60
I-21	2,6-diF	70	70	60	60	80	70	100	100	50	40
I-22	2,6-diCl	60	70	40	40	80	80	90	90	50	50
I-23	3,4-diCl	100	100	90	90	80	80	100	100	60	50
I-24	3-Cl-4F	100	100	100	100	100	100	100	100	100	100
I-25	3-CF_3	100	100	100	100	100	100	100	100	100	100
I-26	4-CF_3	100	100	100	100	100	100	100	100	100	100
I-27	2-OH	70	60	40	30	20	20	80	80	100	100
II-03	3-CH_3	50	50	60	50	80	80	100	100	100	100
II-25	3-CF_3	100	100	100	100	100	100	100	100	100	100
isoxaflutole		30	10[b]	40	40[b]	70	60[b]	60	50[b]	50	50[b]
butachlor		100	100	100	100	80	80	80	80	90	90

[a]Abbreviations: EC for *Echinochloa crusgalli*; DS for *Digitaria sanguinalis*; AR for *Amaranthus retroflexus*; PO for *Portulaca oleracea*; and AT for *Abutilon theophrasti*. [b]Exhibit bleaching symptoms.

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Table 2. Herbicidal Activity of Title Compounds in Petri Dish Tests (10 mg/L)

		inhibition rate (%)									
		EC[a]		DS[a]		AR[a]		PO[a]		AT[a]	
no.	R[1]	root	stem	root	stem	root	stem	root	stem	root	stem
I-01	H	0	0	0	0	30	30	50	40	50	50
I-02	2-CH_3	0	0	0	0	20	20	30	30	30	20
I-03	3-CH_3	0	0	0	0	20	20	30	20	0	0
I-04	4-CH_3	0	0	0	0	20	20	40	40	30	20
I-05	2-OCH_3	0	0	0	0	0	0	20	20	20	20
I-06	3-OCH_3	0	0	0	0	0	0	20	20	0	0
I-07	4-OCH_3	0	0	0	0	0	0	20	20	0	0
I-08	3,4-diOCH_3	0	0	0	0	0	0	20	20	10	10
I-09	3,5-diOCH_3	0	0	0	0	0	0	30	20	20	20
I-10	4-$CH(CH_3)_2$	0	0	0	0	0	0	0	0	0	0
I-11	4-$C(CH_3)_3$	0	0	0	0	0	0	0	0	0	0
I-12	2-F	20	20	20	20	50	40	60	60	60	50
I-13	3-F	30	20	20	20	50	50	70	70	50	50
I-14	4-F	30	30	30	30	70	70	100	100	60	50
I-15	2-Cl	0	0	0	0	0	0	30	30	50	50
I-16	3-Cl	40	40	20	30	50	50	70	70	50	60
I-17	4-Cl	20	20	40	30	80	80	90	90	60	60
I-18	3-Br	30	30	40	40	60	50	60	60	60	60
I-19	2,4-diF	20	10	20	20	50	50	50	30	30	30
I-20	2,4-diCl	20	20	0	0	30	30	40	30	0	0
I-21	2,6-diF	0	0	0	0	0	0	20	20	0	0
I-22	2,6-diCl	0	0	0	0	0	0	20	20	0	0
I-23	3,4-diCl	30	20	30	30	20	20	30	40	30	30
I-24	3-Cl-4F	70	60	60	60	70	70	80	80	70	70
I-25	3-CF_3	70	70	60	60	70	70	80	80	80	80
I-26	4-CF_3	70	70	40	40	90	90	100	100	100	100
I-27	2-OH	0	0	0	0	0	0	0	0	20	20
II-3	3-CH_3	0	0	0	0	20	20	30	30	10	10
II-25	3-CF_3	70	70	60	60	80	70	80	80	80	80
isoxaflutole		40	20[b]	30	10[b]	50	20[b]	40	30[b]	30	20[b]
butachlor		90	80	90	90	60	60	50	50	50	50

[a]Abbreviations: EC for Echinochloa crusgalli; DS for *Digitaria sanguinalis*; AR for *Amaranthus retroflexus*; PO for *Portulaca oleracea;* and AT for *Abutilon theophrasti*. [b]Exhibit bleaching symptoms.

their heights. Around 20 seeds of the tested weeds were sown in each pot and covered with soil to a thickness of 0.2 cm. The pots were maintained at temperatures from 15 to 30 °C in a glasshouse. For pre-emergence treatments, the diluted test solutions (150 g ai/ha) were sprayed on the surface of soil 24 h after the seeds were sown. For postemergence treatments, the weeds were treated with the solutions of tested compounds (150 g ai/ha) at the three-leaf stage. The seedlings treated with the diluted solution of DMSO and Tween 80 were used as the control groups. Each treatment was performed in four replicates. IFT was used as a positive control. The herbicidal activity was evaluated visually at 15 days after treatment.

Effect of Exogenous Homogentisate on *Chlamydomonas reinhardtii*. *C. reinhardtii* was grown autotrophically in the liquid medium as literature described.[24] *C. reinhardtii* cells in the late exponential phase were harvested and transferred to glass tubes (with plastic cover) at a density of 5.0×10^5 cell/mL in a volume of 5 mL culture medium. For the exposures, **I-05** (200 and 100 μM), **I-05** + homogentisate (HGA) (200 + 50 and 100 + 50 μM), and HGA (50 μM) were added to the medium. A chemical-free treatment was used as a blank control. The algae were cultivated in an illumination incubator with a PAR intensity of 3000 lux at 25 °C. The symptoms were observed at 96 h after exposure.

HPPD Enzymatic Assay. HPPD originating from etiolated dark-grown corn seedlings was extracted and purified according to the method described in literature.[25] The inhibitory effects of HPPD by **I-05**, **I-24**, and the active entities of **I-05** and **I-24** (*i.e.*, **II-05** and **II-24**) were evaluated by using a HPLC method according to the procedures described in literature.[25] Mesotrione was used as a positive control. Each treatment was carried out with three replicates. The content of HGA in each assay mixture was measured by HPLC on a Hypersil C_{18} column (5 μm, 4.6 mm × 250 mm) by using methanol/0.1% trifluoroacetic acid (80 + 20, v/v) as a mobile phase. The IC_{50} values of tested compounds were calculated based on the average of three replicates.

Molecular Modeling. The 3D structures of **II-05** and **II-24** were constructed by using ChemBiodraw Ultra 10.0. All compounds were then opened in Discovery studio 4.0, and energy minimization was carried out by CHARMM force field using the ligand partial charge method consistent force field.[26] Minimization was carried out until an energy gradient of 0.01 was reached. The CDOCKER was used for docking of all compounds. A representative *At*HPPD cocrystallized with NTBC (PDB ID: 1TFZ) was taken from the PDB data bank. The water molecules were deleted and hydrogen atoms were added. Final protein was refined with CHARMM in DS 4.0 at physiological pH. To validate the docking reliability, the cocrystallized ligand (DAS869) was first redocked to the binding site of HPPD. Consequently, **II-05** and **II-24** were docked into the same active site, and 20 conformations of each compound were obtained through CDOCKER.[27] The conformations with the lowest energy were selected as the most probable binding conformation. PYMOL was used to analyze the binding modes.

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RESULTS AND DISCUSSION

Chemistry. The title compounds were designed based on the chemical structures of IFT and *N*-isobutyl-*N*-(4-chlorobenzyl)-4-chloro-2-pentenamide. IFT has a complex chemical structure; therefore, a longer synthetic route leads to higher production cost.[28] The synthetic route for the title compounds is much easier than that of IFT. 5-Cyclopropylisoxazole-4-carbonyl acid was the only intermediate needed to be prepared, and all substituted benzylamines used in this study were commercially available. 5-Cyclopropylisoxazole-4-carboxylic acid could be prepared from 1,1-dimethoxy-*N*,*N*-dimethylmethanamine and methyl 3-cyclopropyl-3-oxo-propanoate *via* a one-pot three-step reaction. We observed that the yield of the final product in the pot was strongly affected by the concentration of HCl. Retaining excess water in the second step resulted in low yield of 5-cyclopropylisoxazole-4-carboxylic acid, so removal of solvents before the final reaction was a crucial factor. To further increase the yield, we examined the amount of raw materials and found that the optimal molar ratio of 3-cyclopropyl-3-oxo-propanoate to 1,1-dimethoxy-*N*,*N*-dimethyl-methanamine was 1:1.5. Under the optimal conditions, the yield of the final product was above 80%. For preparation of 5-cyclopropylisoxazole-4-carbonyl chloride, we examined the effect of temperature on the yield, and found that the yield of acyl chloride was close to 100% at room temperature. The preparation of *N*-benzyl-5-cyclopropylisoxazole-4-carboxamides was carried out under an ice-bath condition. Because of the high reactivity of benzylamine, the reaction proceeding at room temperature tended to produce a bisamide. The title compounds could be readily converted to their corresponding DKNs in dichloromethane containing triethylamine at room temperature.

The chemical structures of the synthesized compounds were confirmed by NMR and MS spectrometric analysis.

Herbicidal Activity in Petri Dish Assays. As shown in Table 1, most of the title compounds at 100 mg/L exhibited high herbicidal activity against target weeds, especially broadleaf weeds. The herbicidal activities of **I-16**−**I-18** and **I-24**−**I-26** were stronger than other compounds, and their inhibition rates on all tested weeds were 100%. These results indicated that introduction of electron-withdrawing groups on the benzene ring is more beneficial to herbicidal activity than that of electron donating groups. **I-12**, **I-15**, and **I-19**−**I-22** showed weaker activity than other halogenated compounds, implying that the introduction of halogen atoms to the meta- and para-positions of the benzene ring is better for herbicidal activity than the ortho-position. To further assess the SAR, herbicidal activities of the title compounds at 10 mg/L were evaluated, and the results are listed in Table 2. **I-26** was the most potent compound, and it could effectively inhibit the growth of *A. retroflexus*, *P. oleracea*, and *A. theophrasti* at 10 mg/L. The influence of the substitution position of halogen atoms on the benzene ring could be summarized as: para- > meta- > ortho-position. All derivatives which bear electron-donating groups on the benzene ring including **I-01**−**I-11** and **I-27** showed slight or even no herbicidal activity at this dose.

The symptoms of weeds treated by IFT, butachlor, and **I-26** on *P. oleracea* are illustrated as Figure 4. As an effective HPPD inhibitor, IFT resulted in characteristic bleaching symptoms in the leaves but only slightly inhibited the growth of *P. oleracea*. The synthesized compounds showed strong inhibition against weeds, and the inhibitory rates of some compounds against broadleaf weeds were stronger than that of butachlor. For example, **I-26** completely inhibited the growth of *P. oleracea* at both 100 and 10 mg/L. Its poison symptoms were similar to that of *N*-isobutyl-*N*-(4-chloro-benzyl)-4-chloro-2-pentenamide, one of its lead compounds, and we speculated the title compounds also exhibited herbicidal activity through an inhibition of cell division. However, no bleaching effect was observed in the groups treated by the title compounds. Because the conversion from IFT to DKN is essential for the herbicidal activity, we speculated whether the title compounds were not converted into the active form *in vivo*. To testify this speculation, **II-03** and **II-25**, two DKN derivatives, were prepared from **I-03** and **I-25** in the laboratory, and their herbicidal activities were evaluated by the Petri dish assay (Tables 1 and 2). These two DKN derivatives showed similar inhibitory effects with their corresponding precursor molecules, and no bleaching effect was observed. No bleaching phenomenon might result from poor uptake and translocation of these compounds or their poor stability in seedlings so that their quantity in leaves of seedlings could not accumulate enough.

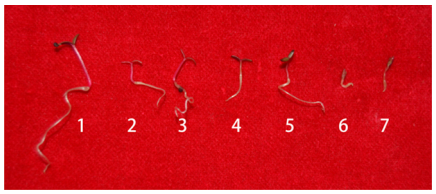

Figure 4. Symptoms of *P. oleracea* treated by IFT, butachlor, and **I-26**. 1: CK; 2, 3: IFT at 100 and 10 mg/L; 4, 5: butachlor at 100 and 10 mg/L; 6, 7: **I-26** at 100 and 10 mg/L.

Pre- and Postemergence Herbicidal Activity. Pre- and postemergence herbicidal activities of the newly prepared compounds against *E. crusgalli* and *A. theophrasti* were assessed at an application rate of 150 g/ha. Unfortunately, none of the tested compounds showed pre-emergence herbicidal activity at this application rate. But several compounds showed good postemergence herbicidal activity, and the results are illustrated as Figure 5. **I-05** and **I-24** exhibited strong herbicidal activity against *E. crusgalli* and *A. theophrasti*, and their activities were comparable to IFT. The inhibition rates of **I-12** and **I-19** against monocotyledon *E. crusgalli* were all above 60%; however, they only showed weak activity against *A. theophrasti*. In contrast, **I-23** showed good herbicidal activity against the broadleaf weed *A. theophrasti*. Furthermore, these active compounds resulted in a bleaching effect similar to that of HPPD herbicides.

The structure−activity relationships of several ortho-substituted derivatives obtained from pot experiments and Petri dish assay had great discrepancies. The inhibitory effects of **I-02** and **I-05** in the Petri dish assay were weaker than those of many meta- or para-halogenated derivatives, whereas they showed stronger postemergence herbicidal activity. It seems that an introduction of electron-donating groups to the ortho-position is helpful for postemergence herbicidal activity. Additionally, the introduction of fluorine and chlorine to the meta- and para-positions on the benzene ring is beneficial to the activity.

Effect of Exogenous HGA on *C. reinhardtii*. HPPD herbicides indirectly inhibit carotenoid biosynthesis, throughout preventing the production of HGA, and this inhibition can be reversed by a supplement of HGA. To assess whether a

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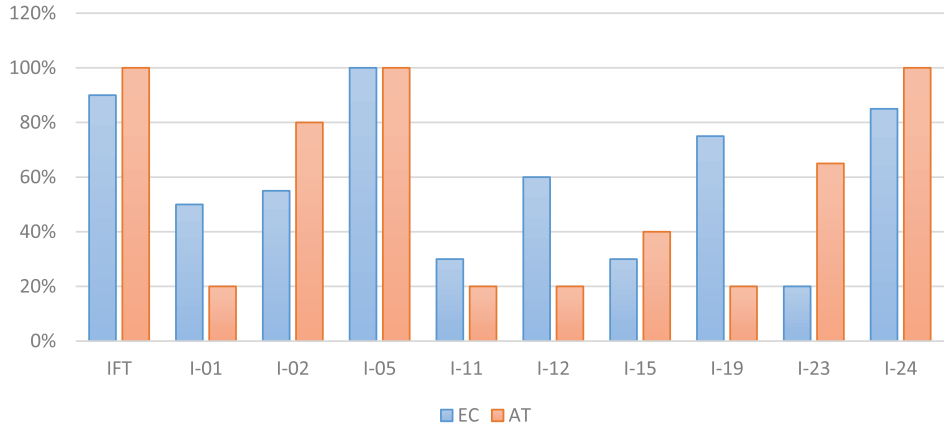

Figure 5. Postemergence herbicidal activity of several title compounds (150 g/ha). EC for *Echinochloa crusgalli*; AT for *Abutilon theophrasti*.

supplement of HGA on the inhibition of *C. reinhardtii* by **I-05**, we ran the following test: *C. reinhardtii* grew normally with green color upon the treatments of HGA and the chemical-free control at 96 h after exposure to **I-05** (Figure 6E,F). However,

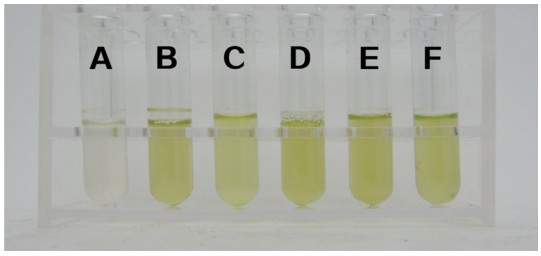

Figure 6. Reversal of bleaching effects of **I-05** on *C. reinhardtii* by adding HGA. (A) **I-05** (200 μM). (B) **I-05** + HGA (200 + 50 μM). (C) **I-05** (100 μM). (D) **I-05** + HGA (100 + 50 μM). (E) HGA (50 μM). (F) control.

the algae treated with 200 μM of **I-05** solution were clearly bleached (Figure 6A), and 100 μM of **I-05** resulted in slight bleaching effect on *C. reinhardtii* (Figure 6C). The algae treated with **I-05** + HGA (Figure 6B, 200 + 50 μM; Figure 6D, 100 + 50 μM) grew normally. The phenomena implied that (1) the bleaching effect induced by **I-05** could be reversed by exogenous HGA; (2) **I-05** showed activity through inhibition of the formation of HGA; (3) **I-05** might be transferred to its active DKN in algae so that the typical bleaching effects could be observed; and (4) a dose−response bleaching phenomenon further suggested that no bleaching effects during the Petri dish assay may come from low quantity of the title compound in seedling leaves.

HPPD Enzymatic Assays. A difference may exist between crop HPPD and weed HPPD (*e.g.*, corn HPPD and *Arabidopsis thaliana* HPPD or *At*HPPD); this leads to selectivity of an herbicide between crops and weeds. Our purpose was to see whether HPPD could be one of the targets of the title compounds (*i.e.*, whether HPPD) and could be inhibited by the title compounds as well as the positive control, a commercial herbicide mesotrione (*i.e.*, a side-by-side comparison). Corn HPPD could be easily prepared in our lab, but *At*HPPD was not readily available in our lab; therefore, corn HPPD was used for this purpose. As shown in Table 3, **II-05**, **II-24**, and mesotrione showed inhibitory effects on HPPD activity, and their IC_{50} values were 1.05, 1.28, and 1.34 μM,

Table 3. Inhibitory Effects of HPPD by Tested Compounds

compounds	IC_{50} (μM)
I-05	>10
II-05	1.05 ± 0.16
I-24	>10
II-24	1.28 ± 0.07
mesotrione	1.34 ± 0.09

respectively. However, **I-05** and **I-24** showed no inhibitory effect on HPPD.

Combined information such as no HPPD inhibition by **I-05**, HPPD inhibition by **II-05**, algae bleaching, and postemergence bleaching phenomena from **I-05** treatments implies that **I-05** could be transferred to **II-05** *in vivo* (*e.g.*, algae and plants) slowly; therefore, **I-05** and other active compounds (*e.g.*, **I-24**) possibly work as prodrugs. For IFT, the ring-opening conversion of isoxazole is a rapid nonenzymatic hydrolysis, happening in both plants and in soil. The rapid hydrolysis may come from the strong effects of the electron-withdrawing carbonyl group located between two rings, which could form a much stable equilibrium among triketones and ketone enols. For title compounds (*e.g.*, **I-05** and **I-24**), the electron-withdrawing effects of the carbonyl in the carboxamide group become weaker because of the stronger electronegative nitrogen. Therefore, the isoxazole ring hydrolysis of title compounds may become very slow or may happen by special metabolic enzymes, which could explain why bleaching symptoms could be observed in the algae assay and postemergence pot assays, but not in the Petri dish assay.

Molecular Simulations. The interactions of **II-05** and **II-24**, the active entities of **I-05** and **I-24**, with HPPD were studied *via* a molecular docking method (Figure 7). As illustrated in Figure 7, **II-05** and **II-24** formed bidentate interactions with Fe^{II}, and the benzene ring could form $\pi-\pi$ interactions with Phe360 and Phe403. The CN group of **II-05** formed a hydrogen bond with the amino group of Asn261, which was suspected to result in an increase in herbicidal activity. By comparing the distances of $\pi-\pi$ interactions, we found that the distance between the benzene ring of **II-05** and Phe360 was approximately equal to that of **II-24**, whereas that between **II-05** and Phe403 (3.7 Å) was smaller than that of **II-24** (4.3 Å). The increased distance might be the reason that the herbicidal activity of **I-05** was slightly stronger than that of **I-24**. In the CDOCKER module of DS, the −CDOCKER interaction energy is used to evaluate the docking results. A

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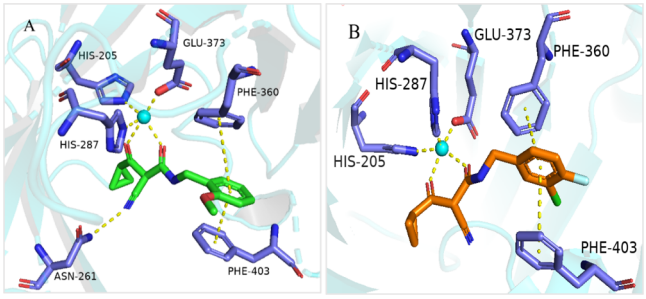

Figure 7. Simulated binding modes of **II-05** and **II-24** with *At*HPPD. The key residues in the active site are shown in blue sticks, and Fe^{II} is shown as a cyan sphere. (A) Binding mode of **II-05** with *At*HPPD. **II-05** is shown in green sticks. (B) Binding mode of **II-24** with *At*HPPD. **II-24** is shown in orange-magenta sticks.

higher score stands for better binding between the drug and acceptor. The −CDOCKER interaction energy values of mesotrione, **II-05**, and **II-24** were 55.98, 49.78, and 38.08, respectively. The score of **II-05** was significantly higher than that of **II-24**. This might explain that **II-05** has one more hydrogen bond interaction than **II-24**. The difference in postemergence herbicidal activity of **I-05** and **I-24** was also consistent with the result of molecular docking.

In summary, a series of *N*-benzyl-5-cyclopropylisoxazole-4-carboxamides were synthesized based on the strategy of a multitarget drug design. The pharmacophores of HPPD and an amide herbicide were incorporated into the structure of the title compounds *via* a merging pharmacophore concept. The title compounds could be readily prepared in good to excellent yields. Some compounds showed strong inhibitory effect on the weeds in the Petri dish assay, and the symptoms were similar to those of the amide herbicide. **I-05** displayed promising postemergence herbicidal activity, and it could work as a prodrug of a HPPD inhibitor. Although its activity is relatively low when compared with newly launched herbicides, this investigation provided a potential multitarget lead compound for the discovery of novel herbicides. Further structural optimization of the lead compound is ongoing.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c03582.

NMR data and spectra of the title compounds (PDF)

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Notes

The authors declare no competing financial interest.

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