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Original Article

Effects of the structures of ecdysone receptor (EcR) and ultraspiracle (USP) on the ligand-binding activity of the EcR/USP heterodimer

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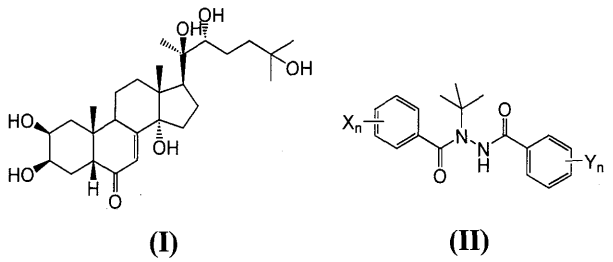
N-tert-Butyl-*N,N'*-diacylhydrazine (DAH) analogs are nonsteroidal ecdysone agonists. The binding activity of DAH analogs to the heterodimer of the ecdysone receptor (EcR) and ultraspiracle (USP) is diverse among insect species, which is probably the main factor causing their selective toxicity. We prepared EcR and USP proteins from lepidopteran *Chilo suppressalis*, dipteran *Drosophila melanogaster* and coleopteran *Leptinotarsa decemlineata*, and measured the binding activity of ecdysone agonists against various hybrid EcR/USP heterodimers. There was a linear relationship between binding activities (pIC_{50} values) before and after replacing native USP with that derived from other insects, suggesting that the selective toxicity of DAH analogs is mainly dependent on the EcR structure and not the USP structure. © Pesticide Science Society of Japan

Keywords: nonsteroidal ecdysone agonists, ecdysone receptor, ultraspiracle, selective toxicity.

Introduction

Insect molting and metamorphosis are regulated by the binding of a molting hormone, 20-hydroxyecdysone (20E, **I**; Fig. 1), to its receptor; the heterodimer of the ecdysone receptor (EcR) and ultraspiracle (USP), a homolog of vertebrate retinoid X receptor (RXR). Both EcR and USP belong to the nuclear receptor superfamily and are constructed from A/B, C (DNA-binding domain; DBD), D, E (ligand-binding domain; LBD), and F regions.[1)] The transactivation of target genes is initiated by the binding of the 20E-EcR/USP (or EcR/RXR) complex to ecdysone response elements (EcREs), which triggers subsequent ecdysone cascade events.[1–4)] In the fruit fly *Drosophila melanogaster*, it has been suggested that ligand-free EcR/USP (or RXR) can also function as a suppressor to regulate a part of steroid-driven development.[5)]

N-tert-Butyl-*N,N'*-diacylhydrazine (DAH) and its analogs (Fig. 1, **II**) are known as synthetic nonsteroidal ecdysone agonists, and four potent DAH analogs, tebufenozide (RH-5992), methoxyfenozide (RH-2485), halofenozide (RH-0345), and chromafenozide (ANS-118), are currently on the market to control agricultural pests.[6–10)] Although 20E is a common molting hormone in all insects, the insecticidal toxicity of DAH analogs varies dramatically among insect species, particularly among insect orders. For example, tebufenozide, methoxyfenozide, and chromafenozide are highly toxic against Lepidoptera but not so toxic to other taxonomic insect orders such as Diptera and Coleoptera.[7)] It has been suggested that the selective toxicity of DAH analogs could be attributed to differences in their receptor binding activity and exclusion mechanisms, rather than a difference in detoxifying activity among insect species.[11–13)] Previously we showed that the



(I) **(II)**

Fig. 1. Structures of 20-hydroxyecdysone (**I**) and nonsteroidal ecdysone agonists (**II**). RH-5849, X_n=Y_n=H; tebufenozide (RH-5992), X_n=3,5-$(CH_3)_2$, Y_n=4-C_2H_5; methoxyfenozide (RH-2485), X_n=3,5-$(CH_3)_2$, Y_n=2-CH_3–3-OCH_3; halofenozide (RH-0345), X_n=H, Y_n=4-Cl; chromafenozide (ANS-118), X_n=3,5-$(CH_3)_2$, Y_n=2-CH_3-3,4-(–$CH_2CH_2CH_2O$–).

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binding activity of DAH analogs against lepidopteran *Chilo suppressalis* EcR/USP (CsEcR/CsUSP) is significantly higher than that against dipteran *D. melanogaster* EcR/USP (DmEcR/DmUSP) or coleopteran *Leptinotarsa decemlineata* EcR/RXR (LdEcR/LdRXR).[14,15] Recently it was reported that the binding activity of tebufenozide to EcR/RXR of hemipteran *Myzus persicae* or *Bemisia tabaci* is much lower than that to lepidopteran or dipteran EcR/USP.[16] These results suggested that the difference in the binding activity of DAH analogs to EcR/USP (or EcR/RXR) could be a significant factor causing selective toxicity among insect species.

The X-ray crystal structures of EcR-LBD have been reported for the lepidopteran *Heliothis virescens*[17] and the hemipteran *B. tabaci*,[16] and the essential amino acids participating in ligand-receptor binding were examined. By analyzing the crystal structures of *H. virescens* EcR-LBD/USP-LBD with Ponasterone A (PonA) and with a DAH analog, BYI06830, Billas *et al.* showed that the ligand-binding cavity used by PonA is only partially shared with that used by DAH.[17] According to Carmichael *et al.*, the ligand-binding cavity of *B. tabaci* EcR was structurally different from that of *H. virescens* EcR.[16] Meanwhile, *in vitro* studies have shown that binding activity between EcR and its ligands is dramatically enhanced in the presence of USP[4,15,18–22]; however, the structural requirements of USP for the enhancement of ligand-EcR binding have not yet been studied in detail. Since the primary structures of USPs are similar to those of EcRs in their diversity among insects,[1] we assumed that USPs from different insects might have various potencies for stabilization of the EcR/USP heterodimer and changing its ligand-receptor binding activity.

In this study, we prepared EcR and USP proteins from lepidopteran *C. suppressalis* (Cs), dipteran *D. melanogaster* (Dm) and coleopteran *L. decemlineata* (Ld) individually, and compared the binding activities of various ecdysone agonists against hybrid-type EcR/USP (or EcR/RXR) heterodimers and wild-type receptors (CsEcR/CsUSP, DmEcR/DmUSP, LdEcR/LdRXR).[14,15,21]

Materials and Methods

1. *Chemicals*

PonA was purchased from Invitrogen Corp. (Carlsbad, CA), and ecdysone (E) and 20E were from Sigma-Aldrich Co. (St. Louis, MO). Chromafenozide was a gift from Sankyo Agro Co., Ltd. and Nippon Kayaku Co., Ltd. Other ecdysteroids and nonsteroidal ecdysone agonists were from our stock samples.[23–30] Tritiated PonA ([^{3}H]PonA, 150 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO).

2. *Protein synthesis and ligand-binding assays*

The expression vectors for DmEcR (pCMA-EcR-B1) and DmUSP (pCMA-USP) proteins[31] were generous gifts from Dr. Lucy Cherbas of Indiana University. Vectors for EcR and USP of *C. suppressalis* and *L. decemlineata* were constructed in our laboratory.[15,32,33] Among EcR isoforms with different N-terminal structures as identified in *C. suppressalis*,[32] *D. melanogaster*,[34] and *L. decemlineata*,[15] we used CsEcR-B1 (encoding 547-aa protein), DmEcR-B1 (878 aa) and LdEcR-A (565 aa) isoforms for ligand-receptor binding assays in this study. *In vitro* transcription/translation of EcR and USP proteins was performed using a T7 promoter-driven TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI) according to the manufacturer's instructions. The efficiency of each protein synthesis reaction was confirmed by the incorporation of [^{35}S]methionine followed by SDS-PAGE and autoradiography (data not shown).

Ligand binding assays were performed as previously reported.[21] Briefly, 2 μl of *in vitro* translated EcR and USP proteins were mixed in a siliconized tube (Bio Medical Equipment Co., Ltd., Tokyo, Japan) in a low-salt buffer (20 mM HEPES, 20 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.9, 1 μg/ml aprotinin, 1 μg/ml pepstatin and 1 μg/ml leupeptin), including [^{3}H]PonA (25,000 dpm; final concentration 5 nM) and a test compound, and were incubated at 25°C for 60 min. To estimate non-specific binding, 500-fold excess PonA was added to the reaction mixture. After incubation, these tubes were placed on ice, and the reaction mixture was filtered immediately through nitrocellulose membrane NC45 (Schleicher & Schuell, Einbeck, Germany) with ice-cold washing buffer (*i.e.* low-salt buffer with 10% glycerol and no protease inhibitors). The radioactivity collected on each membrane was measured in Aquasol-2 (PerkinElmer Life and Analytical Sciences Inc., Wellesley, MA) using a liquid scintillation counter (Aloka LSC-1000). [^{3}H]PonA binding was measured in three replications of each concentration of the test compound, and the average values were used for further analysis. From the concentration-response curve of [^{3}H]PonA binding, the 50% inhibition concentration [IC_{50} (M)] was evaluated using Probit transformation[35,36] for each compound. The reciprocal logarithmic value of IC_{50} (pIC_{50}) was used as an index of binding activity.

3. *Gel mobility shift assays*

Gel mobility shift assays were performed using *in vitro* synthesized proteins and *Drosophila* hsp27 ecdysone response element (hsp27 EcRE: 5′ GATCGACAAGGGTTCAATGCACTTGTC 3′)[37] according to the reported method[32,33,38] with slight modifications as follows. The expressed EcR and/or USP proteins were incubated in 20 mM modified HEPES (pH 7.5) buffer with 10 μM PonA on ice for 30 min, then 1 ng of [α-^{32}P]dCTP-labeled hsp27 EcRE probe was added. The mixture was incubated at 25°C for another 30 min, subjected to non-denaturing polyacrylamide gel electrophoresis, and then analyzed with a bio-imaging analyzer, BAS-2000 (FUJIFILM Corporation, Tokyo, Japan).

Results

1. *Gel mobility shift assays*

In order to see if EcR and USP from different insect species could form a functional, hybrid-type heterodimer, we performed gel mobility shift assays with combinations of EcR and USP from *C. suppressalis* and *D. melanogaster*. Although CsEcR, CsUSP, DmEcR, and DmUSP did not individually bind to the hsp27 EcRE probe, probe binding was detected for CsEcR/DmUSP and DmEcR/CsUSP, as well as CsEcR/CsUSP and DmEcR/DmUSP (Fig. 2). These results indicate that EcR and USP from different insect species can form functional hybrid-type heterodimers that can bind to EcRE.

2. *Binding activity of ecdysteroids and DAH analogs to hybrid EcR/USP heterodimers*

The pIC_{50} values of five ecdysteroids and five DAH analogs for binding to six hybrid-type ecdysteroid receptors (CsEcR/DmUSP, DmEcR/CsUSP, CsEcR/LdRXR, LdEcR/CsUSP, DmEcR/LdRXR, LdEcR/DmUSP) are listed in Table 1. As we reported previously, pIC_{50} values are highly reproducible, usually varying by less than 0.15 standard deviation.

The binding activity of five ecdysteroids to the wild-type receptor DmEcR/DmUSP decreased after replacement of DmUSP with CsUSP or LdRXR. For example, the IC_{50} reduction of 20E was about 2- to 3-fold. On the other hand, the binding activity of ecdysteroids to wild-type ecdysteroid receptors from *C. suppressalis* (CdEcR/CsUSP) and *L. decemlineata* (LdEcR/LdRXR) was slightly increased after replacement of USP with DmUSP (CsEcR/CsUSP *vs.* CsEcR/DmUSP, LdEcR/LdRXR *vs.* LdEcR/DmUSP), although the difference in pIC_{50} values was mostly less than 0.3 and may not be significant.

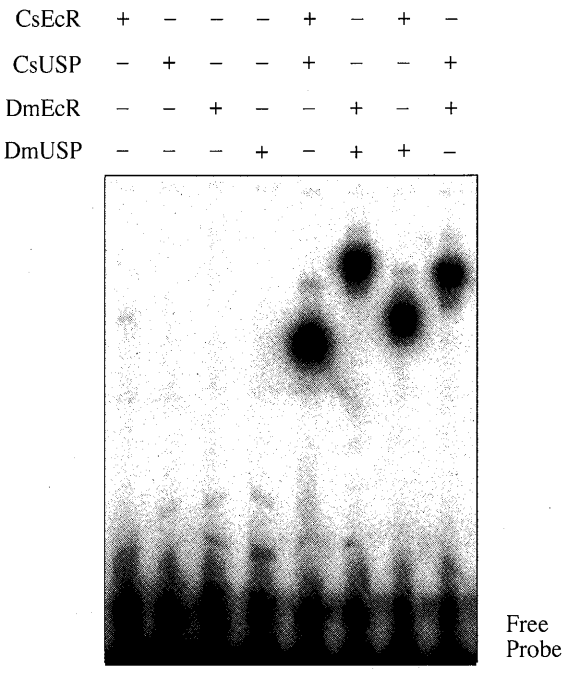

Fig. 2. Binding of *in vitro*-transcribed/translated proteins to ecdysone response element (EcRE). *In vitro*-translated CsEcR, DmEcR, CsUSP and DmUSP proteins were incubated with ^{32}P-labeled hsp27 EcRE probe in the presence of 10 μM PonA, and analyzed on a non-denaturing acrylamide gel.

The binding activity (IC_{50}) of all DAHs to DmEcR/DmUSP was significantly decreased for the hybrid receptor DmEcR/LdRXR; *i.e.* 1/3 for methoxyfenozide, 1/4 for chromafenozide, 1/6 for halofenozide, and 1/12 for tebufenozide. Replacement of DmUSP with CsUSP was not favorable for the binding of DAHs; the ligand-binding activity of DmEcR/DmUSP was slightly higher than that of DmEcR/CsUSP. The activity of RH-5849, tebufenozide and

Table 1. Binding activity (pIC_{50}) of ecdysone agonists against the *in vitro*-translated EcR/USP complex[a)]

No.	Compounds	DmEcR			CsEcR			LdEcR		
		DmUSP[b)]	CsUSP	LdRXR	CsUSP[c)]	DmUSP	LdRXR	LdRXR[d)]	DmUSP	CsUSP
1	Ponasterone A	8.27	8.06	8.00	8.08	8.17	8.10	8.13[e)]	8.21	7.95
2	20-Hydroxyecdysone	7.03	6.54	6.64	6.66	6.90	7.00	6.36[f)]	6.70	6.52
3	Cyasterone	7.07	6.39	6.46	6.65	6.86	6.67	6.29	6.49	6.36
4	Makisterone A	6.87	5.97	6.16	6.33	6.58	6.39	5.74[g)]	5.91	5.75
5	Ecdysone	5.24	4.60	4.58	4.70	5.28	5.25	4.98	5.37	5.14
6	RH-5849	5.16	4.88	4.98	6.50	6.55	6.77	4.97	5.08	5.06
7	Halofenozide	5.95	5.65	5.18	6.92	6.94	7.48	5.23	5.35	5.03
8	Tebufenozide	6.01	5.78	4.93	8.85	8.82	8.76	5.18	5.06	5.19
9	Methoxyfenozide	6.49	6.39	5.99	8.87	8.72	8.74	5.94	5.99	5.98
10	Chromafenozide	6.54	6.38	5.98	9.13	8.88	8.41	5.77	5.73	5.96

[a)] pIC_{50} values from a single replication or the average from two replications are shown unless otherwise noted. [b)] From ref. 14 [c)] From ref. 21 [d)] From ref. 15 [e)] ±0.04 (n=4) [f)] ±0.02 (n=2) [g)] ±0.04 (n=2).

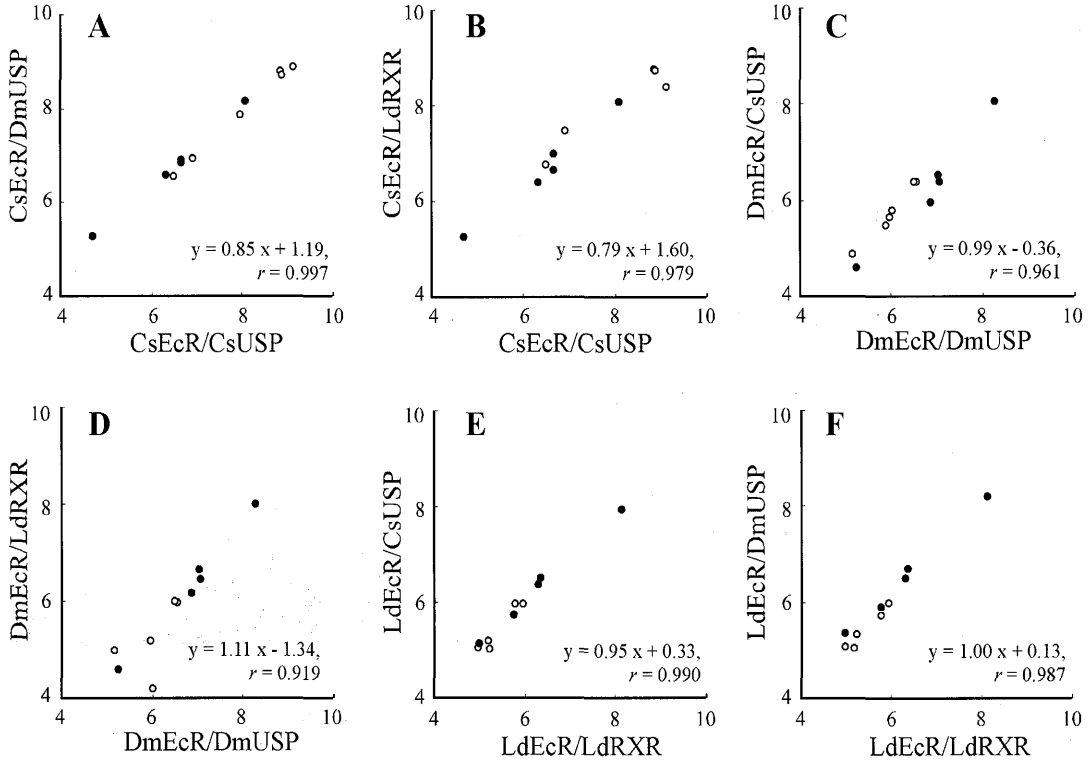

Fig. 3. Relationships of the binding activity (pIC_{50}) of ecdysone agonists against ecdysone receptor/ultraspiracle (EcR/USP) among wild-type (original) and hybrid-type receptors. Solid circles; ecdysteroids. Open circles; diacylhydrazine (DAH) analogs. Binding activities were compared between wild-type CsEcR/CsUSP and hybrid CsEcR/DmUSP (A), between CsEcR/CsUSP and CsEcR/LdRXR (B), between DmEcR/DmUSP and DmEcR/CsUSP (C), between DmEcR/DmUSP and DmEcR/LdRXR (D), between LdEcR/LdRXR and LdEcR/CsUSP (E), and between LdEcR/LdRXR and LdEcR/DmUSP (F). The correlation equation and the correlation coefficient (r) are shown in each graph.

methoxyfenozide against CsEcR/CsUSP was constant regardless of the USP species. The binding activity of halofenozide against CsEcR/CsUSP increased 4-fold when CsUSP was changed to LdRXR, but the activity of chromafenozide decreased 5-fold. The binding of DAHs to LdEcR/LdRXR was very weak with pIC_{50} values of less than 6.0, and binding was not drastically changed by replacement of LdRXR with USPs from other insects.

As shown above, binding activity was slightly changed by the replacement of native USP with that from other insects. In order to investigate the relationship between binding activities in detail, pIC_{50} values of ten ecdysone agonists against wild-type EcR/USP were compared with the values against hybrid-type heterodimers (Fig. 3). The correlation coefficients of the regression lines are fairly high (r=0.961–0.997) except for that in Fig. 3D (r=0.919). The slopes of the regression lines in Figs. 3A and 3B are much less than unity, although those of Figs. 3C, 3D, 3E, and 3F are close to 1. The intercepts in Figs. 3A, 3B, and 3D are far from zero.

Discussion

The significance of the structure of USP has been discussed in many reports.[1] *In vitro* studies have shown that the ligand-binding activity of EcR is dramatically enhanced in the presence of its partner USP *via* an allosteric change of the EcR structure. Two-hybrid experiments, gel mobility shift assays and ligand-binding experiments using point-mutated USP revealed that several amino acid residues in helices 3 and 5 (H3 and H5) in the E/F region of *Drosophila* USP (Leu281, Leu322, Ile323, Cys329, Ser330) are important for ligand-induced heterodimerization of EcR and USP.[39] Moreover, it was reported that complete truncation of the H12 of USP, which is fixed in an antagonistic position,[40] abolished reporter gene induction in a two-hybrid assay, as well as interaction of USP with DNA and the hormone-binding ability of the EcR/USP heterodimer.[41] A point mutation in the H12 of USP reduced the induction of the reporter gene, but did not inhibit the binding of EcR/USP to the ligands.[41] These reports suggest that the E/F region of USP has a significant role in its heterodimerization with EcR, the binding activity of EcR/USP to ligands or EcRE, and the subsequent transactivation of target genes. In this study we found that EcR and USP from different insect species were able to form a hybrid-type heterodimer that could bind to DNA or ligands *in vitro*. There was a slight difference between wild-type and hybrid-type receptors in their binding activity to a set of ligands; the slopes of the regression lines were less than unity, and the intercepts were far from zero in some cases (Fig. 3). We postulate that this effect might be due to the instability of the hybrid EcR/USP that was created from different insect orders. The (in)stability of

hybrid-type EcR/USP could affect its ligand-binding activity. Further studies using point-mutated USP as well as chimeric USP created from different insect species would be helpful to elucidate the structural factors that cause a difference in ligand sensitivity between wild-type and hybrid-type receptors. Although the coefficient was not exactly unity and the constant was not zero in some regression equations, the correlation itself was still high, and more importantly, the linear correlation includes both ecdysteroids and DAH analogs. These results suggest that profiles of structure–activity relationships were very similar even after replacement of USP.

To date, the X-ray crystal structure of EcR-LBD has been reported for lepidopteran *H. virescens*[17] and hemipteran *B. tabaci*,[16] and their tertiary structures have proved to be similar to LBDs of other nuclear receptors such as the liver X receptor-β (LXR-β) and the farnesoid X-activated receptor (FXR). According to Billas *et al.*,[17] EcR-LBDs of *H. virescens* complexed with steroidal and non-steroidal agonists exhibit only partially overlapping ligand-binding cavities; the ligand-binding cavity of *H. virescens* EcR utilized by PonA was reported to be a long and thin L-shape, and that used by BYI06830 was a bulky V-shape with an open cleft extending towards the H8-H9 loop of USP. Amino-acid residues in *H. virescens* EcR participating in hydrogen bonding with PonA exist in H1 (Glu309), H3 (Thr343 and Thr346), H5 (Arg383), H6 (Tyr408) and β-sheet (Ala398), and those binding BYI06830 exist in H3 (Thr343), H6 (Tyr408) and H12 (Asn504). In this study, ligand-binding activity was not affected very much when USP was replaced, probably because the amino-acid residues of EcR participating in ligand-binding are not in the vicinity of the heterodimerization interface.

Carmichael *et al.* reported that the conformation and overall hydrophobic and polar characteristics of EcR LBD in contact with PonA are well conserved between *H. virescens* and *B. tabaci*, while the parts that are not in contact with PonA are structurally different between these two insect species.[16] They suggested that these differences in the ligand-binding pocket may be one of the factors causing the selectivity of DAH insecticides among taxonomic orders. Here, we showed that the replacement of USP in the EcR/USP heterodimer by USP from other insects had no effect on the selective toxicity of DAH analogs among insect orders, although it did have a slight effect on the ligand sensitivity of the EcR/USP heterodimer. In conclusion, our data suggest that ligand-binding profiles of ecdysteroids and DAH insecticides are determined mainly by the structure of EcR, not by the structure of USP.

Acknowledgments

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References

1) L. M. Riddiford, P. Cherbas and J. W. Truman: *Vitam. Horm.* **60**, 1–73 (2001).
2) T.-P. Yao, W. A. Segraves, A. E. Oro, M. McKeown and R. M. Evans: *Cell* **71**, 63–72 (1992).
3) H. E. Thomas, H. G. Stunnenberg and A. F. Stewart: *Nature* **362**, 471–475 (1993).
4) T.-P. Yao, B. M. Forman, Z. Jiang, L. Cherbas, J.-D. Chen, M. McKeown, P. Cherbas and R. M. Evans: *Nature* **366**, 476–479 (1993).
5) M. Schubiger and J. W. Truman: *Development* **127**, 1151–1159 (2000).
6) A. C.-T. Hsu, T. T. Fujimoto and T. S. Dhadialla: "Phytochemicals for Pest Control," ACS Symposium Series 658, ed. by P. A. Hedin, R. M. Hollingworth, E. P. Master, J. Miyamoto and D. G. Thompson, American Chemical Society, Washington DC, pp. 206–219, 1997.
7) T. S. Dhadialla, G. R. Carlson and D. P. Le: *Annu. Rev. Entomol.* **43**, 545–569 (1998).
8) K. Tanaka, Y. Tsukamoto, Y. Sawada, A. Kasuya, H. Hotta, R. Ichinose, T. Watanabe, T. Toya, S. Yokoi, A. Kawagishi, M. Ando, S. Sadakane, S. Katsumi and A. Masui: *Annu. Rep. Sankyo Res. Lab.* **53**, 1–49 (2001).
9) T. Toya, H. Fukasawa, A. Masui and Y. Endo: *Biochem. Biophys. Res. Commun.* **292**, 1087–1091 (2002).
10) Y. Sawada, T. Yanai, H. Nakagawa, Y. Tsukamoto, Y. Tamagawa, S. Yokoi, M. Yanagi, T. Toya, H. Sugizaki, Y. Kato, H. Shirakura, T. Watanabe, Y. Yajima, S. Kodama and A. Masui: *Pest Manag. Sci.* **59**, 49–57 (2003).
11) G. Smagghe and D. Degheele: *Pestic. Biochem. Physiol.* **46**, 149–160 (1993).
12) G. Smagghe and D. Degheele: *Pestic. Biochem. Physiol.* **49**, 224–234 (1994).
13) A. Retnakaran, I. Gelbic, M. Sundaram, W. Tomkins, T. Ladd, M. Primavera, Q. Feng, B. Arif, R. Palli and P. Krell: *Pest Manag. Sci.* **57**, 951–957 (2001).
14) C. Minakuchi, Y. Nakagawa, M. Kamimura and H. Miyagawa: "New Discoveries in Agrochemicals," ACS Symposium Series 892, ed. by J. M. Clark and H. Ohkawa, American Chemical Society, Washington DC, pp. 191–200, 2005.
15) T. Ogura, C. Minakuchi, Y. Nakagawa, G. Smagghe and H. Miyagawa: *FEBS J.* **272**, 4114–4128 (2005).
16) J. A. Carmichael, M. C. Lawrence, L. D. Graham, P. A. Pilling, V. C. Epa, L. Noyce, G. Lovrecz, D. A. Winkler, A. Pawlak-Skrzecz, R. E. Eaton, G. N. Hannan and R. J. Hill: *J. Biol. Chem.* **280**, 22258–22269 (2005).
17) I. M. Billas, T. Iwema, J. M. Garnier, A. Mitschler, N. Rochel and D. Moras: *Nature* **426**, 91–96 (2003).
18) M. Vogtli, M. O. Imhof, N. E. Brown, P. Rauch, M. Spindler-Barth, M. Lezzi and V. C. Henrich: *Insect Biochem. Mol. Biol.*

29, 931–942 (1999).
19) M. Grebe and M. Spindler-Barth: *Insect Biochem. Mol. Biol.* **32**, 167–174 (2002).
20) M. Grebe, S. Przibilla, V. C. Henrich and M. Spindler-Barth: *Biol. Chem.* **384**, 105–116 (2003).
21) C. Minakuchi, Y. Nakagawa, M. Kamimura and H. Miyagawa: *Eur. J. Biochem.* **270**, 4095–4104 (2003).
22) M. Grebe, T. Fauth and M. Spindler-Barth: *Insect Biochem. Mol. Biol.* **34**, 981–989 (2004).
23) N. Oikawa, Y. Nakagawa, K. Nishimura, T. Ueno and T. Fujita: *Pestic. Sci.* **41**, 139–148 (1994).
24) N. Oikawa, Y. Nakagawa, K. Nishimura, T. Ueno and T. Fujita: *Pestic. Biochem. Physiol.* **48**, 135–144 (1994).
25) Y. Nakagawa, K. Nishimura, N. Oikawa, N. Kurihara and T. Ueno: *Steroids* **60**, 401–405 (1995).
26) B. Shimizu, Y. Nakagawa, K. Hattori, K. Nishimura, N. Kurihara and T. Ueno: *Steroids* **62**, 638–642 (1997).
27) Y. Nakagawa, K. Hattori, B. Shimizu, M. Akamatsu, H. Miyagawa and T. Ueno: *Pestic. Sci.* **53**, 267–277 (1998).
28) Y. Nakagawa, G. Smagghe, S. Kugimiya, K. Hattori, T. Ueno, L. Tirry and T. Fujita: *Pestic. Sci.* **55**, 909–918 (1999).
29) Y. Nakagawa, K. Hattori, C. Minakuchi, S. Kugimiya and T. Ueno: *Steroids* **65**, 117–123 (2000).
30) Y. Nakagawa, C. Minakuchi, K. Takahashi and T. Ueno: *Insect Biochem. Mol. Biol.* **32**, 175–180 (2002).
31) X. Hu, L. Cherbas and P. Cherbas: *Mol. Endocrinol.* **17**, 716–731 (2003).
32) C. Minakuchi, Y. Nakagawa, M. Kiuchi, S. Tomita and M. Kamimura: *Insect Biochem. Mol. Biol.* **32**, 999–1008 (2002).
33) C. Minakuchi, Y. Nakagawa, M. Kiuchi, A. Seino, S. Tomita and M. Kamimura: *Insect Biochem. Mol. Biol.* **33**, 41–49 (2003).
34) W. S. Talbot, E. A. Swyryd and D. S. Hogness: *Cell* **73**, 1323–1337 (1993).
35) D. J. Finney: "Probit Analysis," Cambridge University Press, Cambridge, UK, 1971.
36) M. Sakuma: *Appl. Entomol. Zool.* **33**, 339–347 (1998).
37) G. Riddihough and H. R. B. Pelham: *EMBO J.* **6**, 3729–3734 (1987).
38) S. Tomita, Y. Kawai, S. D. Woo, M. Kamimura, K. Iwabuichi and A. S. Imanishi: *In Vitro Cell. Dev. Biol. Anim.* **37**, 564–571 (2001).
39) T. Bergman, V. C. Henrich, U. Schlattner and M. Lezzi: *Biochem. J.* **378**, 779–784 (2004).
40) I. M. L. Billas, L. Moulinier, N. Rochel and D. Moras: *J. Biol. Chem.* **276**, 7465–7474 (2001).
41) S. Przibilla, W. W. Hitchcock, M. Szecsi, M. Grebe, J. Beatty, V. C. Henrich and M. Spindler-Barth: *Biol. Chem.* **385**, 21–30 (2004).