Structural requirement and stereospecificity of tetrahydroquinolines as potent ecdysone agonists

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ABSTRACT

Tetrahydroquinoline (THQ)-type compounds are a class of potential larvicides against mosquitoes. The structure–activity relationships (SAR) of these compounds were previously investigated (Smith et al., *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1943–1946), and one of *cis*-forms (with respect to the configurations of 2-methyl and 4-anilino substitutions on the THQ basic structure) was stereoselectively synthesized. However, the absolute configurations of C2 and C4 were not determined. In this study, four THQ-type compounds with *cis* configurations were synthesized, and two were submitted for X-ray crystal structure analysis. This analysis demonstrated that two enantiomers are packed into the crystal form. We synthesized the *cis*-form of the fluorinated THQ compound, according to the published method, and the enantiomers were separated via chiral HPLC. The absolute configurations of the enantiomers were determined by X-ray crystallography. Each of the enantiomers was tested for activity against mosquito larvae in vivo and competitive binding to the ecdysone receptor in vitro. Compared to the (2*S*,4*R*) enantiomer, the (2*R*,4*S*) enantiomer showed 55 times higher activity in the mosquito larvicidal assay, and 36 times higher activity in the competitive receptor binding assay.

Two major classes of peripheral insect hormones, juvenile hormones (JHs) and molting hormones regulate insect growth. The principal molting hormone of insects is 20-hydroxyecdysone (20E).[1] 20E and its agonist bind to ecdysone receptors (EcRs) in collaboration with the heterodimeric partner, ultraspiracle (USP), and transactivate molting-related genes.[2] In a few arthropods, other steroid compounds such as ponasterone A (PonA), makisterone A, and ecdysone act as molting hormones.[3] To date, the primary sequences of EcRs and USPs have been identified in various insects.[3,4] Three-dimensional structures of the ligand binding domains of EcRs with ponasterone A as the ligand molecule were solved by X-ray crystal analysis in three insects: *Heliothis virescens*,[5] *Bemicia tabacii*,[6] and *Tolibolium castaneum*.[7] The ligand-binding characteristics of 20E, which are similar to that of PonA, were also solved in *H. virescens*.[8] The binding sites of non-steroidal ecdysone agonists for diacylhydrazine (DAH; BYI06830)[5] and an imidazole type compound (BYI08346; PDB 3IXP),[9] were also solved by X-ray analysis. However, these structures differ from those of PonA and 20E.

Compounds that regulate insect molting and metamorphosis are known as insect growth regulators (IGRs) or more recently as insect growth disruptors (IGDs), and some of these compounds are used as insecticides in the agricultural field.[10] IGDs can be classified into three major categories, juvenile hormone agonists, chitin synthesis inhibitors and molting hormone agonists. Among molting hormone agonists, five DAH-type compounds (tebufenozide, methoxyfenozide, chlomafenozide, fufenozide, and halofenozide) are used currently. Most of DAH-type compounds are selectively toxic to Lepidoptera, except for halofenozide, which is registered to control both Lepidoptera and Coleoptera. However, the binding affinity of halofenozide to coleopteran receptors is not stronger than that of other lepidopteran-specific DAHs.[11]

The high degree of specificity of DAHs against Lepidoptera triggered a search for new ecdysone agonists, in both random and rational manners. Although novel non-steroidal ecdysone agonists have been reported, none of these has been used commercially.[12] Among new ecdysone agonists, tetrahydroquinoline (THQ)-type compounds are reported to be dipteran-specific,[13] particularly to the mosquito EcR.[14] These compounds have a unique specificity toward mosquitos, which may offer a more selective and environmentally friendly pest-management option. Therefore, THQs are promising leads to develop into larvicides for mosquito control.

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A previous structure–activity relationship (SAR) study of THQs focused on the optimization of the substituents X and Y, and showed that a fluorinated THQ (X = F, Y = 4-Cl: THQ in Fig. 1) had the highest ecdysone agonistic activity for the *Aedes aegypti* EcR among the 35 compounds tested.[13] We initially synthesized a few THQ analogs according to the published method,[13,15] and compared the binding and insecticidal activity of the analogs with the reported activities as shown in Table 1. We also reported that compounds lacking the benzene ring of the quinoline structure and the aniline moiety, as well as the *trans* isomer of the 2,4-positions of quinoline moiety were inactive.[15]

According to Smith and co-workers[13], one of two possible *cis*-stereoisomers (2*R*, 4*S*) was obtained as a final product by the Doebner-von Miller reaction. However, convincing data regarding the stereochemistry of this compound was not reported. In the present study, we observed that Smith's synthesis method did not yield a stereochemically pure intermediate. Instead, it yields a racemic mixture via Doebner-von Miller reaction. We therefore hypothesized that the THQ reaction mixture prepared by this method also contained the (2*S*, 4*R*) *cis* enantiomer. In our preliminary study, we analyzed the X-ray crystal structure to determine the absolute configuration of the *cis* isomers of compounds **1** (CCDC 984528: Supplement Table S1) and **2** (CCDC 984529: Supplement Table S1), in which the two enantiomers are packed, respectively. Smith et al. reported the stereochemistry of THQs based on H NMR analysis, which is difficult[13,16] without using special chiral analysis techniques such as chiral NMR solvent.

We then asked which enantiomer was biologically active. To determine this, the *cis* product of compound **4** (Y = 4-Br) containing two enantiomers [**4a** (2*S*,4*R*) and **4b** (2*R*,4*S*)] was prepared according to the reported procedure[21], and analyzed by chiral HPLC.[22] A baseline separation of the two enantiomers was achieved with an amylose-based chiral HPLC column,[17] packed with a silica gel bound to tris (3,5-dimethylphenylcarbamate) derivatives of amylose and eluted with hexane/ethanol (90/10, v/v). The enantiomer excess was determined by chiral HPLC and found to be >99%. Each fraction was recrystallized from hexane–ethyl acetate and the absolute configurations were determined by X-ray crystallography as shown in Figure 2 with their chemical structures.[23]

Crystal data and the data collection parameters for compounds **4a** (CCDC 984530) and **4b** (CCDC 984531) are listed in Table 1.

The SARs for compounds **1–3** were consistent with the previously reported SARs.[13] The newly synthesized compound **4** is three times more toxic against mosquitoes than compound **3**, as shown in Table 2. The *cis*-(2*R*,4*S*) enantiomer (**4b**) showed 55 times higher larvicidal activity against mosquito *Culex pipiens pallens* than the *cis*-(2*S*,4*R*) enantiomer (**4a**).[24] The concentration–response relationships for the larvicidal activity of these enantiomers are shown in Figure 3. Consistently, the active compound **4b** had approximately two times higher larvicidal activity than the racemic compound **4**.

The EcR–THQ interaction was then investigated to understand the specific larvicidal activity of these enantiomers in vitro. We

Table 1
X-ray crystallographic data for enantiomers **4a** and **4b**

	Retention time (HPLC)	
	4.3 min **4a**	5.8 min **4b**
Empirical formula	$C_{23}H_{19}BrF_2N_2O$	$C_{23}H_{19}BrF_2N_2O$
Formula weight	457.32	457.32
Crystal system	Orthorhombic	Orthorhombic
Space group	$P2_12_12_1$ (#19)	$P2_12_12_1$ (#19)
a (Å)	7.726 (1)	7.7240 (2)
b (Å)	11.425 (1)	11.423 (3)
c (Å)	23.284 (2)	23.288 (5)
V (Å³)	2055.4 (3)	2054.8 (8)
Z, D_{calc} (g/cm³)	4, 1.478	4, 1.478
F (000)	928.00	928.00
μ (Cu Kα) (mm⁻¹)	3.024	3.025
T (K)	243	243
No. obsd ($I > 2.00\sigma(I)$)	3506	3338
No. parameters	265	265
R_1, wR_2	0.040, 0.156	0.043, 0.145
Flack parameter	0.01(2)	0.01(2)
GOF	1.300	1.031

4a (2*S*,4*R*)   **4b** (2*R*,4*S*)
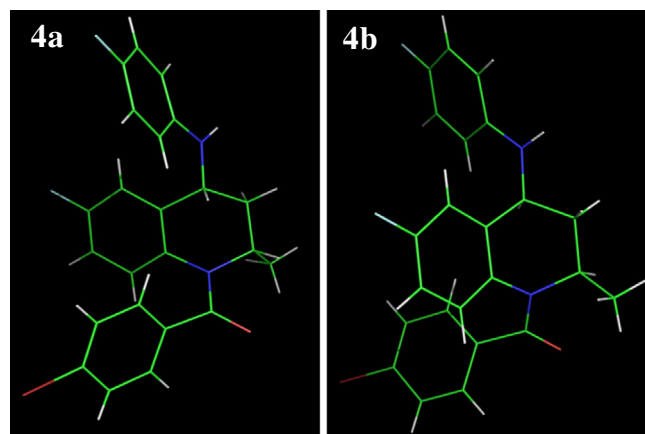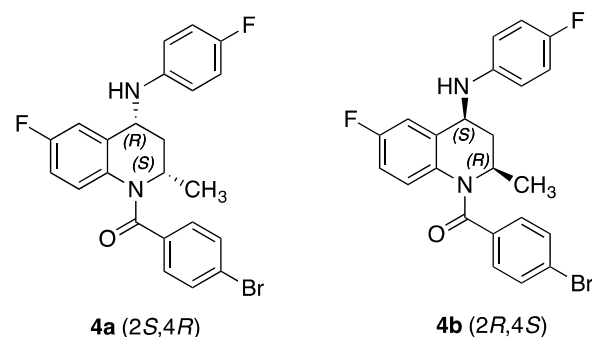
Figure 2. X-ray structures of enantiomer **4a** and **4b**.

R=OH: 20-Hydroxyecdysone (20E)
R=H: Ponasterone A (PonA)

Dibenzoylhydrazine (DBH)

X=Y=H: RH5849
X=3,5-(CH₃)₂, Y=4-Et: Tebufenozide
X=H, Y-4-Cl: Halofenozide
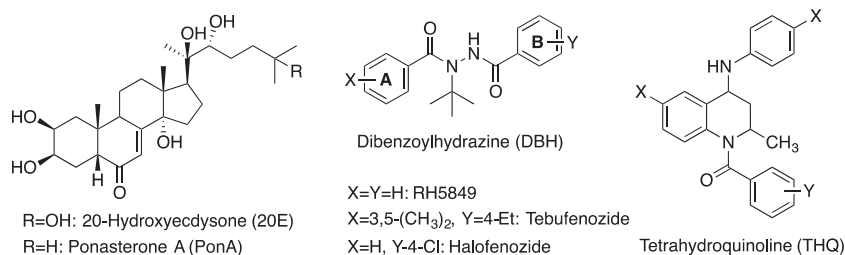
Tetrahydroquinoline (THQ)

Figure 1. Structures of ecdysone agonists.

Table 2
In vivo and in vitro biological activities of ecdysone agonists[a]

Compounds No.	(THQ)[b]		Larvicidal activity pLC$_{50}$ (M)	Binding activity pIC$_{50}$ (M)
	X	Y		
1	CH$_3$	4-CH$_3$	5.33	n.d.[c]
2	CH$_3$	4-Cl	5.38	5.93
3	F	4-Cl	6.52	n.d.
4	F	4-Br	6.92[d]	6.65 ± 0.13 (n = 3)
4a	F	4-Br	5.65 ± 0.42 (n = 2)	5.70 ± 0.16 (n = 2)
4b	F	4-Br	7.40 ± 0.04 (n = 2)	7.26 ± 0.04 (n = 2)
Ponasterone A			n.d.	9.01 ± 0.01 (n = 2)
Tebufenozide			n.d.	7.12 ± 0.03 (n = 2)

[a] Mean values with standard deviation. Number of experiments.
[b] Basic structure is shown in Figure 1.
[c] n.d. not determined.
[d] The trans-type compound (2S,4S + 2R,4R) is inactive (<4.52).
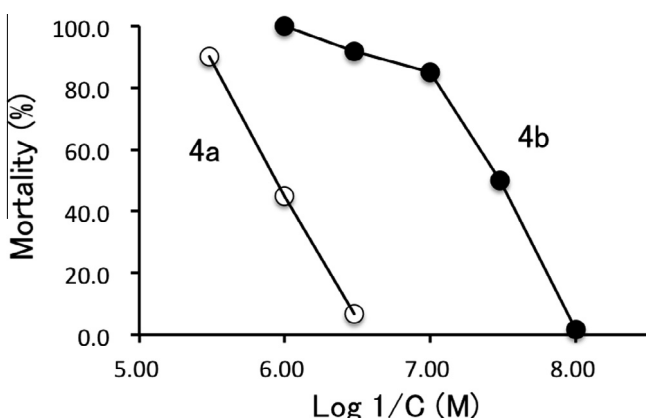
Figure 3. Concentration–response relationships for **4a** (○) and **4b** (●) against the survival of mosquito larvae.

performed a competitive binding assay using [^{3}H]PonA as a radioactive ligand for *Aedes albopictus* (AeAl) cell (NIAS-AeAl-2) binding.[18,19,25] The results revealed that **4b** has a binding affinity for EcR that is 36 times more potent than **4a** (Table 2). These data indicate that differences in binding affinity toward EcR account for the observed larvicidal activity.

Figure 4 shows a summary of the SARs of THQs. The methyl group in position 2 is required to be in the 2R conformation, and the methyl substituent can be replaced with hydrogen. Conversely, the 2S methyl substituent leads to lower activity. This implies that the 2S methyl group has a steric collision with the receptor,
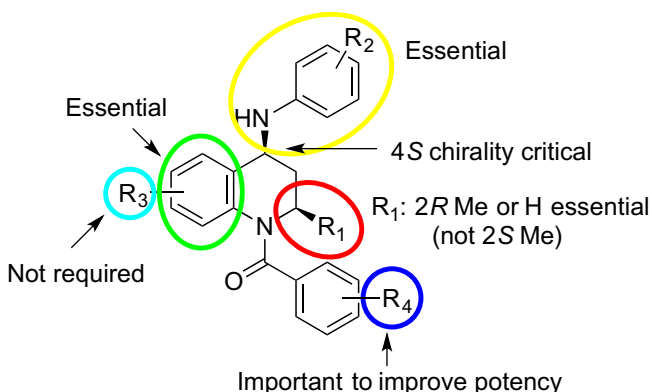
whereas the 2R methyl group and the hydrogen do not. The aniline moiety in position 4 is essential for the larvicidal activity as reported previously[15] and the 4S chiral conformation is also critical for activity. The phenyl group on tetrahydroquinoline ring is also required for activity, but the substituent R$_3$ is not essential.[13] The larvicidal potency was improved approximately 3-fold by changing R$_4$ from 4-chloro to 4-bromo, which can be further optimized in future studies. This SAR summary provides valuable information for the molecular design of novel ecdysone agonists.

Acknowledgments

Supplementary data

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21. Compounds **1–4** were synthesized according to the reported methods.[13] Briefly, ice-cold acetaldehyde (5 ml, 157 mmol) was added dropwise to a mixture of *p*-toluidine (9.57 g, 89.3 mmol) and benzotriazole (2.13 g, 17.9 mmol) in 90 ml of EtOH, and the mixture was stirred at room temperature for 4 days. After evaporating the solvent, the residue was purified by column chromatography (hexane/ethyl acetate = 4:1) to give 2,6-dimethyl-4-(4-methylphenylamino)-1,2,3,4-tetrahydroquinoline (3.49 g, 13.1 mmol, 29%) as a *cis/trans* mixture. 2,6-dimethyl-4-(4-methylphenylamino)-1,2,3,4-tetrahydroquinoline in THF and anhydrous pyridine were added dropwise to a solution of 4-methylbenzoyl chloride

Figure 4. Structural requirements of THQs for ecdysone agonist activity.

(1.14 ml, 7.39 mmol) in anhydrous THF at 0 °C. The reaction mixture was warmed up to room temperature and stirred overnight. After adding 240 ml of THF, the solution was washed with a saturated NaHCO$_3$ aqueous solution and brine. The organic layer was dried over MgSO$_4$ and concentrated. The residue was purified by column chromatography (hexane/ethyl acetate = 4:1) to give *cis* isomer compound **1** (270 mg (0.702 mmol), 19%). Mp: 185 °C. Anal. Calcd for C$_{26}$H$_{28}$N$_2$O C, 81.21; H, 7.34. Found: C, 81.32; H, 7.56. Compound **2**. Mp: 183 °C. Anal. Calcd for C$_{25}$H$_{25}$ClN$_2$O: C, 74.15; H, 6.22. Found C, 74.27; H, 6.36. Compound **3** mp: 178 °C. Anal. Calcd for C$_{23}$H$_{19}$ClF$_2$N$_2$O: C, 66.91; N, 6.79; H, 4.64. Found: C, 66.80; N, 6.82; H, 4.76. Compound **4**: ^{1}H NMR (CDCl$_3$, 400 MHz); δ (ppm) 1.26 (3H, d, J = 6.4 Hz), 1.35 (1H, m), 2.81 (1H, m), 3.72 (1H, br), 4.33 (1H, dd, J = 4.6, 12.0 Hz), 4.88 (1H, m), 6.48 (1H, br), 6.61 (2H, m), 6.68 (1H, m), 6.96 (2H, m), 7.06 (1H, m), 7.12 (2H, d, J = 6.6 Hz), 7.41 (2H, d, J = 6.8 Hz). Mp: 187 °C. Anal. Calcd for C$_{23}$H$_{19}$BrF$_2$N$_2$O: C, 60.41; N, 6.13; H, 4.19. Found: C, 60.21; N, 6.17; H, 4.23.

22. *Separation of enantiomers of compound **4** by chiral HPLC*: The enantiomers were separated on a chiral HPLC column containing a silica gel bound to tris(3,5-dimethylphenylcarbamate) derivatives of amylose (ADMPC) at a 2.0 ml/min flow rate at 40 °C using hexane/ethanol (90/10). The analytes were detected by UV absorption at 254 nm. An ADMPC-bound silica gel column was prepared according to the published method (Method-II in the report by Enomoto and coworkers[17]). The particle size of the column material was 5 µm and was packed in a stainless steel tube (250 mm × 4.6 mm). The retention times for each fraction are 4.3 min (**4a**) and 5.8 min (**4b**), respectively. This HPLC fractionation was repeated 200 times, and all fractions were combined. Enantiomer excess (ee) was determined by the same method and found to be > 99%. After evaporating the solvent, each compound was recrystallized from hexane-ethyl acetate to obtain **4a** and **4b** crystals. Melting points of these compounds were both 201 °C. The HRMS were 456.0647, 458.0631 (**4a**) and 456.0655, 458.0636 (**4b**), (calcd 456.0649, 458.0628). Optical rotations were $[\alpha]_D^{29}$+443 (c = 0.040, ethanol) for **4a**, and $[\alpha]_D^{29}$ −441 (c = 0.037, ethanol) for **4b**.

23. The crystals were mounted on a glass fiber, and measurements were made on a Rigaku RINT RAPID/R with graphite monochromated Cu Kα radiation (λ = 1.5418 Å) at 243 K. All data were processed and corrected for Lorentz and polarization effects. Intensity data within $2\theta \leqslant 136.4°$ were measured using an imaging plate area detector. The structure was solved by direct method using the SHELXS-97 program.[20] Positional parameters of non-H atoms were refined by full-matrix least squares using the SHELXL-97 program[20]. All non-hydrogen atoms were refined with anisotropic thermal parameters. Hydrogen atoms were placed in geometrically idealized positions and standard riding atoms. Crystal data, data collection parameters, and results of the analyses for compound **4a** and **4b** are deposited under the designations 984530 (**4a**) and 984531 (**4b**). Crystal data for compounds **1** and **2** were also deposited under designations 984528 and 984529, respectively.

24. The eggs of the mosquito *Culex pipiens pallens* were kindly provided by Sumitomo Chemical Company (Hyogo, Japan), and reared to the 2nd instar in the laboratory using a 300 cm^2 (approx. 5 cm water depth) tank. Three tablets of Ebios (Asahi Food and Health) were added under conditions of a long-day photo period (16 h light: 8 h dark). Twenty larvae grown for two days after hatching and were transferred to a paper cup containing 30 ml of water and a small amount of Ebios. To this was added 10 µl of DMSO solution with varying concentrations of the test compounds. After rearing for three additional days, the mortality of the mosquitos was evaluated. In each experiment, DMSO was used as a negative control, and tebufenozide treatment (33 µM) was used as a positive control (100% mortality). The median lethal concentration (LC$_{50}$) was evaluated from the concentration-response curves, and the reciprocal logarithm of LC$_{50}$ (pLC$_{50}$) was used as the index of larvicidal activity.

25. The binding assay procedure is the same as that reported previously[18,19]. Insect cells of the forest day mosquito *Aedes albopictus* (NIAS-AeAl-2) were obtained from the National Institute of Agrobiological Sciences (NIAS) GeneBank (http://www.gene.affrc.go.jp/index_en.php). AeAl cells were cultured at 25 °C in 25-cm^3 tissue culture flasks containing approximately 5 ml of the culture medium EX-Cell 401 (SAFC Biosciences) supplemented with 10% fetal bovine serum (FBS). Cell suspensions (400 µl) were added to disposable glass tubes (12 mm × 75 mm) containing 1 µl of the test compound in a DMSO solution at the bottom of the glass tube. Two microliters (ca. 60,000 dpm) of [^{3}H] PonA (140 Ci/mmol; American Radiolabeled Chemicals, Inc., St Louis, MO, USA) solution diluted with 70% ethanol was then added at 1-min intervals and incubated at 25 °C. After 30 min of incubation, 3 ml of water was added to the tubes. The contents were immediately filtered through glass filters (GF-75, ϕ 25 mm; ADVANTEC, Tokyo, Japan) and washed two times with 3 ml of water. The filters were dried under an infrared lamp, and placed into LSC vials. The amount of radioactivity collected on the filters was measured in 3 ml of Aquasol-2 (Packard Instrument Co., Meriden, CT, USA) using an Aloka LSC-5000 counter (Aloka Co., Ltd, Tokyo, Japan). The concentration required to give 50% inhibition of the binding of [^{3}H]PonA (IC$_{50}$, M) was determined from the concentration-response curve, and the reciprocal logarithm of IC$_{50}$ (pIC$_{50}$) was used as the index of binding activity.