J. Pestic. Sci. **49**(4), 303–310 (2024)
DOI: 10.1584/jpestics.J24-02

JOURNAL OF PESTICIDE SCIENCE

Society Awards 2024

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Design, synthesis, and biological evaluation of insect hormone agonists

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(Accepted July 30, 2024)

Agonists of insect hormones, namely molting hormone (MH) and juvenile hormone (JH), disrupt the normal growth of insects and can be employed as insecticides that are harmless to vertebrates. In this study, a series of experiments and computational analyses were conducted to rationally design novel insect hormone agonists. Syntheses and quantitative structure–activity relationship (QSAR) analyses of two MH agonist chemotypes, imidazothiadiazoles and tetrahydroquinolines, revealed that the structural factors important for the ligand–receptor interactions are significantly different between these chemotypes. On the other hand, a virtual screening cascade combining ligand- and structure-based methods identified a piperazine derivative as a novel JH agonist. The results obtained in this study will be useful for the future development of novel insect growth regulators.

Keywords: molting hormone, ecdysone, juvenile hormone, QSAR, virtual screening.

Introduction

Insects grow by repeated molting and metamorphosis. These processes are regulated by two peripheral hormones: the steroidal molting hormone (MH) and the terpenoidal juvenile hormone (JH). The coexistence of MH and JH in insect larvae induces molting, whereas the presence of MH alone triggers metamorphosis. 20-Hydroxyecdysone (20E, Fig. 1) functions as the principal MH in almost all insects. In contrast, a variety of JH molecules have been identified (Fig. 1).[1] JH I and II are the principal hormones in lepidopterans, whereas JH III is predominantly identified in the other insects. Higher dipterans (flies, Brachycera) produce, in addition to JH III, methyl farnesoate (MF)[2] and JH III bisepoxide (JHB_3).[3] A wide range of heteropterans have developed JH III skipped bisepoxide ($JHSB_3$) as their own JH.[4,5]

The molecular mechanism of MH reception was confirmed in the 1990s.[6,7] After entering into target cells, 20E binds to the nuclear receptor complex composed of ecdysone receptor (EcR) and ultraspiracle (USP). The resulting ternary complex binds to the ecdysone response element (EcRE) to trigger molting and metamorphosis. On the other hand, the mechanism of JH reception was established as late as the 2010s.[8,9] In target cells, JH binds to a transcription factor named methoprene-tolerant (Met), a member of the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family. The liganded Met dimerizes with another bHLH/PAS member, taiman (Tai), to bind the JH response element (JHRE), thereby suppressing metamorphosis.

Since molting and metamorphosis are developmental processes specific to arthropods including insects, disruptors of these processes have been considered as promising insecticide candidates with reduced safety concerns.[10] To this end, a variety of insect hormone agonists have been explored, and some compounds, such as tebufenozide, methoprene, and pyriproxyfen, have been successfully launched as insecticides to control agricultural pests and disease vectors (Fig. 2). These compounds, together with chitin synthesis inhibitors, are categorized as insect growth regulators (IGRs) or insect growth disruptors (IGDs).[11] Most of these IGRs, however, were discovered through random screening or analog synthesis; very few examples were found *via* rational approaches.[12]

We have conducted a series of structure–activity relationship (SAR) studies on insect hormone agonists to rationally design novel IGRs.[13–22] This review describes quantitative structure–activity relationship (QSAR) studies on two types of MH agonists and assay development and virtual screening to identify novel JH agonists.

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Published online December 5, 2024

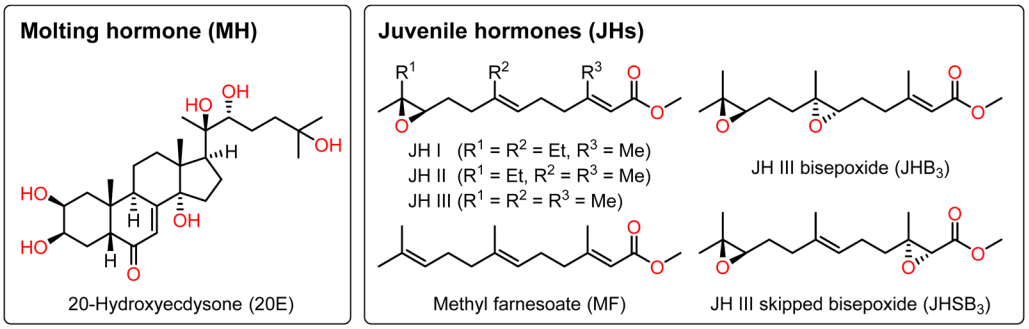


Fig. 1. Chemical structures of insect hormones.

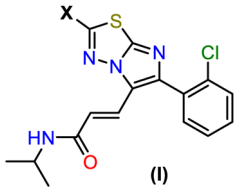
1. MH agonists

1.1. Imidazothiadiazoles[14,15]

Imidazothiadiazoles (ITDs) are a class of nonsteroidal MH agonists discovered by Bayer agricultural chemists (Fig. 2).[23] Structurally, they are characterized by a unique [5,5]-fused ring system and an acrylamide moiety. According to brief SAR data reported by Holmwood and Schindler,[23] some ITD analogs achieved nanomolar potency in an MH-inducible gene expression system. However, the authors did not disclose the experimental details or the target insect species. Further, the substituent effect on the activity, especially at the 2-position of their fused ring system, remained unclear.

To answer these questions, we synthesized ITD congeners and evaluated their activity in a competitive binding assay using [^{3}H]Ponasterone A as the radioactive ligand.[14,15] This assay employs endogenous EcR/USP complexes expressed in cultured insect cells.[24] To investigate insect-order selectivity, we used three insect cell lines: lepidopteran Sf-9 (*Spodoptera frugiperda*), dipteran NIAS-AeAl-2 (*Aedes albopictus*), and coleopteran BCIRL-Lepd-SL1 (*Leptinotarsa decemlineata*). As shown in Table 1, the binding activity of ITD **1** was the strongest in Sf-9 cells, followed by NIAS-AeAl-2 and BCIRL-Lepd-SL1 cells. This selectivity is similar to that of tebufenozide, a diacylhydrazine (DAH)-type MH agonist, and is different from that of 20E. These results suggest that ITDs adopt a mode of binding to the receptor that is similar to that of DAH-type compounds.

Next, we performed the Hansch–Fujita type of QSAR analysis to reveal the effect of substituents at the 2-position (**X**) of the [5,5]-fused bicyclic system.[14,15] Equation (1) was formulated for the binding activity of 15 ITD analogs (**I**):



(I)

$$pIC_{50} = 1.63\mathrm{CLog\,P} + 4.91\sigma_I - 0.32\Delta L - 0.93 \quad (1)$$
$$n = 15,\ s = 0.35,\ r = 0.97$$

In this and the following equations, n is the number of compounds, s is the standard deviation, and r is the correlation coefficient. In Eq. (1), CLogP is the molecular hydrophobicity calculated by the CLOGP algorithm, σ_I is the inductive component

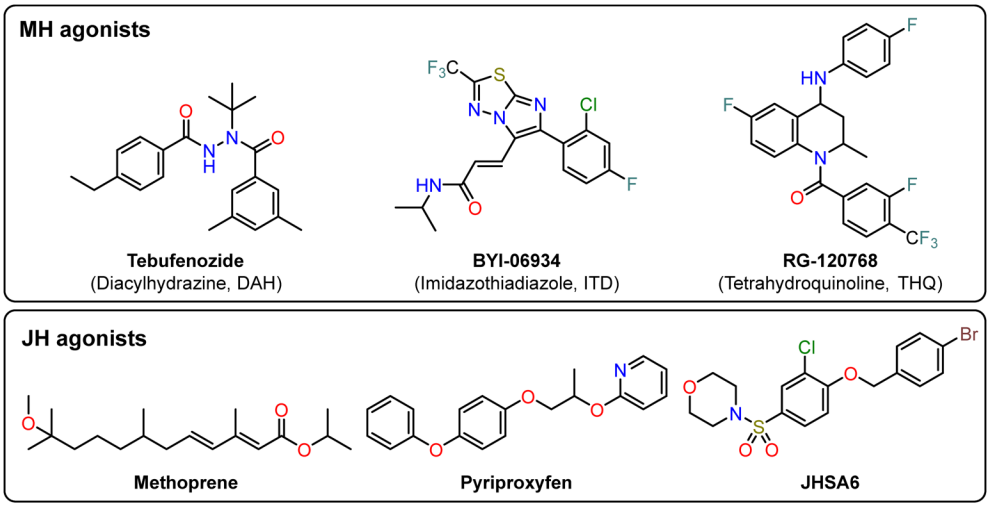
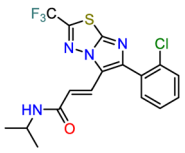
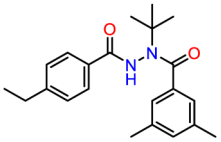
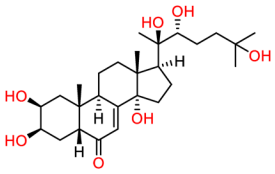


Fig. 2. Chemical structures of synthetic insect hormone agonists.

Table 1. Binding activity of ITD **1** and other MH agonists in three insect cell lines

Compound	Structure	Binding activity [pIC_{50} (M)]		
		Sf-9	NIAS-AeAl-2	BCIRL-Lepd-SL1
1		8.03[a)]	7.10[a)]	4.88[a)]
Tebufenozide		8.81[b)]	7.12[a)]	5.18[c)]
20E		6.78[b)]	7.68[d)]	6.36[c)]

[a)] Taken from ref. 14) [b)] Taken from ref. 24) [c)] Taken from ref. 48) [d)] Taken from ref. 16)

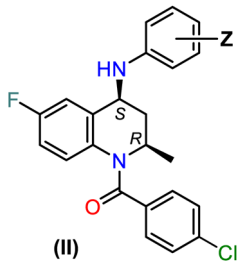
of the Hammett constant σ, and ΔL is the STERIMOL length parameter relative to hydrogen. Equation (1) clearly shows that hydrophobic and inductively electron-withdrawing substituents enhance the binding activity, whereas long substituents are sterically unfavorable. Compound **2** (**X**=CF_2CF_3), satisfying the requirements defined by Eq. (1), showed low nanomolar binding activity (pIC_{50}=8.35) and was 37 times more potent than 20E.

1.2. Tetrahydroquinolines[16,17]

Tetrahydroquinolines (THQs) are a group of nonsteroidal MH agonists that were originally identified at FMC corporation (Fig. 2).[25] THQs were reported to be highly selective to mosquito EcRs,[26] suggesting their potential use as novel mosquito control agents. They have two chiral centers at the 2- and 4-positions of the THQ ring, resulting in four stereoisomers. This feature made their synthesis difficult, leaving their SAR poorly explored. To summarize the SAR data reported, the *cis*-stereochemistry of the THQ core is essential for retaining the MH-like activity, and halogen substituents on the benzoyl moiety are likely to enhance the biological activity.[27] However, the effect of substituents on each benzene ring remained ambiguous, presumably because THQs were tested as racemates in these SAR studies. In 2014, Kitamura *et al.* optically resolved a *cis*-THQ derivative to show that the (2*R*,4*S*)-isomer is about 40 times more potent than its enantiomer.[28] Thus, the SAR associated with the (2*R*,4*S*)-isomers is of particular interest. However, the optical resolution of THQs relied on chiral high-performance liquid chromatography (HPLC), limiting the synthetic throughput required for SAR studies.[28] Furthermore, the previous SAR studies of THQs have focused on *in vitro* MH-like activities; the determinants of their insecticidal activity have remained unclear.

To enable rapid access to THQs with a (2*R*,4*S*)-configuration, we set out to develop a novel asymmetric synthetic route to THQs (Fig. 3).[16] The construction of the THQ core was done with a chiral phosphoric acid-catalyzed Povarov reaction developed by Masson and co-workers.[29,30] This reaction gave compound **6** with an enantiomeric excess (ee) of 89%, which was further enriched to 98% after recrystallization. Next, the reaction of **6** with 4-bromobenzoyl chloride gave **7** in a 95% yield. The deprotection of the carboxybenzyl (Cbz) group and the following Chan–Evans–Lam cross coupling with a phenyl boronic acid gave **8** in a 76% yield. After recrystallization, the enantiomeric purity of **8** was checked by chiral HPLC analysis and proven to be >99% ee. This route enabled the synthesis of over 50 THQ derivatives with a (2*R*,4*S*)-configuration.

The binding activity of the synthesized compounds was evaluated in NIAS-AeAl-2 cells. Then, the Hansch–Fujita type of QSAR analysis was performed to delineate the substituent effects of the THQ analogs.[16] Equation (2) was formulated for the set of compounds with varied substituents **Z** at the anilino moiety **(II)**:



(II)

$$pIC_{50} = 0.33\pi^{meta} - 1.03\Delta B_5^{meta} - 0.39\Delta V_w^{para} + 7.27 \quad (2)$$
$$n = 19,\ s = 0.27,\ r = 0.89$$

where π^{meta} is the hydrophobicity parameter of the *meta*-

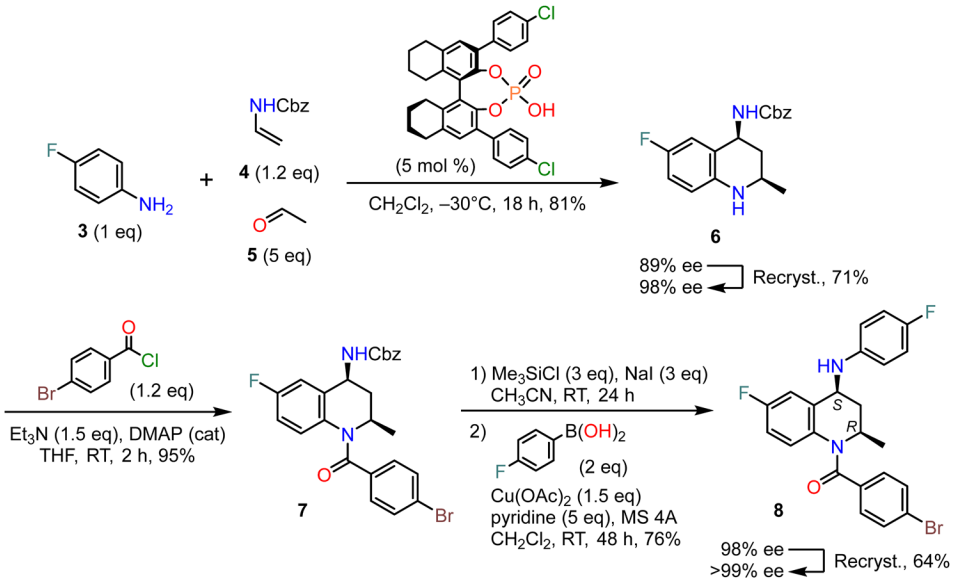
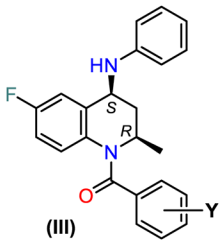


Fig. 3. Asymmetric synthesis of a THQ derivative **8**. Adapted from ref. 16)

substituents, ΔB_5^{meta} is the STERIMOL width parameter of the *meta*-substituents relative to hydrogen, and ΔV_w^{para} is the van der Waals volume of the *para*-substituents relative to hydrogen. Equation (2) indicates that hydrophobic substituents at the *meta*-position enhance the binding activity, whereas both *meta*- and *para*-substitutions are detrimental to the activity for steric reasons.

For the set of compounds with varied substituents **Y** at the benzoyl moiety **(III)**, Eq. (3) was formulated:



$$pIC_{50} = 0.49\Sigma\sigma + 0.39\Delta L^{para} - 1.11\log(\beta 10^{\Delta L^{para}} + 1) + 6.29 \quad (3)$$
$$n = 21,\ s = 0.27,\ r = 0.88,\ \log\beta = -3.05,\ \Delta L^{para}(\text{opt}) = 2.80$$

where $\Sigma\sigma$ is the sum of the Hammett substituent constant σ, and ΔL^{para} is the STERIMOL length parameter of the *para*-substituents relative to hydrogen. The positive coefficient of the $\Sigma\sigma$ term indicates that electron-withdrawing substituents enhance the binding activity. Interestingly, the steric effect at the *para*-position is well described by Kubinyi's bilinear model[31] (Fig. 4): there is the optimum length [ΔL^{para}(opt)] of 2.80, above and below which the slopes of the regression curve are different [0.39 and −0.72 (=0.39 −1.11), respectively]. Compound **9** (**Y**=3-F-4-CN), satisfying the requirements defined by Eqs. (2) and (3), displayed the strongest binding activity (pIC_{50}=8.04) and was twice as potent as 20E.

We next set out to disclose the determinants of larvicidal activity.[17] The mortality rate [*A* (%)] of each test compound was measured against second-instar larvae of *A. albopictus* at 1 μM, and it was logit-transformed to yield logit *A* [=log [*A*/(100−*A*)]]. For the set of compounds with varied benzoyl moieties, Eq. (4) was formulated:

$$\text{logit}\, A = 0.92pIC_{50} + 0.27C\,\text{Log}\,P - 7.48 \quad (4)$$
$$n = 31,\ s = 0.40,\ r = 0.82.$$

As is evident from Eq. (4), the binding activity and the molecular hydrophobicity are key determinants of the larvicidal activity of THQ analogs.

2. *JH agonists*

2.1. *Development of a reporter gene assay*[18]

Reliable assay systems are integral components of SAR studies. To date, various assay methods have been developed to detect

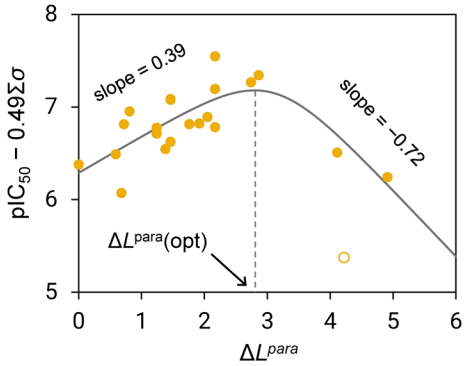
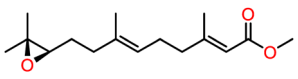
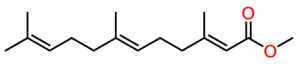
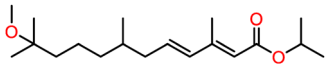
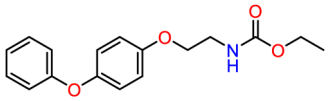


Fig. 4. Partial correlation between the binding activity and ΔL^{para} values of THQ analogs [Eq. (3)]. The open circle indicates an outlier (Y=4-Ph). Adapted from ref. 16)

Table 2. Comparison of pEC_{50} values of JH agonists in three different reporter systems

Compound	Structure	Transactivation activity [pEC_{50} (M)]		
		This study[a)]	Two-hybrid assay[b)]	S2 reporter assay[c)]
JH III		8.40	6.29	6.68
MF		7.93	5.73	5.35
Methoprene		7.44	5.28	N/A
Fenoxycarb		9.17	N/A	8.64

[a)] A reporter assay in HEK293T cells transfected with DmMet/DmTai.[18] [b)] A two-hybrid reporter assay in CHO cells transfected with VP16AD-DmMet and GAL4DBD-DmTai.[39] [c)] A reporter assay in *Drosophila* S2 cells expressing endogenous JH receptors.[38] N/A, not available.

JH-like activities. Historical methods use whole-body insects or cultured tissues to observe JH-dependent morphological changes; however, these methods suffer from low throughput and poor reproducibility.[32,33] The identification of the JH receptor complexes has facilitated the development of more efficient, easier to handle *in vitro* methods.[34] These methods include (i) binding assays using radioactive JH agonists,[35,36] (ii) reporter gene assays in cultured insect cells,[37,38] (iii) two-hybrid reporter gene assays using engineered Met and Tai proteins,[38,39] and (iv) a reporter gene assay using a recombinant yeast strain expressing *Met* and *Tai* genes.[40] Each of these assays, however, still has its own drawback(s). Method (i) requires a radioactive JH analog, which is currently commercially unavailable, and cannot discriminate between agonists and antagonists. Method (ii) suffers from perturbation by endogenous JH-signaling factors, such as JH metabolism and feedback regulation. Method (iii) relies on ligand-dependent heterodimerization between Met and Tai, which does not necessarily reflect transactivation activity. Method (iv) underestimates the activity of hydrophilic compounds, probably due to their poor cellular uptake through yeast cell walls.

With these drawbacks of the existing methods in mind, we set out to develop a new luciferase reporter gene assay.[18] Mammalian HEK293T cells were selected as host cells to eliminate the effects of endogenous JH-signaling molecules. The cells were transiently transfected with expression plasmids carrying the *Drosophila melanogaster Met* (*DmMet*) and *Tai* (*DmTai*) genes, along with a reporter plasmid carrying a firefly luciferase (Fluc) gene downstream of two tandem copies of *Bombyx mori* JHRE (kHJRE). A reference reporter plasmid (pRL-SV40) was co-transfected to correct the experimental variations. This reporter system was able to detect known JH agonists with varied structures (see ref.[18] for details). The pEC_{50} values of the selected JH agonists were compared with those evaluated using the existing methods[38,39] (Table 2). The pEC_{50} values in this study were two orders of magnitude larger than those obtained in the two-hybrid assay that was built on the GAL4–VP16 system,[39] indicating the improved sensitivity of the present method. Interestingly, the pEC_{50} values of natural JHs (JH III and MF) in this study were larger than those obtained in the cultured insect cell (*Drosophila* S2)-based method,[38] despite the similar levels of potency of fenoxycarb in these two assay methods. The underestimated potencies of natural JHs in S2 cells are likely due to endogenous JH-signaling pathways, such as JH degradation by JH esterase and epoxide hydrolase. Thus, our novel reporter system, which overcomes the drawbacks of the existing methods, is a valuable tool for evaluating the intrinsic activity of JH agonists.

2.2. *Virtual screening of novel JH agonists*[19]

To date, various JH agonists have been identified *via* chemical synthesis. Examples of synthetic JH agonists include methoprene, fenoxycarb, and pyriproxyfen, all of which were launched as insecticides in the late 20th century (Fig. 2). These compounds were identified *via* random screening or analog synthesis. Recently, Kayukawa *et al.* discovered several new JH agonists, including JHSA6 (Fig. 2), *via* a high-throughput screening campaign.[41] Thus, all of these JH agonists were identified by chance, and no JH agonist has hitherto been developed *via* a computational approach.

To identify novel JH agonists computationally, we performed a four-step virtual screening campaign against a database of 5 million purchasable compounds (Fig. 5A).[19] The first three steps were conducted *in silico*: (i) the shape-matching against known active compounds using ROCS,[42] (ii) the docking screening against a homology model of DmMet using FRED,[43,44] and (iii) the modified MM/PBSA (molecular mechanics/Poisson–Boltzmann surface area) screening using Amber and Freeform.[45–47] These computational filters reduced the database to 11 compounds, which were subjected to the reporter gene assay in HEK293T cells.[18] Among the compounds tested, only compound **10** induced the reporter activity in a dose-depen-

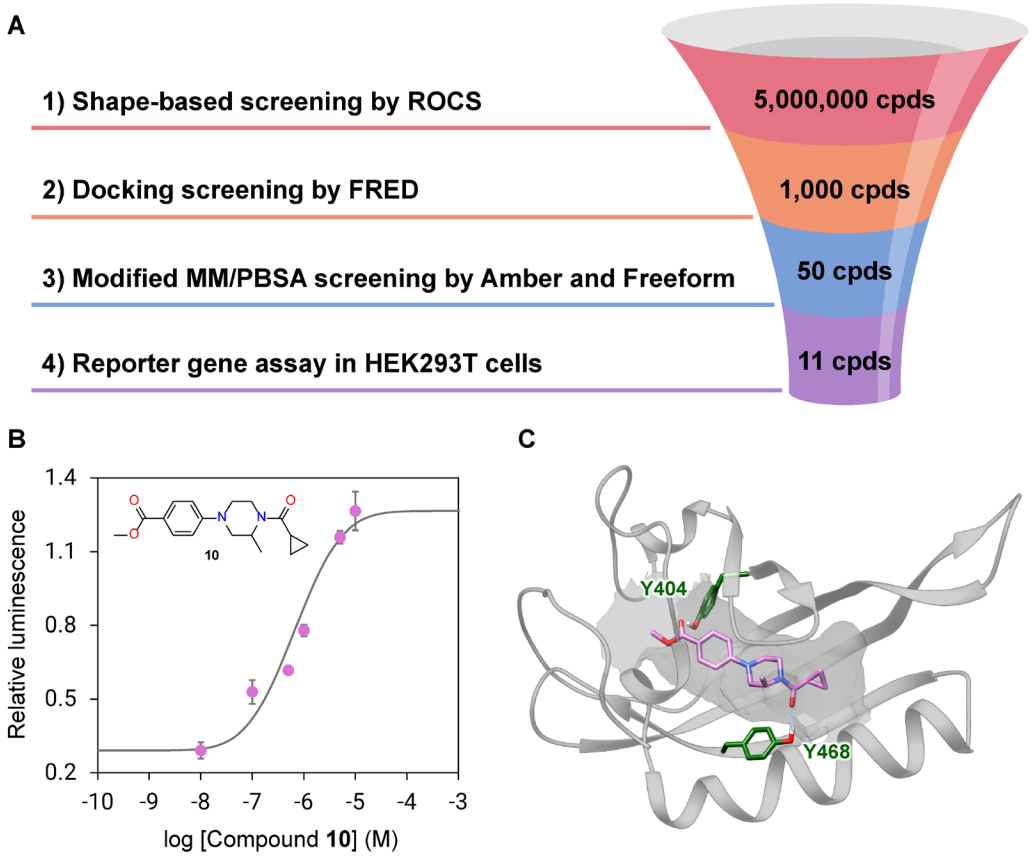


Fig. 5. Identification of compound **10** as a novel JH agonist. (A) Overview of the virtual screening campaign to identify novel JH agonists. (B) A concentration–dependent activation of the reporter gene activity by compound **10**. (C) A representative binding mode of **10** toward the DmMet PAS-B domain.

dent manner (Fig. 5B). The pEC_{50} value of **10** was 6.06, which is 140 times less active than JH I. However, the simple chemical structure of **10** makes its synthesis and optimization easy. Figure 5C shows a representative binding mode of **10** to the receptor extracted from the molecular dynamics simulation. Two hydrogen-bonding interactions with two Tyr residues (Y404 and Y468) are likely to be key contributors to the stable binding of **10**.

Concluding remarks

In this study, we performed QSAR analyses to determine the structural requirements of ITD- and THQ-type MH agonists for their binding to insect EcRs. The QSAR results differed significantly between these two chemotypes, suggesting their different modes of interaction with the receptor. The present QSAR equations will guide the structure optimization of these chemotypes in the future. On the other hand, a multi-step virtual screening campaign identified compound **10** as a novel JH agonist. The simple chemical scaffold of **10** make this compound a useful starting point for the further structure optimization.

Acknowledgements

This study was conducted at the Laboratory of Bioregulation Chemistry, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University. The author would like to express his sincere gratitude to Professors Hisashi Miyagawa, Yoshiaki Nakagawa, Masahiro Miyashita, and Miki Akamatsu for their kind guidance and warm encouragement during this study. The author is also grateful to Professor Ken'ichiro Hayashi (Osaka University), Professor Takashi Yagi (Osaka Metropolitan University, OMU), and Dr. Sayoko Ito-Harashima (OMU) for their invaluable contributions to the development of the reporter gene assay. Lastly, the author would like to thank his co-workers, Ms. Saki Minami, Ms. Chiharu Ishizuka, Mr. Shinri Horoiwa, Mr. Taku Nabe, and Ms. Minori Ueno. Part of this work was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Number JP17J01486.

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