*J. Pestic. Sci.* **49**(2), 87–93 (2024)
DOI: 10.1584/jpestics.D24-004

JOURNAL OF PESTICIDE SCIENCE

**Regular Article**

# Quantitative structure–activity relationship of 2,6-dimethoxy-*N*-(3-(4-substituted phenyl)isoxazol-5-yl)benzamide for the inhibition of chitin synthesis†

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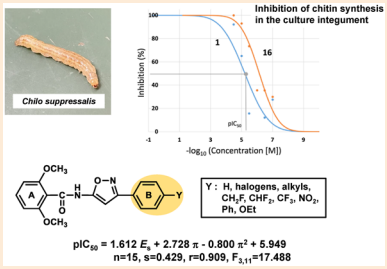
(Received January 25, 2024; Accepted March 17, 2024)

***Supplementary material***

Previously, we found that 5-(2,6-dimethoxybenzoylamino)-3-phenylisoxazoles (IOXs) inhibit chitin synthesis in the cultured integument of *Chilo suppressalis*. In this study, IOXs with various substituents at the *para*-position of the 3-phenyl ring were synthesized, and the concentrations required to inhibit chitin synthesis to 50% ($IC_{50}$) were determined for all compounds. The introduction of halogens—such as F, Cl, and Br—and small alkyls—such as Me, Et, Pr, and *n*-Bu—at the 3-phenyl ring slightly enhanced the activity. However, the activity decreased drastically with the introduction of $NO_2$, $CF_3$, and *t*-Bu. The quantitative analysis of the substituent effect at the 3-phenyl ring on chitin-synthesis inhibition using the Hansch-Fujita method showed that the hydrophobic substituent with the optimum value was favored for the activity, but the bulky substituent in terms of $E_s$ was detrimental to the activity.




***Keywords:*** chitin synthesis inhibitor, 5-benzoylamino-3-phenylisoxazole, QSAR, *Chilo suppressalis*, cultured integument.

## Introduction

About half a century ago, benzoylphenylurea (BPU) was discovered as a novel insecticide in the herbicide-development process in the Netherlands,[1)] which inhibited the chitin synthesis in insects.[2,3)] The first-published BPU was Du19111 (2,6-dichlobenzoyl-3,4-dichlorophenylurea), which was designed from the structures of two herbicides, dichlobenil (a cellulose synthesis inhibitor) and diuron (a photosynthesis inhibitor).[1)] Various substituents were introduced at two benzene rings of Du19111,[4,5)] leading to commercial insecticides such as diflubenzuron, triflumuron, and perfluron (Fig. 1).[6)] As shown in Fig. 1, the *ortho* positions of the benzoyl moiety of these compounds are singly or doubly substituted with F or Cl.[6)] The substituent effects at the benzoyl moiety were quantitatively analyzed,[7)] and the results showed that the introduction of small

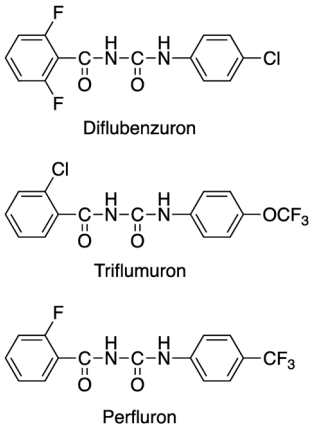



**Fig. 1.** Structures of BPUs commercialized in earlier years.

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Published online May 17, 2024
† 5-(2,6-Dimethoxybenzoylamino)-3-(substituted phenyl)-isoxazole is used in the text as the compound name instead of "2,6-dimethoxy-*N*-(3-(4-substituted phenyl)isoxazol-5-yl)benzamide" shown in the title.

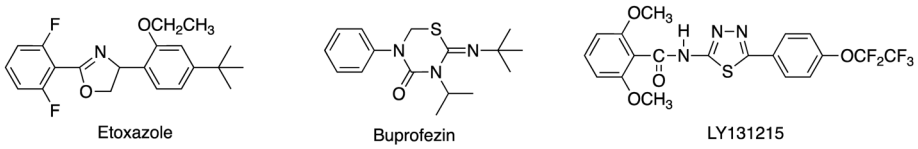



**Fig. 2.** Chemical structures of other chitin-synthesis inhibitors.

and hydrophobic electron withdrawal substituents at *ortho* positions is favorable for the activity, but the bulky substituents at the *meta* and *para* positions were detrimental.[7,8]

The substituent effects at the phenyl (or aniline) moiety of BPUs on the larvicidal activity against the rice stem borer, *Chilo suppressalis*, were quantitatively analyzed using the classical quantitative structure–activity relationship (QSAR).[9,10] This revealed that the important physicochemical property of substituents at the phenyl moiety for the larvicidal activity is hydrophobicity. In addition, we demonstrated that electronic effects on larvicidal activity are varied among insect species—such as *C. suppressalis*, the common cutworm, *Spodoptera litura*, and the silkworm, *Bombyx mori*—that are related to differences in the detoxication power of various insect species.[11] We also found that the oxidative and hydrolytic detoxications are governed by the electronic effects of substituents, and the intrinsic activity is enhanced by the introduction of electron-donating substituents.[12]

After the discovery of BPUs, etoxazole[13] and buprofezin[14] were developed as chitin-synthesis inhibitors that are used as an acaricide and an insecticide in agriculture, respectively (Fig. 2). Thiadiazole (TD)-type compounds, such as LY131215 (Fig. 2), were also reported as new chitin-synthesis inhibitors just after the launching of BPUs; however, they have not been registered in the pesticide market.[15] We synthesized TD analogs with various substituents at both benzoyl and phenyl rings and quantitatively analyzed the substituent effects on chitin-synthesis inhibition.[16] Structure–activity relationships are different between BPUs and TDs. With respect to the substitution at the benzoyl moiety, 2,6-$F_2$ substitution that is the best for BPUs was detrimental for TDs.

Recently, we reported that 2,6-dimethoxybenzoylamino-[3-(4-substituted phenyl)isoxazoles; IOXs) with a Cl, Et, or Ph substituent at the *para*-position of the 3-phenyl ring (B-ring) (Fig. 3) inhibit chitin synthesis.[17] In this study, we synthesized various IOXs with other substituents at the *para*-position of the B-ring, in which the A-ring moiety was fixed with a 2,6-dimethoxy substitution, and we quantitatively measured the chitin synthesis–inhibition activity in the cultured integument. With further study, the substituent effect at the B-ring of the IOXs on the activity was quantitatively analyzed by the classical QSAR (Hansch–Fujita method)[18] to find the physicochemical property essential for chitin-synthesis inhibition and compared with that of BPUs (Fig. 3).

## Materials and methods

*1. Chemicals*

The synthetic method is the same as that previously reported and is schematized in Fig. 4.[17] Methyl benzoates with various substituents (Y) at the *para*-position of the benzene ring (B-ring) were used to synthesize IOXs. Each methyl benzoate was prepared from the corresponding benzoic acid.

The synthetic procedure for compound **1** (Y=H) is shown below, and other analogs were synthesized in a manner similar to that of **1**. Compounds **1**, **2**, **4**–**9**, **11**–**17** were newly synthesized in this study, and three compounds (**3**, **10**, **18**) were synthesized in our previous study.[17] Since benzoic acids with *p*-$CH_2F$ and *p*-$CHF_2$ are not commercially available, they were synthesized from the corresponding benzyl alcohol and benzaldehyde, as shown below. The identification of compounds was done by NMR, elemental analysis and HRMS, as summarized in the supplementary file. Melting points were determined on a Yanako melting point apparatus (Yanagimoto Seisakusho Co. Ltd., Kyoto Japan). Elemental analysis was performed at Kyoto University's Center for Organic Elemental Microanalysis. High-resolution mass spectra (HRMS) were measured using Orbitrap Exploris 240 (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a heated electrospray ionization (ESI) source at a resolution of 240,000 in a positive mode. NMR spectra were recorded on a Bruker AVANCE III 400 spectrometer (Bruker Daltonics Inc., Billerica, MA, USA), in which tetramethylsilane (0 ppm) was used as an internal standard.

*5-(2,6-Dimethoxybenzoylamino)-3-phenyl-isoxazole* (***1***)

Benzoic acid (10 g, 81.9 mmol) dissolved in methanol (300 mL) with a catalytic amount of $H_2SO_4$ (2.0 mL) was refluxed overnight. The reaction mixture was concentrated *in vacuo*, and then diluted with water. The solution was extracted with chloroform twice. The combined organic layers were dried over $Na_2SO_4$ and

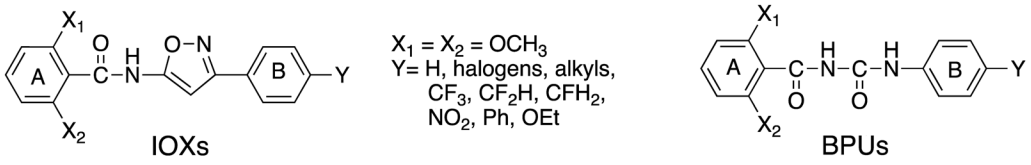



**Fig. 3.** Chemical structures of IOXs and BPUs.

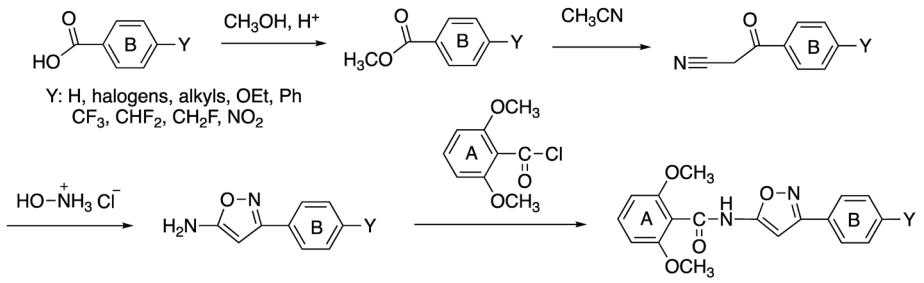



**Fig. 4.** General synthetic scheme for IOXs.

filtered. The filtrate was concentrated to obtain crude methyl benzoate (9.35 g, 83.9%).

Methyl benzoate (9.35 g, 68.7 mmol) and 60% NaH (7.38 g) were placed in a two-necked flask under an Ar atmosphere, and then dissolved in anhydrous toluene (100 mL). To the solution, anhydrous acetonitrile (10.8 mL, 206.1 mmol) was added dropwise within 30 min. After refluxing the mixture for 24 hr, it was quenched by adding water and then acidified to pH 2 with 6 M HCl. The solution was cooled down to obtain a solid material. The mixture was filtered to separate the solid material, and the filtrate was extracted with ethyl acetate three times and washed with brine. The organic layer was concentrated *in vacuo* to obtain crude 3-oxo-3-phenylpropanenitrille (11.0 g, 75.8 mol).

Crude 3-oxo-3-phenylpropanenitrille (11.0 g, 75.8 mmol) and hydroxylamine hydrochloride (5.25 g, 75.6 mmol) were added to the NaOH solution (6.08 g, 152 mmol in 200 mL of water). After refluxing the mixture for 24 hr, it was extracted with chloroform three times. The combined organic layer was dried over anhydrous $Na_2SO_4$ and concentrated *in vacuo* to afford crude 3-phenylisoxazol-5-amine (3.68 g, 23 mmol) as white solids. The yield was 30.4%.

NaH (60%, 1.33 g, 33.6 mmol) was placed in a two-necked flask under an Ar atmosphere, and then anhydrous THF (30 mL) was added. 3-Phenylisoxazol-5-amine (1.8 g, 11.2 mmol) dissolved in anhydrous THF was added dropwise to the NaH in THF solution in the ice bath and stirred for 30 min. To the mixture, 80% 2,6-dimethoxybenzoyl chloride (2.74 g, 11.2 mmol) was added, and it stood for 1 hr in the ice bath. The mixture was then stirred overnight at room temperature. The reaction mixture was quenched by adding a saturated ammonium chloride solution. The mixture was extracted with diethyl ether three times; the combined organic layer was washed with saturated $NaHCO_3$ and brine, and then dried over anhydrous $Na_2SO_4$. The filtrate was concentrated and recrystallized from ethyl acetate to afford 5-(2,6-dimethoxybenzoylamino)-3-phenylisoxazol (0.91 g, 2.81 mol). The yield was 25%. The elemental analysis data and NMR spectra are summarized in the supplementary material.

*Methyl 4-(monofluoromethyl)benzoate*

1,1,2,2-Tetrafluoroethyl-*N*,*N*-dimethyl-1-amine (TFEDMA: 8.86 g, 61 mmol, 2 equiv.) was added to a solution of methyl 4-(hydroxymethyl)benzoate (5.07 g, 30.5 mmol, 1.0 equiv.) in anhydrous $CH_2Cl_2$ (153 mL) within a Teflon® bottle at 0°C. The reaction mixture was stirred at room temperature for 60 min. Then, the mixture was cooled to 0°C, and saturated aqueous $NaHCO_3$ solution was added, followed by extraction with $CH_2Cl_2$. The combined organic layer was washed with brine and dried over anhydrous $Na_2SO_4$, and the filtrate was concentrated under reduced pressure to obtain the crude product. Purification was achieved by column chromatography (hexane/EtOAc, hexane/$Et_2O$, or hexane/ $CH_2Cl_2$) to yield the corresponding fluoride (3.22 g, 63%). The spectral data of the compound matched those reported in the literature.[19]

*Methyl 4-(difluoromethyl)benzoate*

An oven-dried narrow-mouth FEP tube (Nalgene®, 10.0 mL) was loaded with methyl 4-formylbenzoate (1.00 g, 6.09 mmol) and FLUOLEAD® (2.59 g, 10.4 mmol) in 12.2 mL of $CH_2Cl_2$ and stirred. To the mixture, 56 mg (0.06 mmol) of EtOH was added and stirred at room temperature. After 24 hr, the mixture was poured into 1 M aqueous $NaHCO_3$ (3 mL) at 0°C, which was extracted with $CH_2Cl_2$ (3×5 mL) and washed with brine. The combined organic layer was dried over anhydrous $Na_2SO_4$, filtered, and concentrated under reduced pressure. The resulting residue was purified by silica gel flash chromatography (*n*-hexane/AcOEt: 20/1) to yield the title compound (875.5 mg, 77%). The spectral data of the compound matched those reported in the literature.[19]

*2. Measurement of chitin-synthesis inhibition*

The method is same as that previously reported.[17,20] In brief, integument fragments excised from diapause larvae of *C. suppressalis* were incubated in medium containing 20-hydroxyecdysone (0.2 $\mu$M) for 24 hr at 25°C, and then transferred to a hormone-free medium containing *N*-acetyl-D-[1-$^{14}$C]glucosamine ([$^{14}$C]NAG: 0.16–0.24 nmol, 20000–30000 dpm/well), piperonyl butoxide (PB, 20 $\mu$M), and a test compound. After the integument fragments were incubated for 3 d., they were transferred to the vial for liquid scintillation counting by AccuFLEX LSC-8000 (Aloka, Tokyo, Japan) to measure the incorporated radioactivity of the cultured integument.

## Results and discussion

*1. Inhibition of chitin synthesis by IOXs*

Previously, we constructed an *in vitro* bioassay method to quantitatively measure the activity of chitin-synthesis inhibitors in insects using the cultured integument of *C. suppressalis*.[12,20] We also reported that PB has a synergistic effect on the *in vitro*

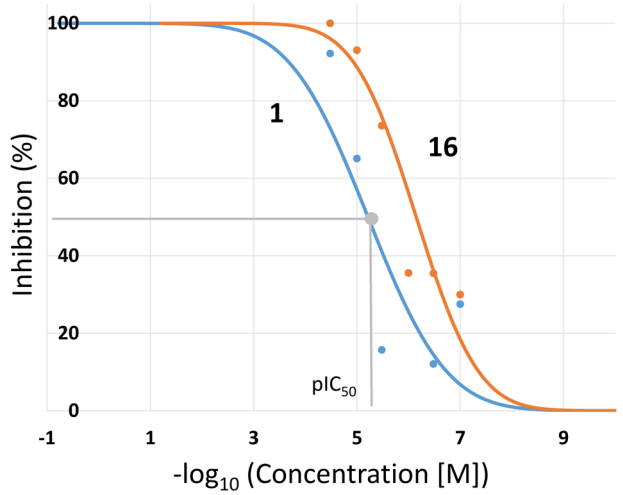


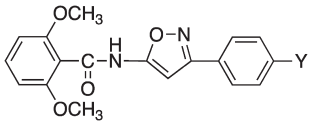

**Fig. 5.** Concentration response relationships for chitin-synthesis inhibition by compounds **1** (blue) and **16** (orange).

activity of BPUs[12] and TDs.[16] Recently, we found the synergistic effect of PB on the activity of IOXs with Et at the B-ring.[17] Therefore, the chitin synthesis–inhibitory activity of all IOXs was measured under synergistic conditions with PB.

Most of IOXs significantly inhibited chitin synthesis in the cultured integument as observed for BPUs and TDs. The concentration required to inhibit the [$^{14}$C]NAG incorporation to 50% ($IC_{50}$ [M]) was determined from each concentration-response curve (Fig. 5), and the reciprocal logarithm of the $IC_{50}$ ($pIC_{50}$) was determined and was used as the index of the activity for chitin-synthesis inhibition. The $pIC_{50}$ values of IOX analogs are listed in Table 1. Some of them were determined from the respective single dose response curve. But, before determination of $pIC_{50}$ values, the chitin synthesis inhibition was measured under the concentrations near the $IC_{50}$. Three compounds (**3, 10, 18**) were previously tested and cited from the previous publication,[17] but the concentration-response of compound **10** was reexamined in this study.

As shown in Table 1, the activity of unsubstituted compound (**1**) was comparable to that of the analog with F (**2**), but it was 1/2 that of the analog with Cl (**3**) or Br (**4**). The introduction of another halogen I (**5**) was not favorable. Et (**10**) is a better substituent than Cl (**3**) for the activity measured under a synergistic condition with PB. Among alkyl substituents, bulky substituents such as *t*-Bu (**14**) and *n*-Hex (**15**) are unfavorable to the activity, but the effects of short, normal alkyl groups—such as $CH_3$ (**9**), *n*-Pr (**11**), and *n*-Bu (**13**)—were equivalent to those of H (**1**) and Cl (**3**). The activity of **12** (*i*-Pr) was slightly lower than that of **11** (*n*-Pr). Electron-donating substituent OEt (**16**) was favor-

**Table 1.** Inhibitory activity of IOXs for chitin synthesis.




| Compounds | | Inhibition of chitin synthesis ($pIC_{50}$) | | |
|---|---|---|---|---|
| No | Substituents | Obsd.[a] | Calcd. (Eq. 2) | Δ[b] |
| **1** | H | 5.58±0.52 (2) | 5.95 | −0.37 |
| **2** | F | 5.51 | 5.57 | −0.06 |
| **3** | Cl | 5.83[b] | 5.92 | −0.09 |
| **4** | Br | 5.96 | 5.83 | 0.13 |
| **5** | I | 4.93 | 5.74 | −0.81 |
| **6** | $CF_3$ | 3.84±0.37 (2) | 3.86 | −0.02 |
| **7** | $CHF_2$ | <4.00 (27.9%) | 4.17 | — |
| **8** | $CH_2F$ | 4.28 | 4.16 | 0.12 |
| **9** | $CH_3$ | 5.64±0.50 (2) | 5.23 | 0.41 |
| **10** | Et | 6.50±0.60 (3) | 5.79 | 0.71 |
| **11** | *n*-Pr | 5.64±0.11 (2) | 5.68 | −0.04 |
| **12** | *i*-Pr | 5.24±0.13 (2) | 5.49 | −0.25 |
| **13** | *n*-Bu | 6.05 | 5.50 | 0.55 |
| **14** | *t*-Bu | 3.45 | 3.73 | −0.28 |
| **15** | *n*-Hex | <4.00 (39.1%) | 4.02 | — |
| **16** | OEt | 6.15 | 5.98 | 0.17 |
| **17** | $NO_2$ | <4.00 (24.2%) | 1.06[c] | — |
| **18** | Ph | 6.43[d] | 6.59 | −0.16 |

[a] The number in parentheses are the number of repetitions. If not stated, it represents the result of a single measurement. [b] Δ=Observed $pIC_{50}$−Calculated $pIC_{50}$. [c] If the less bulky Es value (−1.01) was used in the prediction, the calculated is 3.49. [d] From ref. (Mori *et al.*, 2021)

able to the activity, but electron-withdrawing substituents such as $CF_3$ (**6**) and $NO_2$ (**17**) were detrimental to the activity. Since the activity of compound **6** ($CF_3$) was 100 times lower than that of **3** ($CH_3$), other fluorinated methyl substituents such as $CHF_2$ (**7**) and $CH_2F$ (**8**) were introduced to examine the effect of the F atom. The activity of $CH_2F$ (**8**) is 20 times lower than that of $CH_3$ (**3**), and the activity of $CHF_2$ (**7**) was too low to determine $pIC_{50}$.

*2. QSAR analysis of substituent effects*

The substituent effect on the inhibition of chitin synthesis was quantitatively analyzed using classical QSAR (Hansch–Fujita method).[18] As shown in Table 1, the activities of **6** ($CF_3$) and **14** (*t*-Bu) are very low as compared to those of other substituted compounds. These substituents are bulky in terms of steric parameter $E_s$ (Table 2).[21,22] $E_s$ is defined from the hydrolysis of methyl acetate analogs with various substituents, in which electronic effects affecting hydrolysis are eliminated. $E_s$ values are negative, and $E_s$ with large absolute value means bulky. $E_s$ is the free-energy related parameter that is different from the volume and length parameters. For all compounds with $pIC_{50}$ values, significant correlation Eq. 1 was formulated using $E_s$ and a hydrophobic parameter, $\pi$ (Table 2).[22,23]

$$pIC_{50} = 1.406(\pm0.575)E_s + 0.885(\pm0.576)\pi + 6.351(\pm0.660) \quad (1)$$
$$n = 15, \quad s = 0.537, \quad r = 0.838, \quad F_{2,12} = 14.177$$

In this and the following equations, $n$ is the number of compounds, $s$ is the standard deviation, $r$ is the correlation coefficient, and $F$ is the value of the $F$ ratio between variances of calculated and observed activities. In our previous QSAR study, we used van der Waals volume[16] or STERIMOL[24] parameters as the steric parameter, but these parameters were not significant for QSAR analysis in this study. By adding the squared term of $\pi$ to Eq. 1, the correlation was improved drastically, as shown in Eq. 2. Even though two $E_s$ values (−1.01, −3.82) are available for the phenyl substituent,[21,22] −1.01 was used to derive Eq. 1 and 2. The value −1.01 means sterically less bulky than −3.82. If −3.82 was used instead of −1.01, the significant correlation could not be derived. Therefore, Ph substituent is thought to be coplanar to B-ring.

$$pIC_{50} = 1.612(\pm0.491)E_s + 2.728(\pm1.518)\pi - 0.800(\pm0.628)\pi^2 + 5.949(\pm0.618) \quad (2)$$
$$n = 15, \quad s = 0.429, \quad r = 0.909, \quad F_{3,11} = 17.488, \quad \pi_{opt} = 1.7$$

The $pIC_{50}$ values calculated by Eq. 2 are listed in Table 1, where inactive compounds, such as **7** ($CHF_2$), **15** (*n*-Hex), and **17** ($NO_2$), are predicted. If the less bulky $E_s$ value (−1.01) was used for $NO_2$, the calculated $pIC_{50}$ of compound **17** is 3.49, which is also acceptable. The $NO_2$ substituent may take the conformation between vertical and horizontal toward B-ring. From Eq. 2, the optimum $\pi$ value is calculated to be 1.7, which is close to that of the Pr or Bu group. Previously, we derived Eq. 3 for the substituent effect at the phenyl moiety of BPUs on chitin-synthesis inhibition.[12]

$$pIC_{50} = -0.589\sigma + 1.487\pi - 0.565\pi^2 + 7.189 \quad (3)$$
$$n = 15, \quad s = 0.267, \quad r = 0.916, \quad F_{3,11} = 19.1, \quad \pi_{opt} = 1.32$$

As shown in Eq. 3, electronic substituent parameter $\sigma$ was significant, in addition to the hydrophobic parameter, although the addition of the steric parameter was insignificant. There was an optimum hydrophobicity ($\pi_{opt}$=1.32) for the phenyl substituents, the value of which was not far from that ($\pi_{opt}$=1.7) obtained for IOXs (Eq. 2).

Since the electronic effect was significant in the QSAR for BPUs, the addition of an electronic parameter to Eq. 2 was considered. Even though the $\sigma$ term was only justified at a 73% level by *t*-test, a significant correlation equation (Eq. 4) in terms of the *F*-test was derived. The coefficient of $\sigma$ term is −0.707, meaning that an electron-donating substituent is favorable for the activity, which is close to that (−0.589) derived for BPUs (Eq. 3).

**Table 2.** Substituent parameters used for QSAR analysis.

| Substituent | $\pi$ | $E_s$ | Substituent | $\pi$ | $E_s$ |
|---|---|---|---|---|---|
| H | 0 | 0 | F | 0.14 | −0.46 |
| $CH_3$ | 0.56 | −1.24 | Cl | 0.71 | −0.97 |
| Et | 1.02 | −1.31 | Br | 0.86 | −1.16 |
| *n*-Pr | 1.55 | −1.60 | I | 1.12 | −1.40 |
| *i*-Pr | 1.53 | −1.71 | $CH_2F$ | 0.13[a] | −1.32 |
| *n*-Bu | 2.13 | −1.63 | $CHF_2$ | 0.21[b] | −1.44 |
| *t*-Bu | 1.98 | −2.78 | $CF_3$ | 0.88 | −2.4 |
| *n*-Hex | 3.19[c] | −1.54 | OEt | 0.38 | −0.55 |
| Ph | 1.96 | −1.01[d] | $NO_2$ | −0.28 | −2.52[e] |

[a] Log P value of $PhCH_2F$ is calculated to be 2.26 by MacLog P. [b] Log P value of $PhCHF_2$ is calculated to be 2.34 by MacLog P. [c] $\pi$(*n*-Hex)=2.13 ($\pi$: *n*-Bu)+2×($\pi$: $CH_2$)=2.13+2×0.53=3.19. [d] There are two $E_s$ values (−1.01, −3.82) for Ph. [e] There are two $E_s$ values (—1.01, —2.52) for $NO_2$.

$$pIC_{50} = 1.642(\pm 0.221)E_s + 2.947(\pm 0.706)\pi - 0.937(\pm 0.305)\pi^2 - 0.707(\pm 0.617)\sigma + 6.09(\pm 0.303) \quad (4)$$
$$n = 15, \quad s = 0.423, \quad r = 0.920, \quad F_{4,10} = 13.884, \quad \pi_{opt} = 1.57$$

The reason the steric parameter did not become significant in the QSAR for BPUs is unknown, but these QSAR results derived for BPUs and IOXs will be very helpful when discussing the ligand-enzyme interaction of chitin-synthesis inhibitors.

In conclusion, fifteen IOXs were newly synthesized, and the chitin-synthesis inhibition was quantitatively measured in the cultured integument of *C. suppressalis*. Many IOXs inhibited chitin synthesis at the micromolar level. Concentrations that inhibit chitin synthesis by 50% ($IC_{50}$) were determined from each concentration-response curve, and the reciprocal logarithm of $IC_{50}$ ($pIC_{50}$) was quantitatively analyzed by classical QSAR. The result indicates that the activity is enhanced with the introduction of a hydrophobic substituent with optimum value, although the bulky substituents in terms of $E_s$ were not favorable to the activity. QSAR results also suggested that the binding mode of IOXs resembles that of BPUs. Thus, the substituent effects at the A-ring moiety are expected to resemble those for BPUs. A structure–activity relationship study is in progress for other sets of IOX analogs, especially those with various substituents on the A-ring.

## Acknowledgements

We thank Sumitomo Chemical for providing rice stem borer eggs. This study was partly supported by KAKENHI (19K06051). K. M. was supported by the JSPS Research Fellowship for Young Scientists (23KJ1267).

## Electronic supplementary materials

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

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