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Regular Article

Receptor-binding affinity and larvicidal activity of tetrahydroquinoline-type ecdysone agonists against *Aedes albopictus*

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Ⓢ *Supplementary material*

Tetrahydroquinolines (THQs), a class of nonsteroidal ecdysone agonists, are good candidates for novel mosquito control agents because they specifically bind to mosquito ecdysone receptors (EcRs). We have recently performed quantitative structure–activity relationship (QSAR) analyses of THQs to elucidate the physicochemical properties important for the ligand–receptor interaction. Based on previous QSAR results, here, we newly synthesized 15 THQ analogs with a heteroaryl group at the acyl moiety and evaluated their binding affinity against *Aedes albopictus* EcRs. We also measured the larvicidal activity of the combined set of previously and newly synthesized compounds against *A. albopictus* to examine the contribution of receptor-binding to larvicidal activity. Multiple regression analyses showed that the binding affinity and the molecular hydrophobicity of THQs are the key determinants of their larvicidal activity.

Larvicidal activity
R =
Receptor-binding affinity
Hydrophobicity
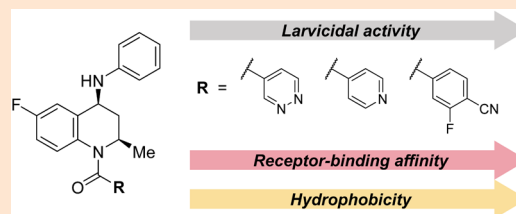
Keywords: tetrahydroquinoline, ecdysone, insecticide, mosquito.

Introduction

Mosquitoes have the capacity to carry many pathogens that infect humans.[1] Anopheline mosquitoes can transmit malaria, which killed 405,000 people in 2018 according to the World Health Organization (WHO).[2] *Aedes* mosquitoes can spread several arboviral diseases such as dengue, chikungunya, Zika, and yellow fever. Neuroactive insecticides, such as pyrethroids and organochlorines, have played a pivotal role in mosquito control, but they have a risk of harming non-targeted organisms.[3,4] In addition, the efficacy of these insecticides has been impaired by the widespread development of insecticide resistance

in mosquitoes.[5,6] Therefore, alternative insecticides with novel modes of action are needed to combat mosquito-borne diseases.

Ecdysone agonists are a class of insect growth regulators (IGRs) that mimic the action of 20-hydroxyecdysone (20E), a master regulator of insect molting and metamorphosis.[7] The target protein of ecdysone agonists is the ecdysone receptor (EcR), a ligand-activated nuclear receptor in insects.[8] EcR works as the heterodimer with ultraspiracle (USP), the homolog of the vertebrate retinoid X receptor (RXR). The binding of ecdysone agonists to EcR triggers the cascade of gene activation, which ultimately results in abnormal and lethal molting. Diacylhydrazines (DAHs) are the first reported nonsteroidal ecdysone agonists,[9,10] and their chemical structures have been successfully optimized to yield several IGRs for controlling lepidopteran pests in agriculture.[11] Other ecdysone agonists with different basic structures have also been reported to date.[12] However, most of them are specific to lepidopterans, and no ecdysone agonists have been commercialized as mosquito control agents.

Tetrahydroquinolines (THQs) are a class of nonsteroidal ecdysone agonists that were originally discovered in a high-throughput screening campaign at FMC Corporation.[13] THQs

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are unique in their ability to bind specifically to mosquito EcRs,[14] which renders them attractive lead compounds for novel mosquito control agents. Structurally, they are characterized by two stereogenic centers at the C-2 and C-4 positions, resulting in four stereoisomers. Preliminary structure–activity relationship (SAR) studies by RheoGene chemists[15,16] revealed that the *cis*-stereochemistry of the THQ scaffold is required for the biological activity. By resolving the racemate of a *cis*-THQ analog *via* chiral column chromatography, we showed that the (2*R*,4*S*)-isomer is about 40 times more active than the (2*S*,4*R*)-isomer.[17] More recently, we performed quantitative structure–activity relationship (QSAR) analyses of THQ analogs with the (2*R*,4*S*)-configuration and identified several physicochemical properties of THQs that are important for binding affinity.[18] However, only a few studies[14,17] have investigated the larvicidal activity of THQs, and the determinants of larvicidal activity still remain unclear.

In this study, we newly synthesized 15 THQ derivatives that have a heteroaryl group instead of the benzene ring of the benzoyl moiety and evaluated their binding affinity against *A. albopictus* EcRs. These newly synthesized compounds, together with 20 THQ analogs prepared in our previous study,[18] were tested for their larvicidal activity against *A. albopictus*. By performing multiple regression analyses, we found that the binding affinity and the molecular hydrophobicity are key determinants of the larvicidal activity of THQs.

Materials and methods

1.   Chemistry

1.1.   General

Compounds **1–20** (Series I) were prepared in our previous work.[18] The other THQ derivatives (**21–35**, Series II) were newly synthesized according to the synthetic route shown in Scheme 1. In brief, compound **36** was prepared in 97% ee from

4-fluoroaniline, acetaldehyde, and benzyl vinylcarbamate in accordance to the procedure reported in the literature.[18] The carboxybenzyl (Cbz) group of **36** was removed by catalytic hydrogenolysis, and the resulting diamine was selectively arylated *via* a Chan–Evans–Lam coupling reaction to give the common intermediate **37**. Compounds **21**, **24**, **25** and **26** were synthesized *via* HATU-assisted condensation of **37** with carboxylic acids (Method A), and the other compounds were synthesized *via* acylation of **37** with acid chlorides (Method B). The following spectrometric and analytical instruments were used for the chemical characterization: melting points (mp), Yanaco MP-S3 (Kyoto, Japan); optical rotations, Jasco P-1010 (Tokyo, Japan); NMR, Bruker Avance III 400 or 500 (Billerica, MA, USA); and high-resolution mass spectrometry (HRMS), Thermo Fisher Scientific Exactive Plus (Waltham, MA, USA) equipped with an electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) source. The synthetic procedures and characterization data of the selected compounds are described below, and those of the others are described in the Supplementary Information.

1.2.   Preparation of (2R,4S)-6-fluoro-2-methyl-N-phenyl-1,2,3,4-tetrahydroquinolin-4-amine (**37**)

Under a hydrogen atmosphere, a suspension of **36** (3.32 g, 10.6 mmol) and 5% palladium on carbon (2.26 g, 1.06 mmol) in EtOH (106 mL) was vigorously stirred at room temperature for 2 hr. The mixture was then filtered through a pad of Celite, which was washed with EtOH. The filtrate was concentrated under reduced pressure, and the residue was dissolved in anhydrous CH_2Cl_2 (65 mL). To this solution were added phenylboronic acid (2.37 g, 19.4 mmol), copper(II) acetate (2.64 g, 14.5 mmol), powdered 4A molecular sieves (4.0 g, preactivated by flame-drying), and anhydrous pyridine (3.9 mL, 48.4 mmol). The resulting slurry was vigorously stirred at room temperature for 2 days. The mixture was then diluted with CH_2Cl_2 and
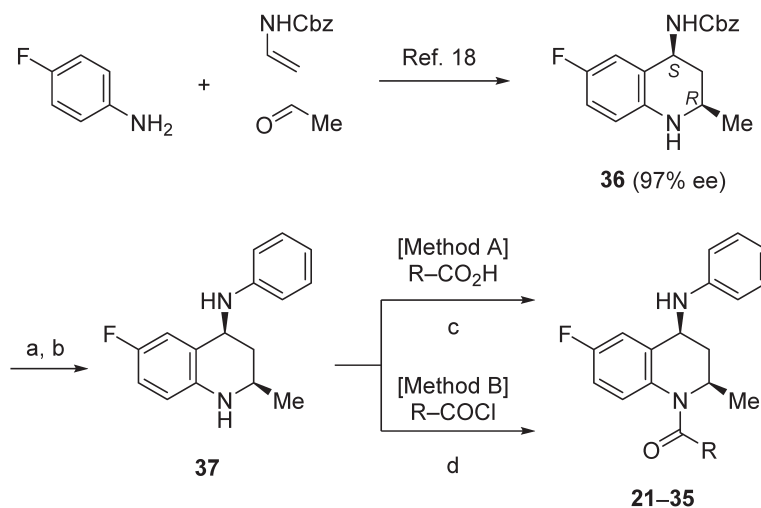
Scheme 1.   Synthesis of compounds **21–35**. Reagents and conditions: (a) H_2 (balloon), 5% Pd–C (10 mol%), EtOH, RT, 3 hr; (b) PhB(OH)$_2$ (2 eq), Cu(OAc)$_2$ (1.5 eq), pyridine (5 eq), 4A molecular sieves, CH_2Cl_2, RT, 2 days, 57% yield (in two steps); (c) R–CO$_2$H (1.5 eq), HATU (1.7 eq), DIPEA (3 eq), CH_2Cl_2, RT; (d) R–COCl (1.7 eq), Et$_3$N (3 eq), DMAP (cat), THF, 0°C to RT.

filtered through a pad of Celite. The filtrate was successively washed with 1 M aqueous NH_3 solution (three times) and brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane/EtOAc=95/5–50/50) to give **37** (1.56 g, 57% yield) as a yellowish oil. ^1H NMR (400 MHz, $CDCl_3$): δ 1.19 (3H, d, J=6.6 Hz), 1.46 (1H, ddd, J=11.9, 11.9, 11.9 Hz), 2.32 (1H, ddd, J=12.6, 5.5, 2.3 Hz), 3.55 (1H, m), 3.62–3.68 (2H, br), 4.75 (1H, br), 6.41 (1H, dd, J=8.7, 4.7 Hz), 6.64–6.66 (2H, m), 6.70–6.76 (2H, m), 7.11 (1H, ddd, J=9.8, 2.9, 1.0 Hz), 7.17–7.21 (2H, m).

1.3. Representative procedure for Method A: preparation of (2R,4S)-6-fluoro-2-methyl-4-(phenylamino)-1-(2-pyridyl-carbonyl)-1,2,3,4-tetrahydroquinoline (21)

Picolinic acid (92.7 mg, 0.75 mmol), *N,N*-diisopropylethylamine (DIPEA, 0.26 mL, 1.49 mmol), and HATU (335 mg, 0.88 mmol) were added to a solution of **37** (130 mg, 0.51 mmol) in anhydrous CH_2Cl_2 (2.5 mL). The mixture was stirred at room temperature for 18 hr and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane/EtOAc=85/15–0/100) and recrystallization from EtOAc/hexane to furnish **21** as brown needles (53 mg, 13% yield). Mp: 135–136°C (EtOAc/hexane). $[\alpha]_D^{18}$ −334 (*c* 0.225, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$): δ 1.29 (3H, d, J=6.4 Hz), 1.40 (1H, ddd, J=12.0, 12.0, 8.3 Hz), 2.79 (1H, ddd, J=12.4, 8.5, 4.3 Hz), 3.86 (1H, br s), 4.49 (1H, br d, J=10.8 Hz), 4.95 (1H, br s), 6.50–6.59 (2H, br s), 6.70–6.72 (2H, m), 6.77–6.81 (1H, m), 7.05 (1H, dd, J=8.9, 2.2 Hz), 7.21–7.26 (3H, m), 7.40 (1H, d, J=7.7 Hz), 7.67 (1H, ddd, J=7.7, 7.7, 1.5 Hz), 8.45 (1H, br d, J=4.4 Hz). ^{13}C NMR (101 MHz, $CDCl_3$): δ 21.2, 40.7, 48.3, 49.7, 111.3 (d, J_{C-F}=24.6 Hz), 113.3, 113.4 (d, J_{C-F}=23.3 Hz), 118.3, 123.4, 124.3, 127.4 (d, J_{C-F}=8.1 Hz), 129.5, 132.5, 136.4, 139.3, 146.8, 148.9, 154.1, 160.6 (d, J_{C-F}=246 Hz), 167.7. ^{19}F NMR (377 MHz, $CDCl_3$): δ −116.75 (s). HRMS–ESI (*m/z*): calcd for $C_{22}H_{20}FN_3ONa$ [M+Na]$^+$, 384.1483; found, 384.1490.

1.4. Representative procedure for Method B: preparation of (2R,4S)-6-fluoro-2-methyl-4-(phenylamino)-1-(4-pyridyl-carbonyl)-1,2,3,4-tetrahydroquinoline (23)

A solution of isonicotinoyl chloride (113 mg, 0.80 mmol) in anhydrous THF (3 mL) was added to an ice-cooled solution of **37** (122 mg, 0.47 mmol) and triethylamine (0.21 mL, 1.5 mmol) in anhydrous THF (3 mL). A catalytic amount of 4-(dimethylamino)pyridine (DMAP) was added, and the resulting slurry was stirred at room temperature for 2 hr. The mixture was diluted with EtOAc and successively washed with 10% aqueous citric acid solution, 10% aqueous K_2CO_3 solution, and brine. The organic layer was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane/EtOAc=85/15–0/100) to give **23** (106 mg, 62% yield) as a colorless amorphous solid. $[\alpha]_D^{18}$ −232 (*c* 0.220, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$): δ 1.28 (3H, d, J=6.3 Hz), 1.39 (1H, ddd, J=12.1, 12.1, 8.6 Hz), 2.81 (1H, ddd, J=12.5, 8.5, 4.2 Hz), 3.86 (1H, d, J=6.8 Hz), 4.41 (1H, br s), 4.91 (1H, br s), 6.49 (1H, br s), 6.63–6.70 (3H, m), 6.80–6.85 (1H, m), 7.09–7.12 (3H, m), 7.24–7.29 (2H, m), 8.58 (2H, br d, J=5.0 Hz).

^{13}C NMR (101 MHz, $CDCl_3$): δ 20.9, 40.8, 48.5, 50.0, 111.8 (d, J_{C-F}=24.5 Hz), 113.2, 114.0 (d, J_{C-F}=23.1 Hz), 118.7, 122.4, 128.1 (d, J_{C-F}=8.8 Hz), 129.6, 131.7 (d, J_{C-F}=2.3 Hz), 139.4 (d, J_{C-F}=8.1 Hz), 143.2, 146.6, 150.0, 161.0 (d, J_{C-F}=247 Hz), 166.8. ^{19}F NMR (377 MHz, $CDCl_3$): δ −115.16 (s). HRMS–ESI (*m/z*): calcd for $C_{22}H_{20}FN_3ONa$ [M+Na]$^+$, 384.1483; found, 384.1490.

2. Bioassay

2.1. Materials

Tritium-labeled ponasterone A ([^3H]PonA, 95 Ci/mmol) was custom synthesized by American Radiolabeled Chemicals (St. Louis, MO, USA) and diluted to 30,000 dpm/μL using 70% aqueous EtOH. Cold PonA was obtained from Enzo Biochem (New York, NY, USA) and dissolved in EtOH (1.1 mM). Tebufenozide was from our stock samples. The THQ derivatives and tebufenozide were dissolved in dimethyl sulfoxide (DMSO). The NIAS-AeAl-2 cell line[19] was obtained from NIAS Genebank (currently known as NARO Genebank, Tsukuba, Japan). The cells were maintained in EX-CELL 420 medium (SAFC Biosciences, Lenexa, KS, USA) supplemented with 10% fetal bovine serum. The eggs of *A. albopictus* were purchased from Sumika Technoservice (Takarazuka, Japan). The eggs were hatched in a styrofoam box (20 cm wide×30 cm deep×20 cm tall) containing distilled water (2 cm deep). The hatched larvae were fed with crushed Ebios tablets (Asahi Group Foods, Tokyo, Japan) and reared at 25±2°C for 4–6 days under a long-day photoperiod (16 hr light, 8 hr dark) until they molted to the second instar.
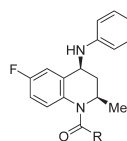
2.2. Binding assay

The binding assay was performed according to the previously reported method[20,21] with some modifications. A test compound solution in DMSO (1 μL) was added to the NIAS-AeAl-2 cell suspension (400 μL, 0.7–1.0×10^7 cells/mL) in a disposable glass tube (12 mm×75 mm). To the suspension was added a [^3H]PonA solution (2 μL, *ca.* 60,000 dpm). After incubating the suspension for 30 min at 25°C, it was diluted with water (3 mL) and quickly filtered through a glass fiber filter GF/B (Whatman, Maidstone, Kent, UK). The filter was washed with water (2×3 mL), dried under an infrared lamp, and then put into a glass vial. To this vial was added 3 mL of Insta-Gel Plus (PerkinElmer, Waltham, MA, USA), and the radioactivity was measured with an Aloka LSC-8000 counter (Tokyo, Japan). Treatments with DMSO and PonA (final concentration: 2.7 μM) were used to determine the total and non-specific binding, respectively. The 50% inhibition concentration for the [^3H]PonA binding [IC$_{50}$ (M)] was determined by probit analyses using PriProbit 1.63,[22] and the logarithm of its reciprocal, pIC$_{50}$, was used as the index of binding affinity.

2.3. Larvicidal assay

Twenty second-instar larvae of *A. albopictus* were transferred to a plastic cup (top diameter=75 mm, bottom diameter=50 mm, height=75 mm) containing distilled water (50 mL) and the diet. Then, a test compound solution in DMSO (5 μL) was added to the cup. The final concentration of each test compound was set to 1 μM. Treatments with DMSO and tebufenozide (final con-

Table 1. Biological activity and molecular hydrophobicity of THQ analogs.

| Series I	R= (Y-phenyl)	pIC$_{50}$ (M)[a]	pLC$_{50}$ (M)[b]	Mortality (%) at 1 µM		Logit A		CLogP[f]
No.	Y			Obsd.[c]	Calcd.[d]	Obsd.[e]	Calcd.[d]	
1	H	6.38	ND	56±31 (2)	38	0.11	−0.49	4.19
2	4-F	6.52	5.68	27±11 (2)	42	−0.44	−0.31	4.36
3	4-Cl	7.20	ND	68±11 (2)	61	0.32	0.47	4.93
4	4-Br	6.93	ND	80±1 (2)	56	0.61	0.26	5.08
5	4-Me	6.87	ND	83±4 (2)	52	0.70	0.10	4.69
6	4-Et	6.82	ND	91±2 (2)	55	1.03	0.20	5.22
7	4-*n*-Pr	7.28	ND	81±8 (2)	68	0.64	0.76	5.75
8	4-*n*-Bu	6.43	ND	32±5 (2)	53	−0.33	0.13	6.28
9	4-*n*-Pentyl	6.17	ND	50±3 (2)	51	0.00	0.03	6.80
10	4-CF$_3$	6.98	ND	33±20 (2)	58	−0.31	0.31	5.11
11	4-CN	7.52	ND	74±4 (2)	60	0.47	0.42	3.70
12	4-NO$_2$	6.93	ND	34±23 (2)	49	−0.28	−0.03	4.00
13	4-NH$_2$	6.49	ND	6±6 (2)	36	−1.24	−0.58	3.47
14	4-OMe	6.69	ND	41±9 (2)	46	−0.16	−0.15	4.38
15	4-OEt	7.15	6.08	47±0 (2)	60	−0.05	0.42	4.91
16	3,4-Cl$_2$	6.92	6.28	75±25 (2)	59	0.48	0.37	5.53
17	3-F-4-Cl	7.36	6.70	92±3 (2)	66	1.06	0.65	5.08
18	3-F-4-CF$_3$	7.21	ND	77±3 (3)	64	0.52	0.57	5.26
19	3-F-4-CN	8.04	6.65	95±0 (2)	72	1.28	0.94	3.85
20	3-Cl-4-CN	7.29	ND	70±15 (2)	59	0.36	0.37	4.29

Series II	R	pIC$_{50}$ (M)[c]	pLC$_{50}$ (M)[b]	Mortality (%) at 1 µM		Logit A		CLogP[f]
No.				Obsd.[c]	Calcd.[d]	Obsd.[e]	Calcd.[d]	
21	2-pyridyl	5.93±0.08 (2)	ND	0 (1)	23	—	−1.23	2.99
22	3-pyridyl	6.76±0.15 (2)	ND	0 (1)	39	—	−0.47	2.99
23	4-pyridyl	7.29±0.03 (2)	5.59	36±14 (3)	50	−0.24	0.02	2.99
24	pyrimidinyl	6.67±0.03 (2)	ND	10 (1)	30	−0.95	−0.82	1.98
25	pyridazinyl	6.51±0.13 (2)	ND	10 (1)	27	−0.95	−1.02	1.81
26	pyrimidinyl	6.37±0.02 (2)	ND	5 (1)	25	−1.28	−1.10	1.98
27	2-Cl-pyrimidinyl	6.11±0.12 (2)	ND	16 (1)	24	−0.72	−1.14	2.71
28	2-Me-pyridyl	6.64±0.08 (2)	ND	11 (1)	39	−0.91	−0.44	3.49

Table 1. Continued

Series II No.	R	pIC$_{50}$ (M)[c]	pLC$_{50}$ (M)[b]	Mortality (%) at 1 μM		Logit A		CLogP[f]
				Obsd.[c]	Calcd.[d]	Obsd.[e]	Calcd.[d]	
29	(pyridin-2-yl-Cl structure)	7.09±0.07 (2)	6.34	80 (1)	51	0.60	0.04	3.73
30	(pyridin-2-yl-CF$_3$ structure)	7.03±0.00 (2)	6.02	44±24 (2)	51	−0.10	0.04	3.92
31	(pyridin-2-yl-CN structure)	7.61±0.08 (2)	6.29	77±19 (2)	56	0.51	0.23	2.70
32	(pyridin-2-yl-F structure)	7.47±0.08 (2)	6.33	74±11 (2)	56	0.44	0.23	3.16
33	(pyridin-2-yl-Cl structure)	7.95±0.02 (2)	6.00	60 (1)	70	0.18	0.83	3.73
34	(pyridin-2-yl-CF$_3$ structure)	6.56±0.04 (2)	ND	0 (1)	40	—	−0.40	3.92
35	(pyridin-2-yl-CN structure)	6.55±0.07 (2)	ND	0 (1)	32	—	−0.74	2.70

[a] Cited from ref.[18] [b] Determined from a single dose–response experiment. ND, not determined. [c] Mean±standard deviation. Values in parentheses are the number of replications. [d] Calculated by Eq. 4. [e] Logit-transformed mortality based on Eq. 1. [f] Calculated using CLOGP for Windows ver. 4.0.

centration: 10 μM) were also performed as negative and positive controls, respectively. The cups were kept at 25±2°C for 3 days under a long-day photoperiod, after which mortality was determined. The observed mortality was corrected by Abbott's formula.[23] The selected compounds (**2**, **15–17**, **19**, **23**, **29–33**) were subjected to dose–response experiments to determine the 50% lethal concentrations (LC$_{50}$, M). LC$_{50}$ values were calculated by probit analyses using PriProbit 1.63[22] and converted to the logarithm of its reciprocal, pLC$_{50}$.

3. Regression analyses
Regression analyses were performed using the Analysis ToolPak in Microsoft Excel (Redmond, WA, USA). For the analyses, the corrected mortality of each compound at 1 μM was logit-transformed according to Eq. 1.

$$\text{Logit A} = \log[\text{mortality} \% / (100 - \text{mortality} \%)] \quad (1)$$

If the mortality of a given compound was less than 3% or more than 97%, it was not considered for the regression analyses. In this study, compounds **21**, **22**, **34**, and **35** were excluded. The pIC$_{50}$ values of compounds belonging to Series I were taken from our previous study.[18] Molecular hydrophobicity, CLogP, was calculated using CLOGP for Windows ver. 4.0 (Biobyte, Claremont, CA, USA). In all regression equations, values in parentheses are the 95% confidence intervals of the regression coefficients, n is the number of compounds, s is the standard deviation, and r is the correlation coefficient. $F_{x,y}$ is the ratio between regression and residual variances, where x and y represent the regression and residual degrees of freedom, respectively.

Results and discussion

1. Binding affinity
We previously obtained the following QSAR equation for the binding affinity of THQs with various *meta*- and *para*-substituents at the benzoyl moiety[18]:

$$\text{pIC}_{50} = 0.49\Sigma\sigma + 0.39\Delta L^{para} - 1.11\log(\beta 10^{\Delta L^{para}} + 1) + 6.29,$$
$$n = 21, \quad s = 0.27, \quad r = 0.88, \quad F_{4,16} = 13.07, \quad \log\beta = -3.05, \quad (2)$$

where $\Sigma\sigma$ is the electronic parameter that represents the sum of the Hammett constants for *meta*- and *para*-substituents ($\Sigma\sigma = \sigma_m + \sigma_p$), and ΔL^{para} is the STERIMOL length parameter for *para*-substituents relative to hydrogen. According to Eq. 2, the electron deficiency at the benzoyl moiety is favorable to the binding affinity, and the optimum length for *para*-substituents [ΔL^{para} (opt)=2.80] is found at the benzoyl moiety. The compound set used for the analysis (Series I members plus 4-OH analog) was biased toward hydrophobic ligands (CLogP ≥3.47).

To enhance the electron deficiency at the benzoyl moiety and reduce the molecular hydrophobicity, we newly designed compounds **21–35** (Series II), in which the benzene ring of the ben-

zoyl moiety was replaced with a pyridine or diazine ring. The binding affinities of these compounds are listed in Table 1.

We first examined the effect of the benzene-to-pyridine conversion of the benzoyl moiety (**21–23**). The *ortho* CH-to-N conversion (**21**) caused a threefold loss in potency as compared to that of the parent compound (**1**). By contrast, converting the *meta-* or *para*-CH bond to nitrogen (**22** or **23**) resulted in a two- or eightfold gain in potency, respectively. Next, we introduced an additional ring nitrogen to make the benzoyl moiety more electron deficient (**24–26**). However, their potencies were not as high as that of the 4-pyridyl analog (**23**). We introduced an electron-withdrawing Cl group into compound **26**, but the binding affinity was not enhanced (**27**). These results suggest that, upon introducing the ring nitrogen atom(s), the change in potency does not depend solely on the electron deficiency of the benzoyl moiety.

We also synthesized 3-pyridyl analogs with varied *para*-substitutions (**28–31**). The methylated compound (**28**) was equipotent to the parent compound (**22**), but the introduction of electron-withdrawing substituents (**29–31**) enhanced the binding affinity. In particular, sevenfold gain in potency was observed upon the introduction of a CN group (**31**), whose length ($\Delta L = 2.17$) is close to the optimum value predicted by Eq. 2.[18] Thus, the substituent effect within the 3-pyridyl analogs is similar to that observed for Series I.

The substituent effect of 4-pyridyl analogs was also briefly examined (**32–35**). As compared to the parent compound (**23**), substitution with F (**32**) and Cl (**33**) groups at the *meta*-position enhanced the binding affinity. In particular, the potency of **33** ($IC_{50} = 11$ nM) was close to that of the most potent compound (**19**, $IC_{50} = 9.1$ nM) in our THQ library. On the other hand, the introduction of CF$_3$ (**34**) and CN (**35**) groups was detrimental to potency. The lowered potency of **34** and **35** may be attributed to the increased bulkiness or length of these substituents. Since the introduction of CF$_3$ and CN groups at the *para*-position did not decrease the potency (**1** *vs.* **10** and **11**; **22** *vs.* **30** and **31**), the steric hindrance at the *meta*-position is larger than that at the *para*-position.

2.   Larvicidal activity
All 35 compounds (Series I and II) were tested for their insecticidal activity against *A. albopictus* larvae at 1 μM, and mortalities were transformed to Logit A values (Table 1). We also measured pLC$_{50}$ values for 11 THQ analogs (**2**, **15–17**, **19**, **23**, **29–33**) to examine their correlation with the Logit A values. As shown in Fig. 1, the Logit A values were linearly correlated with the pLC$_{50}$ values ($r = 0.95$). Therefore, Logit A can be used as the index of larvicidal activity instead of pLC$_{50}$.

The Logit A values were determined for all compounds belonging to Series I, but four compounds from Series II (**21**, **22**, **34**, **35**) did not show larvicidal activity, which rendered their Logit A values unavailable. In addition, only four compounds from Series II showed positive Logit A values (**29**, **31**, **32**, **33**), whereas more than half of Series I members exhibited positive
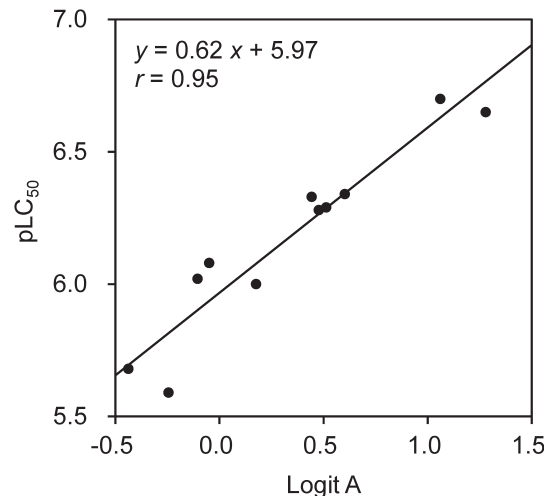

Fig. 1.   Relationship between Logit A and pLC$_{50}$ values of the selected THQ analogs. The solid line indicates the regression line.

Logit A values. These results suggest that the benzene-to-(di)-azine conversion at the benzoyl moiety is generally unfavorable to larvicidal activity.

3.   Regression analyses
To disclose the factors affecting larvicidal activity, we performed regression analyses using Logit A as the objective variable. For the combined set of Series I and II members, Eq. 3 was formulated using the binding affinity, pIC$_{50}$, as the descriptor:

$$\text{Logit A} = 0.90(\pm 0.40)\text{pIC}_{50} - 6.26(\pm 2.83),$$
$$n = 31, \quad s = 0.52, \quad r = 0.65, \quad F_{1,29} = 20.89. \tag{3}$$

Although Eq. 3 was justified above the 99.9% level by the *F*-test, it explained only 42% of the total activity variance ($r^2 = 0.42$). In our previous QSAR studies for the larvicidal activity of DAHs, molecular hydrophobicity, log P, was proven to be the most important parameter.[24,25] Therefore, we added the hydrophobicity parameter, CLogP, to obtain Eq. 4:

$$\text{Logit A} = 0.92(\pm 0.31)\text{pIC}_{50} + 0.27(\pm 0.12)\text{CLogP} - 7.48(\pm 2.23),$$
$$n = 31, \quad s = 0.40, \quad r = 0.82, \quad F_{2,28} = 28.01. \tag{4}$$

There is no collinearity ($r = 0.02$) between the two descriptors (pIC$_{50}$ and CLogP) in Eq. 4. These descriptors and the calculated Logit A values by Eq. 4 are listed in Table 1. The relationship between the calculated and observed Logit A values is shown in Fig. 2.

The positive coefficient of the pIC$_{50}$ term in Eq. 4 means that the stronger the binding affinity to EcR, the stronger the larvicidal activity. Moreover, the coefficient of the pIC$_{50}$ term (0.92) is close to unity. These results suggest that the larvicidal activity of THQs results from the EcR agonism, not from interaction with other insecticidal targets.

The positive coefficient of the CLogP term in Eq. 4 indicates that increased hydrophobicity is favorable to larvicidal activity.
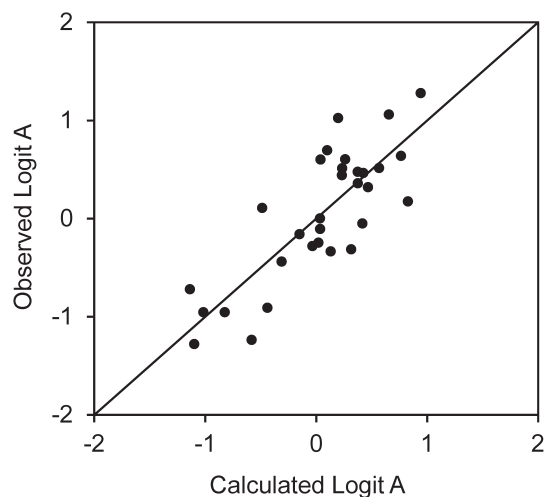
Fig. 2. Relationship between the observed and calculated Logit A values (Eq. 4). The solid line is the regression line.

This term probably reflects the membrane permeation process: *i.e.*, hydrophobic compounds penetrate the larval cuticle more easily and are distributed more densely in the larval body than in water. However, it is generally accepted in the QSAR community that, when compounds partition into biological membranes, the slope of the log P term takes a value close to unity.[26] In Eq. 4, the coefficient of the CLogP term (0.27) is much smaller than 1. Therefore, this CLogP term may also reflect processes other than membrane permeation, such as metabolic degradation and non-specific binding.

To compare the contributions of the pIC_{50} and CLogP terms to larvicidal activity, the normalized values (Z-score) of these parameters (Table S1) were used to obtain Eq. 5:

$$\text{Logit A} = 0.44(\pm0.15)Z_{pIC_{50}} + 0.33(\pm0.15)Z_{CLogP} + 0.04(\pm0.15)$$
$$n = 31, \quad s = 0.40, \quad r = 0.82, \quad F_{2,28} = 28.01. \tag{5}$$

According to Eq. 5, the receptor-binding affinity is likely more important than the molecular hydrophobicity.

We also performed regression analyses for each series of compounds:

‹Series I›
$$\text{Logit A} = 1.05(\pm0.49)pIC_{50} + 0.28(\pm0.26)CLogP - 8.40(\pm4.01),$$
$$n = 20, \quad s = 0.43, \quad r = 0.74, \quad F_{2,17} = 10.44. \tag{6}$$

‹Series II›
$$\text{Logit A} = 0.78(\pm0.61)pIC_{50} + 0.26(\pm0.46)CLogP - 6.53(\pm3.76),$$
$$n = 11, \quad s = 0.41, \quad r = 0.85, \quad F_{2,8} = 10.04. \tag{7}$$

In Eq. 6, derived for Series I, the coefficient of each term is almost identical to that in Eq. 4. By contrast, in Eq. 7, derived for Series II, the CLogP term was statistically insignificant (justified at the 77.6% level by the *t*-test). This insignificance is probably due to (i) the moderate collinearity ($r = 0.53$) between the pIC_{50}

and CLogP values of Series II members and (ii) the limited data points used for the analysis ($n = 11$).

For compounds whose Logit A cannot be defined (**21**, **22**, **34**, **35**), we calculated mortality rates using Eq. 4 (Table 1). The prediction accuracy was not so good, especially for compounds **22**, **34**, and **35**. These results may be inevitable given the moderate correlation in Eq. 4 ($r = 0.82$) and the relatively large variability in the observed mortality (*e.g.*, SD = 31% for the mortality of compound **1**). In addition, factors not considered in Eq. 4, such as metabolic degradation, should play significant roles in determining larvicidal potency. In this context, examining the effect of synergists is of particular interest and may be covered in our future work.

Conclusion

In this study, we expanded our THQ library and evaluated the receptor-binding affinity and larvicidal activity against *A. albopictus*. Multiple regression analyses of larvicidal activity disclosed that the binding affinity and the molecular hydrophobicity of THQs mainly contribute to larvicidal activity. These results should provide a useful guideline for designing novel mosquito control agents with ecdysone agonist activity.

Electronic supplementary materials

The online version of this article contains supplementary materials (Characterization data for compounds **21**–**35**, Table S1), which are available at http://www.jstage.jst.go.jp/browse/jpestics/.

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