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

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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 [\(-C(CH_3)(CH_2CH_3)\)] 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 15, 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
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 BPU 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 BPU. 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 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.\(^{46}\) The structure of LY131215 is rather different from that of BPU 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-diethyl[1-(C(CH\(_3\)) \_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**

**Synthesis**

IOXs were synthesized from corresponding benzoic acids, CH\(_3\)CN, and NH\(_2\)OH 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.

**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\(\mu\)mol of 20-hydroxyecdysone (H5142, Sigma-Aldrich) and transferred to fresh medium containing test compounds and \(N\)-acetyl-\([1,14\text{C}]\) glucosamine ([\(14\text{C}\)NAG]). In some experiments, the medium contained 20\(\mu\)M of piperonyl butoxide (PB) in addition to the two media mentioned above. The original [\(14\text{C}\)]NAG (0.1 mCi/mL; specific activity: 55 mCi/mmol, ARC, USA) was diluted with 70% EtOH to make 20,000–30,000 dpm/\(\mu\)L, and 1\(\mu\)L of [\(14\text{C}\)]NAG EtOH solution was added to each culture well containing 1 mL medium. After culturing the integument fragments for three days in medium containing [\(14\text{C}\)]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 [\(14\text{C}\)]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)
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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, IC50 (M), was determined by probit analysis.20) The IC50 values of three IOXs determined in this study are shown in Table 1; previously measured pIC50 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 OCH3 and OCH2CH3. However, compounds with electron-withdrawing substituents, such as Cl (σ = 0.23, σI = 0.47) and NO2 (σ = 0.78, σI = 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

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

| No | Core structure | Y | pIC50 (M) | pIC50 (M) | ΔpIC50 |
|----|----------------|---|-----------|-----------|--------|
| 1  | OCH3           | Cl| 5.83      | 5.83 ± 0.27∥ (n = 2) | 0.00   |
| 2  | OCH3           | Et| 5.57      | 7.04 ± 0.34∥ (n = 3) | 1.47   |
| 3  | OCH3           | Ph| 5.40      | 6.43       | 1.03   |
| 4  | OCH3           | Cl∥ | 7.69∥ | 7.72∥ | 0.03   |
| 5  | OCH3           | Et| 6.54∥ | 8.12∥ | 1.58   |
| 6  | OCH3           | Cl∥ | 7.27∥ | 7.23∥ | −0.04   |
| 7  | OCH3           | Me| 5.91∥ | 7.07∥ | 1.16   |
| 8  | OCH3           | Ph| 6.71∥ | 6.91∥ | 0.10   |

∥ pIC50 (PB) – pIC50 (none). ∥ Mean ± S.D. for two or three replications.
∥ Diflubenzuron. ∥ From Ref. [17]. ∥ From Ref. [16].
synthesis inhibition of certain CSIs, it has no effect in the measurement of molting hormone activity. In the molting hormonal activity test, integument fragments are treated with compounds one day, and the cultured integuments were further cultured for three days in the [14C]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 (pIC50 = 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.

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Electronic supplementary materials

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

References

1. T. Sparks and R. Nauen: IRAC: Mode of action classification and insecticide resistance management. *Pestic. Biochem. Physiol.* 121, 122–128 (2015).
2. J. J. van Daalen, J. Meltzer, R. Mulder and K. Wellinga: A selective insecticides with a novel mode of action. *Naturwissenschaften* 59, 312–313 (1972).
3. T. Haga, T. Toki, T. Koyanagi and R. Nishiyama: Quantitative structure–activity relationships of benzoyl(pyridyl)oxophenyl)urea insecticides. *Japanese J. Pestic. Sci.* 10, 217–223 (1985) (in Japanese).
4. J. Suzuki, T. Ishida, T. Shibuya and K. Toda: Development of a new acaricide, etoxazole. *Japanese J. Pestic. Sci.* 26, 215–223 (2001) (in Japanese).
5. T. Yamada, K. Kaeriyama, M. Natsui and H. Yoneda: Development of a new miticide, hysynthiazox. *Japanese J. Pestic. Sci.* 12, 327–335 (1987) (in Japanese).
6. K. Ikeda, M. Yasui, H. Kanno and S. Maekawa: Development of a new insecticide, buprofezin. *Japanese J. Pestic. Sci.* 11, 287–295 (1986) (in Japanese).
7. L. C. Post and W. R. Vincent: A new insecticide inhibits chitin synthesis. *Naturwissenschaften* 60, 431–432 (1973).
8. F. Matsumura: Studies on the action mechanism of benzoylurea insecticides in to inhibit the process of chitin synthesis in insects: A re-
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view on the status of research activities in the past, the present and the future prospects. *Pestic. Biochem. Physiol.* 97, 133–139 (2010).
9. T. Van Leeuwen, P. Demaeght, E. J. Osborne, W. Dermauw, S. Gohlke, R. Nauen, M. Gribic, L. Tirry, H. Merzendorfer and R. M. Clark: Population bulk segregant mapping uncovers resistance mutations and the mode of action of a chitin synthesis inhibitor in arthropods. *Proc. Natl. Acad. Sci. U.S.A.* 109, 4407–4412 (2012).
10. V. Douris, D. Steinbach, R. Panteleri, I. Livadaras, J. A. Pickett, T. Van Leeuwen, R. Nauen and J. Vontas: Resistance mutation conserved between insects and mites unravels the benzoylurea insecticide mode of action on chitin biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 113, 14692–14697 (2016).
11. Y. Suzuki, T. Shiotsuki, A. Jouraku, K. Miura and C. Minakuchi: Benzoylurea resistance in western flower thrips *Frankliniella occidentalis* (Thysanoptera: Thripidae): The presence of a point mutation in chitin synthase 1. *J. Pestic. Sci.* 42, 93–96 (2017).
12. K. Ogata and H. Uneyama: An automatic homology modeling method consisting of database searches and simulating annealing. *J. Mol. Graph. Model.* 18, 258–272, 305–306 (2000).
13. J. L. W. Morgan, J. Strumillo and J. Zimmer: Crystallographic snapshot of cellulose synthesis and membrane translocation. *Nature* 493, 181–186 (2013).
14. H. Merzendorfer: Insect chitin synthases: A review. *J. Comp. Physiol.* B 176, 1–15 (2006).
15. F. Huggenberger, E. A. Jennings, P. I. Ryan and K. W. Burrow: El-107, a new selective herbicide for use in cereals. *Proc. 1982 British Crop Protection Conference-Weeds* 1, 47–52 (1982).
16. J. S. Ward: N-[1,3,4-thiadiazol-2-yl]benzamides, US Patent 4114984 (1979).
17. Y. Nakagawa, K. Nishimura, K. Izumi, K. Kinoshita, T. Kimura, N. Kurihara and T. Fujita: Quantitative structure–activity relationships of larvicidal N-[5-(substituted phenyl)-1,3,4-thiadiazol-2-yl]-benzamides in the inhibition of N-acetylglucosamine incorporation into a cultured integument system. *J. Pestic. Sci.* 21, 195–201 (1996).
18. Y. Nakagawa, M. Matsutani, N. Kurihara, K. Nishimura and T. Fujita: Quantitative structure–activity studies of benzoylphenylurea larvicides. VIII. Inhibition of N-acetylglucosamine incorporation into the cultured integument of *Chilo suppressalis* Walker. *Pestic. Biochem. Physiol.* 43, 141–151 (1992).
19. Y. Nakagawa, M. Matsutani, N. Kurihara, K. Nishimura and T. Fujita: Inhibition of N-acetylglucosamine incorporation into the cultured integument of *Chilo suppressalis* by diflubenzuron. *Pestic. Biochem. Physiol.* 42, 242–247 (1992).
20. M. Sakuma: Probit analysis of preference data. *Appl. Entomol. Zool.* 33, 339–347 (1998).
21. Y. Nakagawa, K. Nishimura, N. Oikawa, N. Kurihara and T. Ueno: Activity of edysone analogs in enhancing N-acetylglucosamine incorporation into the cultured integument of *Chilo suppressalis*. *Steroids* 60, 401–405 (1995).
22. Y. Nakagawa, Y. Soya, K. Nakai, N. Oikawa, K. Nishimura, T. Ueno, T. Fujita and N. Kurihara: Quantitative structure–activity studies of insect growth regulators. XI. Stimulation and inhibition of N-acetylglucosamine incorporation in a cultured integument system by substituted N-tert-butyl-N,N′-dibenzoylhydrazines. *Pestic. Sci.* 43, 339–345 (1995).