Steroids

Relationships between structure and molting hormonal activity of tebufenozide, methoxyfenozide, and their analogs in cultured integument system of *Chilo suppressalis* Walker☆

Yoshiaki Nakagawa*, Kazunari Hattori, Chieka Minakuchi, Soichi Kugimiya, Tamio Ueno

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

Abstract

The molting hormonal activity of methoxyfenozide (RH-2485), tebufenozide (RH-5992), five analogs with various alkyl groups, and 18 acyl analogs was measured by using cultured integument of rice stem borers, *Chilo suppressalis* Walker. The hormonal activity of methoxyfenozide was remarkably high ($EC_{50} = 1.1 \times 10^{-9}$ M), being equivalent to that of tebufenozide (RH-5992). The hormonal activity of several tebufenozide analogs with varying alkyl groups such as CH_3, n-C_3H_7, i-C_3H_7, n-C_4H_9 and n-C_5H_{11} at the *para*-position of the benzene ring furthest from the *tert*-butyl group was lower than that of tebufenozide (alkyl group is C_2H_5). The activity decreased to varying degrees as a result of replacement of the 3,5-dimethylphenyl moiety of tebufenozide with either a phenyl, naphthyl, or cyclohexyl group. Both 1- and 2-naphthyl derivatives were very active ($EC_{50} = 4.3 \times 10^{-8}$ M and 3.2×10^{-8} M, respectively) without any significant difference between them. The activity of the 1-cyclohexenyl analog ($EC_{50} = 1.0 \times 10^{-7}$ M) was about $40\times$ that of the corresponding 3-cyclohexenyl analog ($EC_{50} = 4.4 \times 10^{-6}$ M), but 1/100 that of tebufenozide. The activity varied parabolically with respect to the molecular hydrophobicity, and decreased with longer acyl moieties. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Tebufenozide (RH-5992); Methoxyfenozide (RH-2485); QSAR; Ecdysteroids; Diacylhydrazines; Hydrophobicity

1. Introduction

N-Benzoyl-*N*-*t*-butyl-*N′*-benzoylhydrazine [RH-5849, **I**: $X_n = Y_n = H$] was reported as the first synthetic nonsteroidal ecdysteroid agonist that induced premature molting in Lepidoptera [1,2]. Tebufenozide [RH-5992, **I**: $X_n = 3,5$-$(CH_3)_2$, $Y_n = 4$-C_2H_5] was first registered in 1994 in Europe and Japan to control lepidopterous insects, followed by the U.S.A. [3,4]. Recently, a more potent analog with lower mammalian toxicity, methoxyfenozide [RH-2485, **I**: $X_n = 3,5$-$(CH_3)_2$, $Y_n = 2$-CH_3-3-OCH_3], has been developed to control Lepidoptera [5,6]. However, tebufenozide and methoxyfenozide have extremely low potency against coleopterous insects such as Colorado potato beetles (*Lepti-*

notarsa decemlineata) [7–10]. On the other hand, their analogs such as RH-5849 (**I**: $X_n = Y_n = H$) and halofenozide (RH-0345, **I**: $X_n = H$, $Y_n = 4$-Cl) are rather potent against Coleoptera [8,11].

(I)
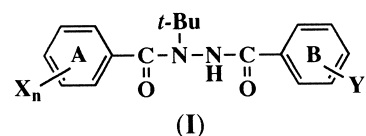
Even though all insects use 20-hydroxyecdysone (20E) in their endocrine systems, the susceptibility to nonsteroidal ecdysteroid agonists varies drastically among insect species. To explain the differences in the toxicity spectrum among these compounds, the pharmacokinetics and metabolic detoxification mechanisms were compared by using radio–labeled tebufenozide [7] and RH-5849 [12]. However, significant differences were not observed [7,12]. Currently, it is thought that the selec-

tivity is caused by differences in the amino acid sequence in the 20E-binding site of the ecdysteroid receptor (EcR) among insect species. The amino–acid sequences of several EcRs are known to demonstrate interspecies variation [13–18]. However, the binding modes between the EcR and ecdysteroid analogs are unknown.

To gain insight into the molecular mechanism of nonsteroidal ecdysteroid agonists, we quantitatively analyzed the relationship between structure and activity [10,19–24] by using quantitative structure-activity relationship (QSAR) techniques such as the Hansch–Fujita approach [25] and comparative molecular field analysis (CoMFA) [26]. Recently, CoMFA was applied to the structure-activity relationship study of steroidal ecdysteroid agonists [27]. We found that with increasing molecular hydrophobicity there was both greater hormonal [21–23] and insecticidal activity [19,20] against lepidopterous rice stem borer *Chilo suppressalis* Walker. A similar QSAR was obtained for the insecticidal activity between rice stem borers and beet army worms *Spodoptera exigua* [24], which both are lepidopterous insects. However, the results were different from the QSAR derived for the larvicidal activity against the coleopterous insect, the Colorado potato beetle [10]. The insecticidal activity against the two lepidopterous insects increased with molecular hydrophobicity within a limited range of log P (log P < 4.5), but the activity against potato beetles varied in a bilinear manner with respect to log P. The activity of mono–substituted compounds generally increased with log P, except for several hydrophobic compounds. However, activity decreased with log P for disubstituted and hydrophobic mono–substituted compounds [10]. In an additional structure-activity study we found that the alkyl side chain of 20-hydroxyecdysone corresponds to the B-ring moiety in structure **I** [28]. By synthesizing and assaying a number of alkanoyl analogs, this correspondence was confirmed through the use of three-dimensional QSAR [23]. The aim of this study was to obtain more precise information on the structural factors that enhance hormonal activity. We therefore quantitatively analyzed the hormonal activity of various acylhydrazines, including alkanoyl analogs, by using physicochemical parameters such as molecular hydrophobicity and acyl moiety length.

2. Experimental

2.1. Chemicals

Compounds **8**, **13**, **14**, **16**, **22–25** (Table 1) were newly synthesized according to the conventional methods previously reported [10,19,20,23,24,28,29]. Methoxyfenozide **15** was also synthesized according to the reported method as shown in Fig. 1 [5]. Chemicals used for organic syntheses were obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA), Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan), Wako Pure Chemical Industries,

Ltd. (Osaka, Japan), and Nacalai Tesque Inc. (Kyoto, Japan). Racemic 3-cyclohexene-1-carboxylic acid used to synthesize compound **25** was purchased from Tokyo Kasei Kogyo Co. Ltd. *N*-Acetyl-[1-^{14}C]glucosamine ([^{14}C]GluNAc, 58.7 mCi/mmol) was purchased from Amersham International plc (Buckinghamshire, UK). The proportion of enantiomers of compound **25** was 1:1 by HPLC analysis with a Chiralcel OD-RH column (Daicel Chemical Industries, Ltd., Tokyo, Japan). The intermediates and final compounds were purified by either recrystallization or column chromatography by using Wakogel C-200 (Wako Pure Chemical Industries, Ltd.). The structures and the purity of newly synthesized compounds were confirmed by both [^{1}H]NMR and elemental analyses. The analytical values for C, H, and N agreed with the calculated values within $\pm$ 0.3%. [^{1}H]NMR spectra were recorded on a Bruker AC-300 NMR spectrometer at 300 MHz in deuteriochloroform (CDCl$_3$) with tetramethylsilane as the internal standard. Other compounds are identical to those used in our previous studies [20,23,28]. Melting points of all compounds were determined on a Yanako melting point apparatus (Yanagimoto Seisakusho Co. Ltd., Kyoto, Japan) and were uncorrected. Melting points of final compounds are listed in Table 1.
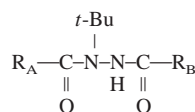
2.2. 3-Methoxy-2-methylbenzoic acid

Concentrated H$_2$SO$_4$ (2.9 g, 29.8 mmol) was added dropwise to 3-amino-2-methylbenzoic acid (3.0 g, 19.8 mmol) suspended in methanol (21.6 ml) in an ice bath. After warming up the suspension to 55°C, 33.3% aqueous NaNO$_2$ (4.3 g, 89.3 mmol) was slowly added over a period of 45 min. After letting the mixture stand for 1 h, 10 ml of 25% aqueous NaOH was slowly added over a period of 2 h, then dimethylsulfate (5 g, 39.6 mmol) was added over 45 min. Throughout the reaction the temperature was kept between 55 and 65°C. After evaporating the methanol, the residue was dissolved in ethyl acetate (250 ml). The organic layer was washed with 1 M H$_2$SO$_4$ and brine. After drying the organic layer over anhydrous magnesium sulfate, the solvent was removed under reduced pressure. The residue was dissolved in a minimal amount of hot methanol and poured into 1 M H$_2$SO$_4$ (24 ml) to yield a solid material. The solid was collected by filtration and manipulated from benzene to afford a brown powder (1.76 g, yield 53.4%).

2.3. N′-t-Butyl-N′-(3,5-dimethylbenzoyl)-3-methoxy-2-methylbenzohydrazine (RH-2485; 15)

N-t-Butyl-*N*-3,5-dimethylbenzohydrazine (1.20 g, 5.5 mmol), prepared from 3,5-dimethylbenzoic acid and *t*-butylhydrazine according to conventional methods [23], was suspended in anhydrous ether (9 ml). This ether solution along with triethylamine (3 ml) was simultaneously added

Table 1

Molting hormonal activity, melting points, and QSAR parameters for diacylhydrazine analogs

$$R_A-\overset{\overset{\textit{t-Bu}}{|}}{\underset{\underset{O}{\|}}{C}}-\underset{\underset{H}{|}}{N}-N-\underset{\underset{O}{\|}}{C}-R_B$$

No.	Compounds		pEC$_{50}$ (M)		log P[b]	Length (Å)[c]		m.p. (°C)[d]
	R$_A$	R$_B$	Obsd[a]	Calcd		D$_A$	D$_B$	
1	C$_6$H$_3$[3,5-(CH$_3$)$_2$]	n-C$_3$H$_7$	5.53[e]	5.72	2.60[g]	4.28	3.86	162–163[e]
2	C$_6$H$_3$[3,5-(CH$_3$)$_2$]	n-C$_4$H$_9$	7.10[f]	6.84	3.13[g]	4.28	5.03	161–162[h]
3	C$_6$H$_3$[3,5-(CH$_3$)$_2$]	n-C$_5$H$_{11}$	8.05[f]	7.45	3.66[g]	4.28	6.34	136–137[h]
4	C$_6$H$_3$[3,5-(CH$_3$)$_2$]	i-C$_5$H$_{11}$	7.97[f]	7.95	3.53[g]	4.28	5.03	115–116[h]
5	C$_6$H$_3$[3,5-(CH$_3$)$_2$]	n-C$_6$H$_{13}$	8.13[f]	7.69	4.19[g]	4.28	7.56	91–92[h]
6	C$_6$H$_3$[3,5-(CH$_3$)$_2$]	i-C$_6$H$_{13}$	7.96[f]	8.23	4.06[g]	4.28	6.34	144–145[h]
7	C$_6$H$_3$[3,5-(CH$_3$)$_2$]	n-C$_7$H$_{15}$	6.35	7.48	4.72[g]	4.28	8.83	89–90[e]
8	C$_6$H$_3$[3,5-(CH$_3$)$_2$]	n-C$_9$H$_{19}$	5.50	5.80	5.78[g]	4.28	11.34	114
9	C$_6$H$_3$[3,5-(CH$_3$)$_2$]	C$_6$H$_4$(4-CH$_3$)	8.07	8.39	3.95	4.28	5.78	240–241[i]
10	C$_6$H$_3$[3,5-(CH$_3$)$_2$]	C$_6$H$_4$(4-C$_2$H$_5$)	8.94[f]	8.53	4.39	4.28	6.62	194–195[i]
11	C$_6$H$_3$[3,5-(CH$_3$)$_2$]	C$_6$H$_4$(4-n-C$_3$H$_7$)	8.35	8.02	4.86	4.28	8.07	175–176[i]
12	C$_6$H$_3$[3,5-(CH$_3$)$_2$]	C$_6$H$_4$(4-i-C$_3$H$_7$)	8.28	8.92	4.86	4.28	6.59	202–203[i]
13	C$_6$H$_3$[3,5-(CH$_3$)$_2$]	C$_6$H$_4$(4-n-C$_4$H$_9$)	7.29	7.45	5.39	4.28	9.05	142–143
14	C$_6$H$_3$[3,5-(CH$_3$)$_2$]	C$_6$H$_4$(4-n-C$_5$H$_{11}$)	6.80	6.17	5.92	4.28	10.49	178–181
15	C$_6$H$_3$[3,5-(CH$_3$)$_2$]	C$_6$H$_4$(2-CH$_3$-3-OCH$_3$)	8.95	9.26	3.93	4.28	4.28	206–208
16	C$_6$H$_5$	C$_6$H$_4$(4-C$_2$H$_5$)	8.24	6.92	3.51[g]	4.28	6.63	214–215
17	n-C$_4$H$_9$	C$_6$H$_4$(4-C$_2$H$_5$)	5.40[e]	5.29	3.16[g]	5.04	6.62	145–146[e]
18	cyc-C$_4$H$_7$	C$_6$H$_4$(4-C$_2$H$_5$)	4.14[e]	4.37	2.49[g]	3.42	6.64	190–191[e]
19	n-C$_5$H$_{11}$	C$_6$H$_4$(4-C$_2$H$_5$)	5.09[f]	5.50	3.69[g]	6.35	6.62	112–113[f]
20	n-C$_6$H$_{13}$	C$_6$H$_4$(4-C$_2$H$_5$)	5.17[f]	5.40	4.22[g]	7.54	6.62	140–141[f]
21	cyc-C$_6$H$_{11}$	C$_6$H$_4$(4-C$_2$H$_5$)	5.06[e,j]	7.08	3.60[g]	4.34	6.63	210–211[e]
22	1-C$_{10}$H$_7$	C$_6$H$_4$(4-C$_2$H$_5$)	7.37[j]	8.87	4.69[g]	4.30	6.48	177–179
23	2-C$_{10}$H$_7$	C$_6$H$_4$(4-C$_2$H$_5$)	7.50	6.90	4.69[g]	6.51	6.48	205–206
24	1-cyc-C$_6$H$_9$	C$_6$H$_4$(4-C$_2$H$_5$)	7.00	7.12	3.57[g]	4.32	6.48	204–205
25	3-cyc-C$_6$H$_9$	C$_6$H$_4$(4-C$_2$H$_5$)	5.36	5.78	3.12[g]	4.34	6.63	202–203

[a] Newly measured unless otherwise noted.

[b] Empirically estimated from the corresponding benzamides or hydrazine analogs (see Refs. [20,35]), unless otherwise noted.

[c] The distance between the terminal carbon of each acyl moiety and the corresponding carbonyl carbon in structure **I**.

[d] Newly measured unless otherwise noted.

[e] From Ref. [23].

[f] From Ref. [28].

[g] Calculated by CLOGP method.

[h] Melting point data from previous reports (Refs. [23,28]) was printed out of sequence and has been corrected in this publication.

[i] From Ref. [20].

[j] Not included in the correlation analysis.

to anhydrous ether (9 ml) containing 3-methoxy-2-methyl-benzoyl chloride (0.87 g, 4.7 mmol), prepared from the corresponding benzoic acid shown above, and stirred in an ice bath. After stirring for 4 h, the precipitate was collected by filtration and dissolved in ethyl acetate. The organic layer was washed with 1 M NaOH, 1 M HCl, and brine successively, then dried over anhydrous magnesium sulfate. After evaporating the solvent, the residue was recrystallized from ethyl acetate to afford colorless needles 1.07 g (yield 61.6%). [^{1}H]NMR δ (ppm): 1.60 (9H, s), 1.90 (3H, s), 2.27 (6H, s), 3.78 (3H, s), 6.15 (1H, d), 6.81 (1H, d), 6.98–7.03 (2H, m), 7.07 (2H, s), 7.41 (1H, s). Analysis for calculated C (71.71), H (7.66), and N (7.60), Found C (71.54), H (7.62), N (7.64). m.p. 206–208°C.

2.4. Computations and physicochemical parameters for QSAR

Computations were performed with the molecular modeling software package SYBYL (ver 6.5; Tripos Co., St. Louis, MO, USA). The initial conformation of each compound was derived from the X-ray diffraction structure of RH-5849 previously reported [22]. Each structure was optimized by the semi-empirical molecular orbital method, PM3, by using the program package MOPAC 5.0 contained in the SYBYL module. The initial structures of alkanoyl analogs were constructed from the dibenzoylhydrazines by replacing each ring part with the corresponding alkyl group. The distance (D$_B$ with the unit of Å) between the carbonyl
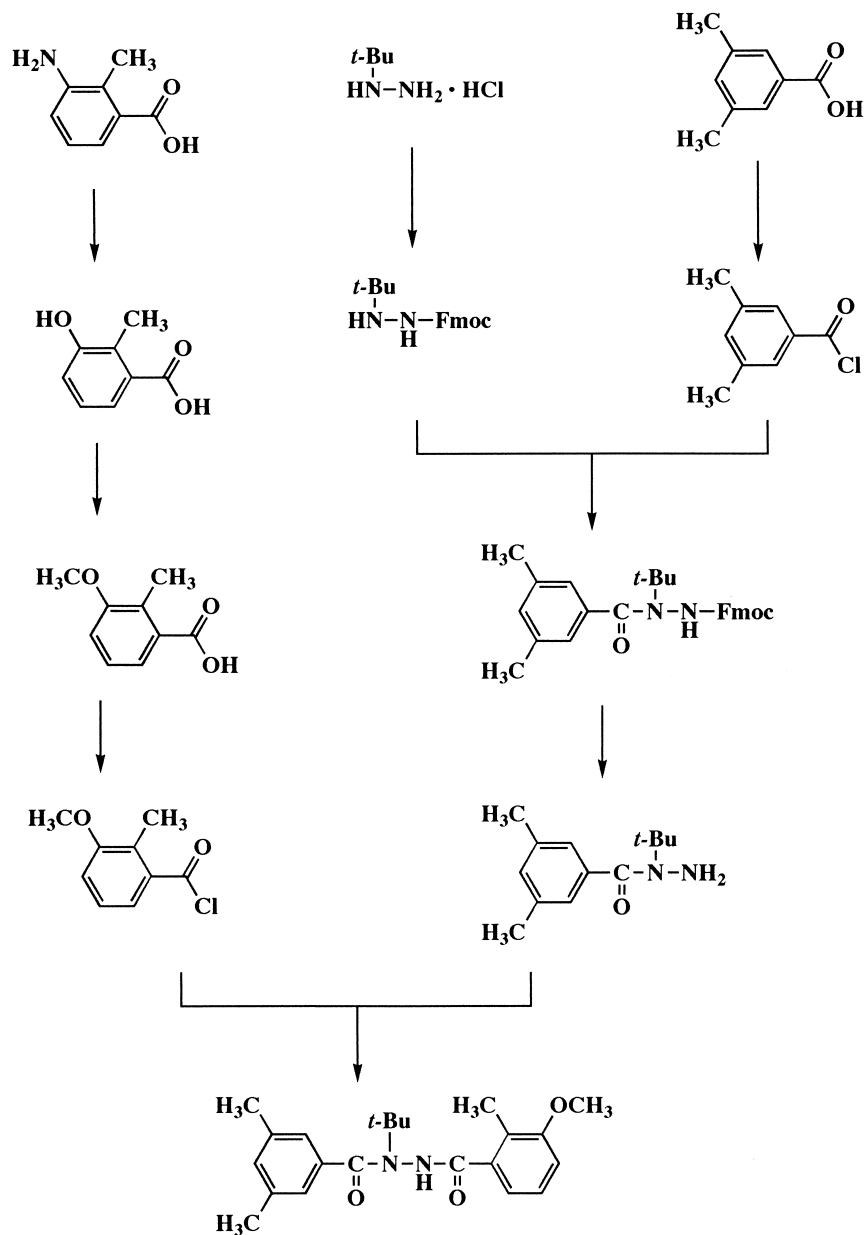
Fig. 1. Synthetic scheme of methoxyfenozide **15**.

carbon and the terminal carbon of the B-ring substituents for the dibenzoylhydrazine or the acyl terminal carbon in the alkanoyl analogs was measured with the SYBYL basic module. For methoxyfenozide **15**, the distance between the carbonyl carbon atom and the *para*-carbon atom of the B-ring was measured. The length of the acyl group for the A-ring moiety (D_A) was also measured in a similar manner as that for D_B. These D_A and D_B values are listed in Table 1. The molecular hydrophobicity values, in terms of log P, were estimated empirically [20,35] or calculated by CLOGP (MacLogP 2.5, BioByte Corp., Claremont, CA, USA), and are listed in Table 1. The log P values calculated by the CLOGP method are considered to be close to the experimental values because the calculated value (2.45) of unsub-

stituted dibenzoylhydrazine (**I**, $X_n = Y_n = H$) was identical to the experimental value determined with an octanol/water partitioning system [19]. Hansch–Fujita-type analysis [25] was executed by using the QREG program (Ver. 2.05; Tanabe Seiyaku Co., Ltd., Osaka, Japan) developed by Asao et al. [36].

2.5. Bioassays

The molting hormonal activity of all compounds was evaluated in the cultured integument as reported previously [21,30]. Briefly, integument fragments excised from the diapause larvae of rice stem borer were cultured for 24 h in the medium containing compounds at various concentra-

tion. Fragments were then transferred to fresh medium containing [^{14}C]GluNAc (6000–8000 d.p.m./ml). After culturing for 72 h, the medium was removed and the integument fragments were washed with distilled water. Scintillation cocktail (Aquasol II; Packard Instrument Co., Meriden, CT, USA) was added and the radioactivity incorporated into the fragments was measured with a liquid scintillation counter (Aloka LSC1000; Aloka Co. Ltd., Tokyo, Japan). In each assay, treatments with 20-hydroxyecdysone (20E) and dimethyl sulfoxide (DMSO) were also performed as positive and negative controls, respectively. Various concentrations of the stock solution of each compound were prepared with DMSO. The highest and lowest radioactivity levels were set using the concentration–response relationship for the incorporation of [^{14}C]GluNAc from 20E (100%) and DMSO (0%) treatments. From each curve, the half-effective concentration, EC_{50} (M), at the ascending phase was evaluated by probit analysis [31–33]. The reciprocal logarithm values of the EC_{50} (pEC_{50}) were used as an index of the molting hormonal activity and are listed in Table 1.

3. Results and discussion

3.1. Molting hormonal activity

We previously found that, to retain high hormonal activity, the optimum length of the alkyl chain of alkanoyl analogs is C_5 to C_6 [23,28]. This length approximately corresponds to the alkyl side chain of 20E. To confirm the requirement for the optimum chain length with respect to the acyl moiety corresponding to the B-ring, we measured the activity of an alkanoyl analog with a longer alkyl (C_9) chain and benzoyl analogs with various alkyl groups at the *para*-position of the B-ring. As shown in Table 1, the activity of alkanoyl analog **8** with a nonyl group (C_9) was drastically decreased as predicted from our previous study [23,28]. Its potency was about 1/300 to 1/400 of other alkanoyl analogs, such as compounds **3**–**6**. Benzoyl analogs, **11**, **13**, and **14** that have alkyl chain lengths longer than the ethyl C_2 group, were less potent than tebufenozide; i.e. C_2H_5 (**10**) > n-C_3H_7 (**11**) > n-C_4H_9 (**13**) > n-C_5H_{11} (**14**). The replacement of the C_2H_5 group of tebufenozide with CH_3 lowered the activity to 1/7 of tebufenozide (cf. compound **9**). Although the length of i-C_3H_7 is identical to C_2H_5 with respect to the STERIMOL length parameter [34], compound **12** (i-C_3H_7) was less potent than tebufenozide **10** (C_2H_5) and equipotent to compound **11** (n-C_3H_7). Methoxyfenozide (**15**), which was recently developed to be more active than tebufenozide, was highly potent in this *Chilo* integument system. However, methoxyfenozide proved to be equipotent to tebufenozide.

The activity of compound **16**, which does not carry the 3,5-dimethyl groups of the A-ring of tebufenozide **10**, is about 1/5 that of tebufenozide. The activity of the naphthyl analogs (**22** and **23**) was about 1/30 that of tebufenozide,

whereas no significant difference could be found for the activity between them. Interestingly, the 1-cyclohexenyl analog (**24**) was more potent than the 3-cyclohexenyl (**25**) analog by a factor of 40, whereas both analogs were less potent than the corresponding phenyl analog **16**. Other compounds (**17**–**21**) with various alkyl groups at the A-ring moiety demonstrated low activity [23,28].

3.2. QSAR analyses of molting hormonal activity

Our previous QSAR study of substituted dibenzoylhydrazine indicated that the molting hormonal activity against rice stem borers increased with molecular hydrophobicity and decreased with the introduction of bulkier substituents at the A- and B-rings [21]. Furthermore, 3-D QSAR by using CoMFA derived for the combined set of ecdysones and diacylhydrazines visualized the sterically unfavorable region at the *para*-position of both the A- and B-rings [23]. For the A-ring moiety, there were unfavorable regions around one of the *ortho*-positions, whereas there were sterically favorable regions around both of the *meta*-positions [23]. The optimum chain length at the B-ring moiety seems to be a requirement for high activity, as shown above. However, the factors for this higher activity have not yet been proven due to a high collinearity ($r = 0.813$) between the length of the B-ring moiety and the hydrophobicity. Thus, we quantitatively analyzed the structure-activity relationship by using the Hansch–Fujita method [25] to scrutinize the physicochemical factors governing the activity. The result is shown in the following equation.

$$pEC_{50} = 7.779 \, (\pm 2.576) \log P - 0.755 \, (\pm 0.325) \, (\log P)^2$$
$$- 0.893 \, (\pm 0.300) \, D_A - 0.609 \, (\pm 0.278) D_B$$
$$- 3.227 \, (\pm 5.518)$$
$$n = 23, \, s = 0.577, \, r = 0.929, \, F = 28.291$$

In the above equation, n is the number of compounds, s is the standard deviation, r is the correlation coefficient, and F is the value of the ratio between regression and residual variances. The figures in the parentheses are the 95% confidence intervals of the regression coefficients. Compounds **21** and **22** were not included to formulate the equation due to their high deviation. This relationship is shown in Fig. 2, and the values calculated from the equation are listed in Table 1. The equation indicates that there is an optimum hydrophobicity ($\log P = 5.15$) favorable to the activity, whereas the introduction of longer groups at both A- and B-ring moieties is unfavorable.

The optimum molecular hydrophobicity has not yet been estimated for the insecticidal activity against Lepidoptera [19,20,24]. However, an optimum $\log P$ value (3–4) seems to be required for the insecticidal activity against coleopterous Colorado potato beetles [10]. The reason why the optimum $\log P$ value was not derived for the activity against lepidopterous *C. suppressalis* and *S. exigua* was due to the
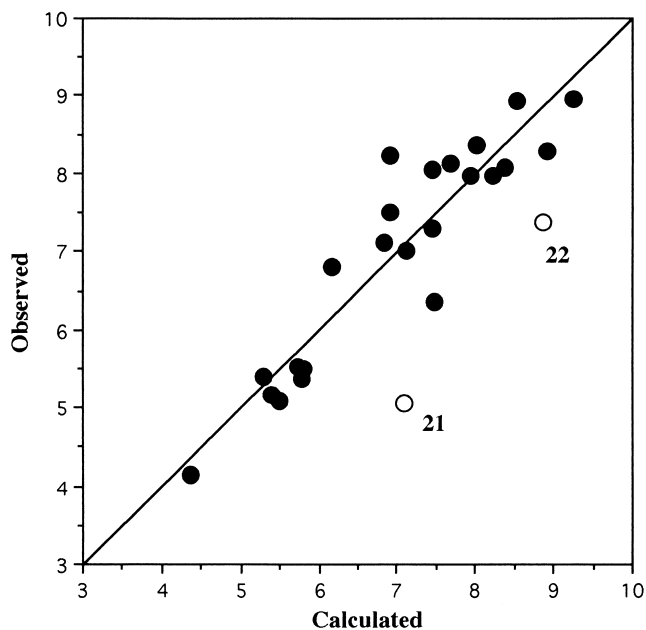
Fig. 2. Plot of the observed hormonal activity (pEC_{50}) versus the calculated value from the equation. Compounds **21** and **22** are marked with open circles and are not included in the correlation analysis.

fact that this value was greater than that for the most hydrophobic compound used in this QSAR analyses. The highest log P values used in QSAR analyses for the insecticidal activity against rice stem borers and beet army worms were 4.69 and 4.49, respectively. Therefore, the optimum log P value (=5.15) evaluated from this QSAR analysis is reasonable.

As described above, the 1-naphthyl analog (**22**) was not included to derive the correlation equation. This is probably due to the unaccountability of the sterically unfavorable effects of *ortho-* and *meta*-substituents, which was reported previously [21]. Although the optimum length at the acyl moiety seems to be required as described above, the addition of the squared terms of D_A and D_B did not give any significant correlation together with log P and its squared term. However, the high standard deviation value ($s = 0.577$) of the equation suggests that other steric and electronic factors are involved in the variations of the activity. The low activity of compound **21** might be due to the fact that the cyclohexyl group is not as flat as benzene and naphthalene and lacks the π electron system.

Notes

Quantitative structure-activity studies of insect growth regulators XVII. The former paper of this series is published in *Pestic. Sci.* **55**, 909–918 (1999), which is entitled 'Quantitative structure-activity studies of insect growth regulators XVI. Substituent effects of dibenzoylhydrazines on the insecticidal activity to Colorado potato beetle *Leptinotarsa decemlineata.*'

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