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Brief Report

Isoxaben analogs inhibit chitin synthesis in the cultured integument of the rice stem borer *Chilo suppressalis*

Kotaro Mori, Hideya Tokuoka, Hisashi Miyagawa and Yoshiaki Nakagawa*

Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto 606–8502, Japan

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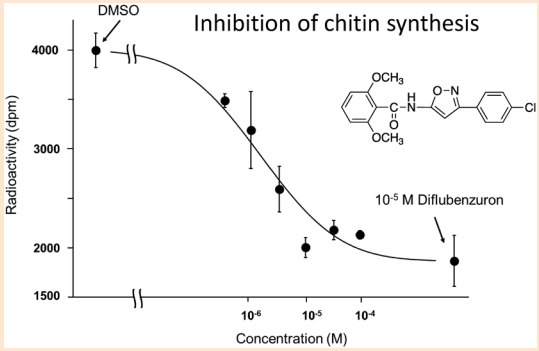
Supplementary material

Benzoylphenylureas (BPUs) were discovered as novel type insecticides about a half century ago; many analogs have been launched as insecticides and acaricides. BPUs are known to inhibit chitin synthesis in insects and other arthropods, but they have no effect against microorganisms such as fungi. We designed new chitin synthesis inhibitors based on the hypothesis that biomolecules that play important roles in cellulose and chitin biosynthesis are similar. In the full automatic modeling system (FAMS), the cellulose synthase was selected as a template three-dimensional structure. Thus, we focused on the structure of cellulose synthase inhibitor, isoxaben, to develop new chemistry. The 1,1-diethylethyl [-C(CH_3)(CH_2CH_3)$_2$] group of isoxaben was changed to a 4-substituted phenyl group bearing Cl, Et, or Ph. These compounds significantly inhibited chitin synthesis in the cultured integument of the rice stem borer *Chilo suppressalis*. The activity of the 4-ethylphenyl analog was enhanced 30-fold by adding piperonyl butoxide to the culture medium.



Keywords: chitin synthesis inhibitors, chitin synthase, cellulose synthase, isoxazole, *Chilo suppressalis*, benzoylphenylurea.

Introduction

Arthropods, such as insects, mites, and crustaceans, must molt in order to grow. When molting, they must shed their skin constructed from chitin and protein and reconstruct a new one. Chitin is the polymer of *N*-acetylglucosamine, which is not synthesized in vertebrates such as mammals. Therefore, chitin synthesis inhibitors (CSIs) are thought to be mammal-friendly insecticides, although they may not be safe to animals in the environment such as daphnia. The body weight of certain insects increases thousands of times during their larval stages by repeated molting. Therefore, they consume large quantities of food to grow during the larval stage, damaging agricultural crops. To protect crops from pests, we use insecticides with different modes of action. These are categorized by the insecticide resistance action committee (IRAC),[1] in whose classification system, chitin synthesis inhibitors (CSIs) belong to Groups 10, 15, and 16. The chemical structures of representative CSIs are shown in Fig. 1. Found in 1972,[2] benzoylphenylureas (BPUs), categorized in Group 15, are the earliest CSIs registered as insecticides and acaricides. Among them, one of the BPUs, diflubenzuron (Fig. 1), was the first launched as an agricultural insecticide, followed by various analogs such as chlorfluazuron.[3] Two decades later, etoxazole[4] and hexythiazox,[5] categorized in Group 10, were developed as acaricides. Buprofezin[6] is also known as a CSI but is categorized in a different IRAC group (Group 16).

Although many CSIs with various core structures have been developed, their molecular modes of action have not been solved yet. About a half century ago, it was reported that BPUs inhibit chitin synthesis *in vivo*,[7] but the direct target of BPUs was not solved for a long time.[8] However, a few years ago, a mutation in the *chitin synthase 1* (*CHS1*) gene was found in the etoxazole-resistant *Tetranychus urticae*.[9] A similar mutation was

* To whom correspondence should be addressed.
E-mail: yoshiaki.nakagawa7@gmail.com
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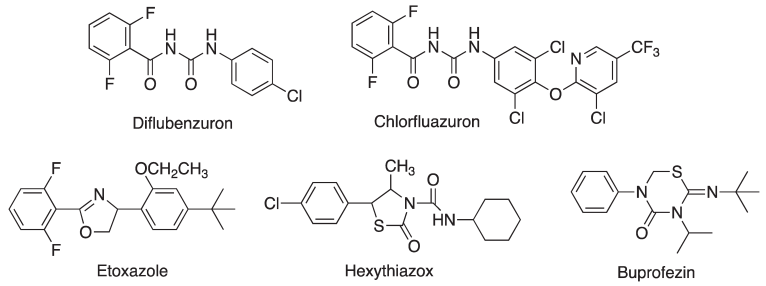


Fig. 1. Chemical structures of launched insecticides and acaricides

also detected in *CHS1* genes of BPU-resistant *Plutella xylostella*[10] and BPU-resistant *Frankliniella occidentalis*.[11] These findings suggested that the target protein of BPUs and etoxazole is CHS1, but the target sites of these CSIs are still unknown. Therefore, we constructed a three-dimensional model of CHS1 to find the binding site of BPUs. Among various protein modeling systems, we used a full automatic modeling system (FAMS; In-Silico Sciences Inc.) developed by Ogata and Umeyama.[12] FAMS suggested using a bacterial cellulose synthase of *Rhodobacter sphaeroides* (PDB:4HG6)[13] as the template three-dimensional (3D) structure in the comparative modeling of CHS1.

Since cellulose synthase was selected as the template 3D protein structure for CHS1 modeling, we thought that the inhibitor of cellulose synthase can be the inhibitor of CHS1. Actually, the similarity between chitin synthases and cellulose synthases has been pointed out by another group.[14] Therefore, we planned to modify the structure of isoxaben (Fig. 2), which is the inhibitor of cellulose synthase.[15] In the meantime, LY131215 (Fig. 2) was reported as a chitin synthesis inhibitor.[16] The structure of LY131215 is rather different from that of BPUs but similar to that of isoxaben. In our previous study, we synthesized LY131215 analogs with various substituents at both benzene rings and measured their chitin synthesis inhibition against cultured integument.[17] Since LY131215 and isoxaben have similar chemical structures, the 1,1-diethylethyl[-C(CH_3)(CH_2CH_3)$_2$] moiety of isoxaben was replaced with a substituted phenyl moiety. In this study, we synthesized three 5-benzoylamino-3-phenylisoxazole analogs (IOXs) with Cl, Et, and phenyl groups at the phenyl moiety (Fig. 2) and quantitatively measured their chitin synthesis inhibitory activity using cultured integument of *Chilo suppressalis*.[18,19]

Materials and methods

1. Synthesis

IOXs were synthesized from corresponding benzoic acids, CH_3CN, and NH_2OH according to the conventional synthetic route (Scheme 1). The details of synthesis are described in the supplementary materials. Synthesized compounds were identified by nuclear magnetic resonance (NMR) and elemental analysis.

2. Measurement of chitin synthesis

The bioassay procedure is identical to that reported previously.[18,19] In brief, diapause larvae reared on an artificial diet under sterilized conditions were used. Integuments excised from larvae were cultured in serum-free Grace's medium that was prepared manually from amino acids and other essential ingredients. The integument fragments were cultured for 24 hr in medium containing 0.2 μmol of 20-hydroxyecdysone (H5142, Sigma-Aldrich) and transferred to fresh medium containing test compounds and *N*-acetyl-D-[1-^{14}C]glucosamine ([^{14}C]NAG). In some experiments, the medium contained 20 μM of piperonyl butoxide (PB) in addition to the two media mentioned above. The original [^{14}C]NAG (0.1 mCi/mL; specific activity: 55 mCi/mmol, ARC, USA) was diluted with 70% EtOH to make 20,000–30,000 dpm/μL, and 1 μL of [^{14}C]NAG EtOH solution was added to each culture well containing 1 mL medium. After culturing the integument fragments for three days in medium containing [^{14}C]NAG, the cultured integument fragments were transferred to the vial for liquid scintillation counting (LSC).

Results and discussion

Three IOXs (**1**–**3**) were synthesized from 3-(4-substituted phenyl)isoxazole-5-amine and 3,5-dimethoxybenzoyl chloride in about 50% yields. 3-(4-Phenyl)isoxazole-5-amine analogs with varied substituents at the phenyl ring were constructed from corresponding 4-substituted benzoyl chloride, acetonitrile, and hydroxylamine in about a 10% yield.

The incorporation of [^{14}C]NAG to the cultured integument was inhibited by compound **1** (4-Cl) in a concentration-dependent manner, as shown in Fig. 3. For other compounds, similar sigmoidal curves were obtained. Radioactivity incorporated into the cultured integument fragments treated with solvent (control)

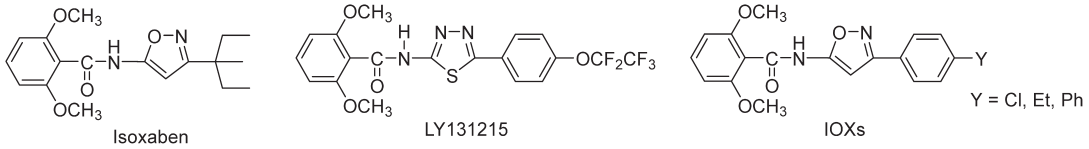
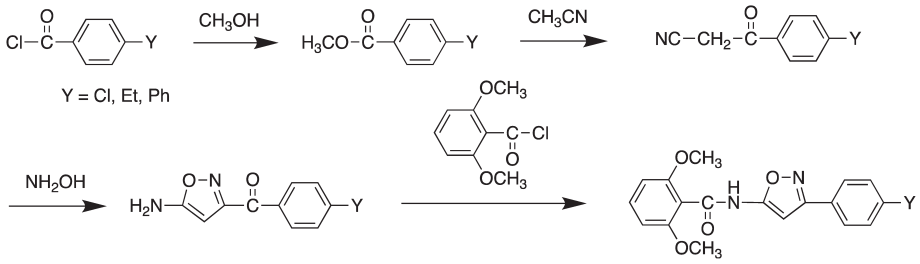


Fig. 2. Structures of isoxaben, LY131215 and newly synthesized IOXs



Scheme 1.

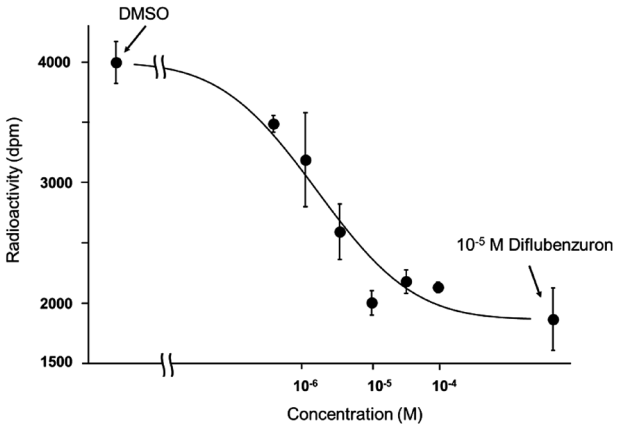


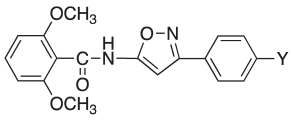
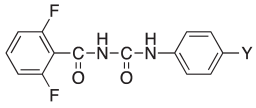
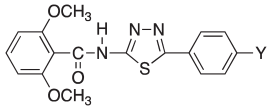
Fig. 3. Concentration-response curve for the inhibition of [^{14}C]NAG incorporation to the cultured integument of compound **1**

and diflubenzuron (positive control) were also measured in each experiment. Counts obtained for control and diflubenzuron treatment were set as 100% and 0%, respectively. From each response curve, the concentration required to give 50% inhibition, IC_{50} (M), was determined by probit analysis.[20] The IC_{50} values of three IOXs determined in this study are shown in Table 1; previously measured pIC_{50} values for other CSIs are listed as well.

In the previous study, we showed that the activity of BPU with 4-Et (**5**; Table 1) substitution at the phenyl ring was enhanced 30 times by adding an inhibitor of oxidative detoxification, piperonyl butoxide (PB).[18] PB also showed a synergistic effect for compounds with other electron-donating groups, such as OCH_3 and OCH_2CH_3. However, compounds with electron-withdrawing substituents, such as Cl (σ=0.23, σ_I=0.47) and NO_2 (σ=0.78, σ_I=0.67), were not synergized with PB. We discussed that electron-donating groups accelerate the oxidation (hydroxylation) of the benzene ring using quantitative structure–activity relationship (QSAR) analysis. Therefore, we measured the activity of IOXs under synergistic conditions with PB. As shown in Table 1, the activity of compound **2** with Et was dramatically enhanced by the addition of PB, but PB did not show any synergistic effect for compound **1** with Cl. These results were consistent with those observed for the activity of BPUs. Similar synergistic effects were also observed in the previous measurement of the chitin synthesis inhibition of *N*-[5-(Substituted phenyl)-1,3,4-thiadiazol-2-yl]-benzamides.[17]

The *Chilo* cultured integument system has also been used to quantitatively measure the molting hormone activity.[21,22] Although PB showed a significant synergistic effect on the chitin synthesis inhibition of certain CSIs, it has no effect in the measurement of molting hormone activity. In the molting hormonal

Table 1. Inhibition of chitin synthesis by IOXs and other CSIs in the cultured integument

Compound			pIC_{50} (M)		
No	Core structure	Y	None	with PB	$\Delta^{a)}$
1		Cl	5.83	5.83±0.27[b] (n=2)	0.00
2		Et	5.57	7.04±0.34[b] (n=3)	1.47
3		Ph	5.40	6.43	1.03
4		Cl[c]	7.69[d]	7.72[d]	0.03
5		Et	6.54[d]	8.12[d]	1.58
6		Cl	7.27[e]	7.23[e]	−0.04
7		Me	5.91[e]	7.07[e]	1.16
8		Ph	6.71[e]	6.91[e]	0.10

[a] pIC_{50} (PB) – pIC_{50} (none). [b] Mean±S.D. for two or three replications. [c] Diflubenzuron. [d] From Ref. [17]. [e] From Ref. [16].

activity test, integument fragments are treated with compounds one day, and the cultured integuments were further cultured for three days in the [^{14}C]NAG-containing medium without compounds. Probably, since molting hormone agonists are required to trigger the chitin synthesis in a short time, the oxidative detoxication of compounds was not significant in the evaluation of hormone activity.

In conclusion, three IOXs designed from isoxaben, an inhibitor of cellulose synthase, were newly synthesized. These new compounds inhibited chitin synthesis in the cultured integument of *C. suppressalis*. The 50% effective concentration of the most potent IOX compound was 0.1 μM (pIC_{50}=7.04). The objective of our study is the *in silico* identification of the target site of BPU and related CSIs. Docking simulations and molecular dynamics (MD) simulations are in progress.

Acknowledgements

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Electronic supplementary materials

The online version of this article contains supplementary materials (Synthesis of compounds), which are available at https://www.jstage.jst.go.jp/browse/jpestics/.

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