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Target-based design, synthesis and biological activity of new pyrazole amide derivatives

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Abstract

Based on the similarities in the conformations of VS008 (N-(4-methylphenyl)-3-(tert-butyl) -1-(phenylmethyl)-1H-pyrazole-5-carboxamide) and BYIO6830 (N′-(3,5-Dimethylbenzoyl)-N′-tert-butyl-5-methyl-2,3-dihydro-1,4-benzodioxine-6-carbohydrazide) bound to the active site of the EcR subunit of the ecdysone receptor (EcR)-ultraspiracle protein (USP) heterodimeric receptor, a series of new pyrazole amide derivatives were designed and synthesized. Their structures were confirmed by IR, ^{1}H NMR and elemental analysis. Results from a preliminary bioassay revealed that two of the pyrazole derivatives exhibited promising insecticidal activity. Specifically, compounds **6e** and **6i** exhibited good activity against Helicoverpa armigera (cotton bollworm) at low concentration. Symptoms displayed by tebufenozide-treated H. armigera were identical with thoseode displayed by its treated counterpart. **6i** showed the same poisoning symptoms as those of tebufenozide. In addition, results from molecular docking result indicated that the binding m of **6e** and **6i** at the active site of the EcR subunit of the heterodimeric receptor is similar to that of the bound tebufenozide.

Keywords

Moulting hormone, Pyrazole amide, Rational design, Bioactivity, Molecular docking

1. Introduction

20-Hydroxyecdysone (20E), the molting hormone of several insects, initiates moulting through the heterodimeric ecdysone receptor (EcR)-ultraspiracle protein (USP) receptor [1, 2]. Design of other ligands targeting the EcR-USP heterodimer remains a prominent approach towards the development of environmentally benign insect growth regulators [3]. For example, non-steroidal ecdysone agonists, dibenzoylhydrazine insecticides (DBHs), bind to the EcR subunit of the heterodimer and induce the insect moulting. These agents, which bear the common dibenzoylhydrazine core structure, exhibit an excellent activity against lepidopteran pests but have no effect on mammals and environment [4-7].

However, their relatively narrow spectrum of activity and emergence of DBH-resistant insects have prompted the efforts towards the design of compounds containing different structural motifs or identification of newer molecular targets. With the progress towards the structural resolution of target EcRs over last few decades, it is now possible to adopt a target structure-guided approach towards the design of new compounds with molting hormone activity. Such efforts have led to the identification of highly active and novel ecdysone agonists (I–IV, Fig. 1) in recent years [8, 9].

Previously, using a technique of virtual screen against the EcR-USP crystal structure, we obtained a compound library and identified ligands that interact with the EcR subunit of the heterodimer. Analysis of the binding results revealed that the identified pyrazole-based compound VS008 (*N*-(4-methylphenyl)-3-(*tert*-butyl)1-(phenylmethyl)-1*H*-pyrazole-5-carboxamide) has an active conformation that is similar to that of BYIO6830 (*N′*-(3,5-dimethylbenzoyl)-*N′-tert*-butyl-5-methyl-2,3-dihydro-1,4-benzodioxine-6-carbohydrazide), a DBH analog (Fig. 2), at the target site of the EcR subunit[10]. Inspired by these observations, a series of pyrazole amides based on the lead compound, VS008, were designed and synthesized in this study. Subsequently, their insecticidal activity against a few lepidopteransis was evaluated. Furthermore, interactions between these newly synthesized compounds and the target site on the EcR subunit were also investigated by molecular docking.

2. Experimental

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Melting point of all compounds were determined on an X-5 binocular (Fukai Instrument Co., Beijing, China), and were not corrected. ^{1}H NMR spectra were recorded on a Bruker AM-300 (300 MHz) spectrometer with $CDCl_3$ as the solvent and TMS as the internal standard. Chemical shifts were reported in δ (parts per million) values. IR spectra were recorded on a Perkin Elmer Spectrum 100 FT-IR spectrometor (KBr presser method). Elemental Analysis was obtained with an ST-Carloerba. Co instrument. All the reagents were obtained commercially and used after further purification. VS008 was bought from J&K Scientific. Column chromatography purification was carried out using sillca gel.

2.1 General procedure for synthesis of compounds **6a-p**

Pinacolone **1** (0.087 mol) and diethyl oxalate **2** (0.087 mol) were added dropwise to a solution of ethanol (55mL) containing thinly sliced sodium (0.047 mol) at 0°C. The mixture was stirred overnight at room temperature. Next morning, the mixture was acidified (pH 3.0, with 20% H_2SO_4) and filtered to remove the formed solid. The filtrate was extracted with dichloromethane, dried, and concentrated under vacuum to yield an orange red viscous liquid **3** (12.98 g; 74.6% y). A solution of the 1,3-diketone **3** (0.025 mol) in methanol (10 mL) was added dropwise to a cooled solution (0°C) of hydrazinobenzene (0.025 mol) in methanol (30 mL). The mixture was warmed to room temperature by stirring for an hour and then refluxed for 2h. The resulting cooled mixture was concentrated under vacumm and the pyrazole ester **4** was obtained after purification by column chromatography (a 5% gradient of ethyl acetate in hexanes over a column of silica gel). To saponify the ester, a solution of **4** (0.007 mol) was combined with an aliquot of 6 mol/L NaOH(aq) (7 mL) and the mixture was stirred at 80°C. Ice water (50 mL) was added at the end of 2 h and the mixture was acidified (pH 1–2) with concentrated HCl. The formed solid was collected by filtration and the filter-cake was dried. The carboxylic acid was purified by recrystallization (methanol:water, 1:1) to afford **5** (3.57g; 83.2% y).

The amide derivatives **6a–p** were prepared through the acyl chlorides derived from **5.** A solution of **5** (0.004 mol) in thionyl chloride (10mL) was refluxed for 5h [11] and then concentrated under vacumm. The formed crude acyl chloride was added dropwise to a cooled solution (0 °C) of substituted aniline (0.004 mol) and TEA (0.008 mol) in dichloromethane (10 mL). The resulting mixture was stirred overnight at room termparature to produce the crude product, which was purified on a column of silica using a gradient of ethyl acetate in hexanes to afford the pure products **6a**–**p**.

2.2 Insecticidal test of target compounds **6a-p**

Their biological activities against *Mythimna separate, Helicoverpa armigera* and *Pyrausta nubilalis* were evaluated using the reference method [12, 13]. The poisoning symptoms of *Helicoverpa armigera* treated with **6i** were tested *using* the reference method [14].

2.3 Molecular docking

A Molecular Operating Environment (MOE) software [15] was used for the molecular docking. All synthesized compounds were built and optimized using the MMFF94 force field and charges. The low energy conformation of each compound was selected as the initial docking conformation. A crystal structure of ecdysone receptor (EcR) complexed with BYIO8346, which was obtained at 2.85 Å, was downloaded from the protein data bank (PDB ID: 3IXP). Waters and other solvent molecules (such as phosphatidylethanolamines) were removed and the modified structure was protonated. The active site was defined by the residues with a radius of 6 Å around BYIO8346. The ligand molecules were placed in the site with the Triangle Matcher method and ranked with the London dG scoring function. 30 Docking poses per ligand molecules were retained and further refined by energy minimization in the pocket. Then they were rescored with the GBVI/WSA dG scoring function, a force field-based scoring function, which was used to estimate the binding free energy of these ligands with EcR.

2.4 Molecular dynamics simulations

The molecular dynamics (MD) simulations were performed using the AMBER 12 program [16] by using the AMBER ff99SB force field. The protein was solvated using explicit TIP3P water models and the water molecules extended about 10 Å from the protein atom in a cubic periodic box. Before the MD simulations, three energy minimization steps were applied to the system. First, the solute was kept fixed and waters and counterions were minimized. Second, the backbone atoms of the protein were fixed with the ligand, while side chains and other atoms were free to move. Finally, the entire system was fully minimized without any constraint. In each step, energy minimization was first performed using the steepest descent algorithm for 2000 steps and then the conjugated gradient algorithm for another 3000 steps.

The MD simulation was performed under periodic boundary conditions using the Sander module of the AMBER 12 program. First, the system was fixed to make the heating only for waters and counterions for 10 ps to ensure the solute was fully solvated; second, the whole system was gradually heated from 10 to 298 K by a weak-coupling method and equilibrated for 100 ps with the protein backbone fixed; last, the system was switched to a constant pressure equilibration for 20 ns. During the MD simulation, the particle mesh Ewald (pmE) algorithm was used to manage long-range electrostatic interactions with a cutoff distance of 10 Å, which was also used for the van der Waals (vdW) energy terms. All of the angles and bonds involving hydrogen atoms were constrained using the SHAKE algorithm. The binding free energy between ligands and receptor is computed as a sum of the gas-phase molecular mechanics energies, and solvation energy, and conformational entropy based on the MM-PBSA method.

3. Results and discussion

The route adopted for the synthesis of the targeted compounds **6a**–**p** is summarized in Scheme 1. Compound **3** is synthesized following a previously described protocol [11]. The pyrazole core of the key intermediate **4** is prepared by the cyclization of the 1, 3-diketone moiety in **3** with phenylhydrazine. The saponification of **4,** followed by the activation of the resulting carboxylic acid **5,** and subsequent amidation using substituted anilines afford the targeted amides **6a–p** in moderate yields.

The structures of the targeted compounds are confirmed through the analyses of their melting points, ^{1}H NMR spectra, ^{13}C NMR spectra, IR spectra, and elemental analysis. The ^{1}H and ^{13}C NMR spectra for Compound **6e**, **6i** are confirmed for instance. These data are included in the supporting information. The methyl protons of the *t*-butyl groups are observed at δ~1.20 in the ^{1}H NMR spectra of the compounds. Signals corresponding to the C–H and the N–H protons of the pyrazole ring in compounds **6a–p** are observed at δ 6.85 and δ 8.66, respectively and signal for the protons on the benzene ring are observed at δ 6.63–8.96. In the IR spectra of compounds **6a–p**, strong absorptions at 3200–3400 cm^{-1}, attributed to the presence of the secondary amide functionality, are observed. In addition, other strong absorptions bonds representing the carbonyl and unsaturated pyrazole groups are observed at ~1700 cm^{-1} and ~1500 cm^{-1}, respectively. The elemental analyses of the compounds **6a–p** are in good agreement with the theoretical data calculated based on the chemical formulae.

The insecticidal activity of these compounds against lepidopteran pests *Mythimna separata*, *Helicoverpa armigera* (cotton bollworm), and *Pyrausta nubilalis* are listed in Table 1. Compounds containing the *t*-butyl substituent at positions 2 (**6g**) and 4 (**6i**) relative to the aniline group exhibited superior activity. The increased activity observed for the **6g** and **6i** can be attributed to the hydrophobic effect of the *t*-butyl substituent. Other substituents at positions 2 and 4 are not as effective as *t*-butyl substitution. Amongst the analogues containing a substituent at position 3, the compound **6e**, harboring a methoxy substituent, exhibits the highest observed activity. The factors contributing to the observed high activity can be the hydrophobic effect and the relatively small size of the methoxy substituent. It is likely that the bulky *t*-butyl substituent is not optimally accommodated at the active site of the EcR subunit. At 600μg/mL, the insecticidal activity of compounds **6e** and **6i** is similar to that exhibited by the positive control tebufenozide. Even at lower concentrations, **6e** and **6i** exhibit activity against *H.armigera* (see Table 2); this indicates that an electron-donating substituent is more desirable than an electron-withdrawing substituent to generate a compound with better insecticidal activity. It is worth mentioning here that the skeletal structure of this series of compounds (**6a–p)** is similar to that of the pesticide chlorantraniliprole, a compound that induces a convulsion-free gradual contraction, thickening, and shortening of the insect body by binding to the ryanodine receptor [17, 18]. In order to gain insights into the mode of action of the pyrazole based compounds, the effects of treating *H.armigera* with **6i** are evaluated based on a method previously reported in literature [14]. Analysis of the results (Fig. S1, S2 in Supporting information) reveals that symptoms, such as lipped head capsule and extrusion of the hindgut, exhibited by a cotton bollworm treated with **6i** are similar to those exhibited by its tebufenozide-treated counterpart. These results indicate that it is more likely that, akin to tebufenozide, **6i** binds to the EcR subunit of the heterodimeric receptor [19] rather than the ryanodine receptor. The binding conformation of compounds **6e**, **6i**, and tebufenozide are evaluated by docking these compounds into the active site pocket of the EcR subunit (Fig. 3 (a)–(c)). Important amino acid residues forming hydrogen bonding interactions with tebufenozide are Asn504, Tyr408, and Thr343. The nitrogen atom of the pyrazole group for compound **6e** establishes a hydrogen bond interaction with Tyr408 (see Fig. 3(b)). For compound **6i**, the amide group forming hydrogen bonding interactions with both Asn504 and Tyr408, while the nitrogen atom of the pyrazole group interacts with the Thr343 through a hydrogen bond (see Fig. 3(c)). The docking results reveal that the binding conformations of **6e** and **6i** are similar to that of tebufenozide at the active site of the EcR subunit.

The calculated average free energies for the complexes of **6e** and EcR, **6i** and EcR are –54.18 kJ/mol and –71.35 kJ/mol, respectively (see Fig. 2S in Supporting information). Under the same conditions, the calculated average free energy of binding of tebufenozide to the EcR subunit is determined to be –69.46 kJ/mol. These results show that the binding free energy values of compounds **6e** and **6i** results in the higher activity in the enzymatic assay. Further studies on the binding assay and structure-activity relationship are currently in progress.

4. Conclusion

In summary, based on the conformational similarities observed in the binding modes of VS008 and BYIO6830 at the active site of the EcR subunit of the heterodimeric receptor, a series of novel pyrazole amides were designed and synthesized. Bioassay-guided studies revealed that when compared to the activity displayed by the tebufenozide, two of the synthesized analogues, **6e** and **6i**, displayed potent activity against lepidopteran pests *Mythimna separata, Pyrausta nubilalis* and *Helicoverpa armigera* even at low concentrations. Similarities in the observed symptoms when *H. armigera* was treated either with tebufenozide or the pyrazole analogues (**6e** or **6i**) indicated that these compounds might have the same mode of action, *i.e.*, by binding to the EcR subunit of the ecdysone EcR-USP heterodimeric receptor. Furthermore, molecular docking and molecular dynamics studies revealed that the binding modes of **6e** or **6i** with the EcR subunit were similar to that of tebufenozide. These results gave useful guides to discovering new IGRs.

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Fig. 1 Structures of new ecdysone agonists

Fig. 2 The design strategy of target compound 6 based on the binding conformation of VS008 and BYIO6830 with EcR

Scheme 1 Scheme displaying the synthetic route for obtaining the pyrazole-based analogues **6**. Reagents and conditions: (a) sodium, ethanol, 0 $^{\circ}$C, r.t. (overnight); (b) phenylhydrazine (1 equiv.), methanol, 0 $^{\circ}$C (30 min), r.t. (1 h), reflux (2 h); (c) (i) NaOH, 80 $^{\circ}$C (2 h), (ii) HCl; (d) (i) $SOCl_2$, (ii) substitutedaniline (1.0 equiv.),TEA (2.0 equiv.), DCM, 0 $^{\circ}$C (10 min), r.t. (overnight).

Fig. 3 Hydrogen bonding interactions between the binding pocket in the target EcR subunit and the compounds (a) tebufenozide, (b) **6e**, and (c) **6i.** Carbons in **6e, 6i,** and tebufenozide are represented by light blue spheres, oxygens as red spheres and nitrogens as dark blue spheres. H-bonds are indicated by blue dotted lines.

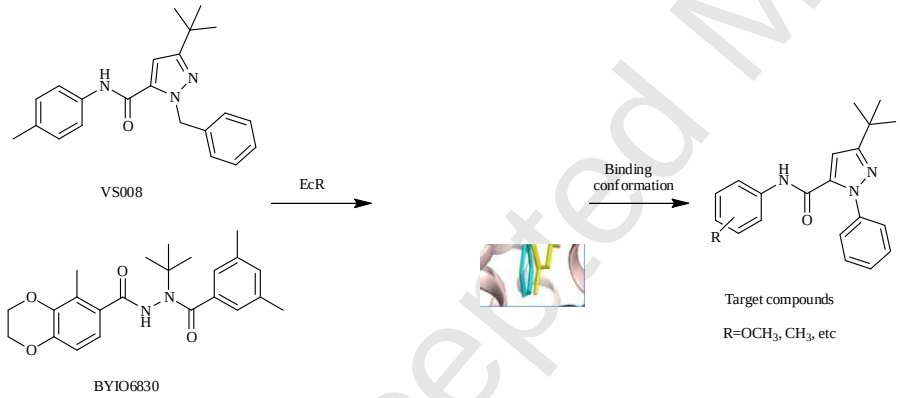
Table 1 Observed *in vivo* insecticidal activity (mortality) of pyrazole analogues (600 μg/mL)

Compounds	R	Mortality (%)		
		Mythimna Separate	*Helicoverpa armigera*	*Pyrausta nubilalis*
6a	2-Me	16.7	15	10
6b	3-Me	13.3	0	0
6c	4-Me	10	15	15
6d	2-OMe	0	10	0
6e	3-OMe	100	100	100
6f	4-OMe	40	20	25
6g	2-*t*-Bu	35	65	45
6h	3-*t*-Bu	10	15	55
6i	4-*t*-Bu	83.3	85	75
6j	2-NO_2	13.3	0	0

6k	3-NO_2	20	20	25
6l	4-NO_2	30	15	15
6m	2-Cl	23.3	20	10
6n	3-Cl	43.3	30	35
6o	4-Cl	26.7	35	50
6p	H	26.7	25	30
VS008		10	25	25
tebufenozide		100	100	100

Table 2 Dosage-dependent *in vivo* insecticidal activity (mortality) of **6e**, **6i,** and tebufenozide against *H. armigera*

Compd.	Mortality (%) at different concentrations (μg/mL)					
	200	100	50	25	5	1
6e	100	72.2	61.1	58.3	30.6	19.4
6i	77.8	63.9	58.3	52.8	36.1	25
tebufenozide	94.4	86.1	80.6	72.2	61.1	33.3



Based on the binding conformation of ligands with the ecdysone receptors (EcRs), a series of new pyrazole amide derivatives were designed and prepared. Several compounds, for example **6e** and **6i,** had good insecticidal activity. *Helicoverpa armigera* treated with **6i** showed the same poisoning symptoms as those of tebufenozide. Furthermore, molecular docking and molecular dynamics studies revealed that the binding modes of **6e** or **6i** with the EcR subunit were similar to that of tebufenozide. These results gave the useful guide to discover new IGRs.

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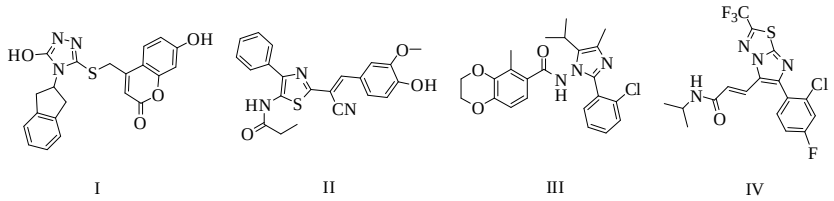


Fig.1 Structures of new ecdysone agonists

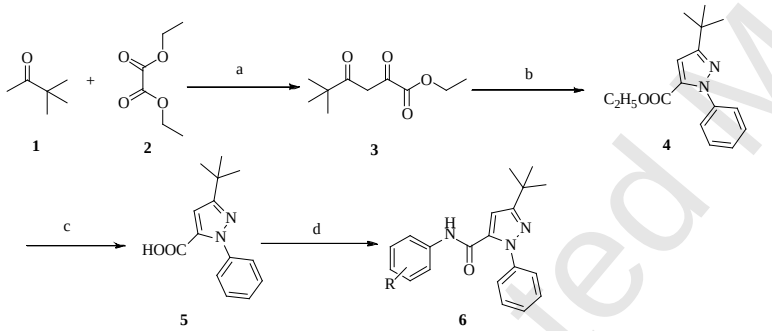
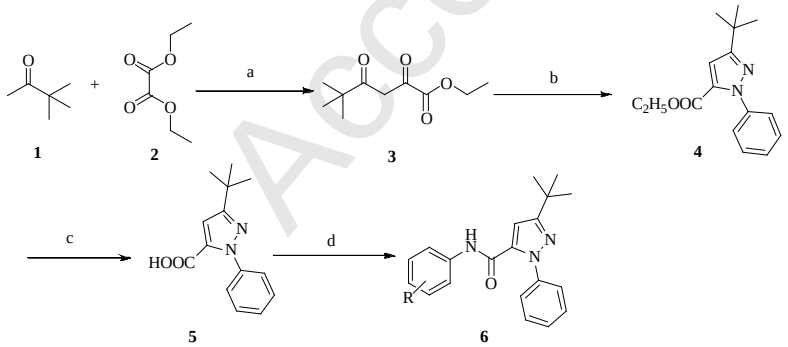
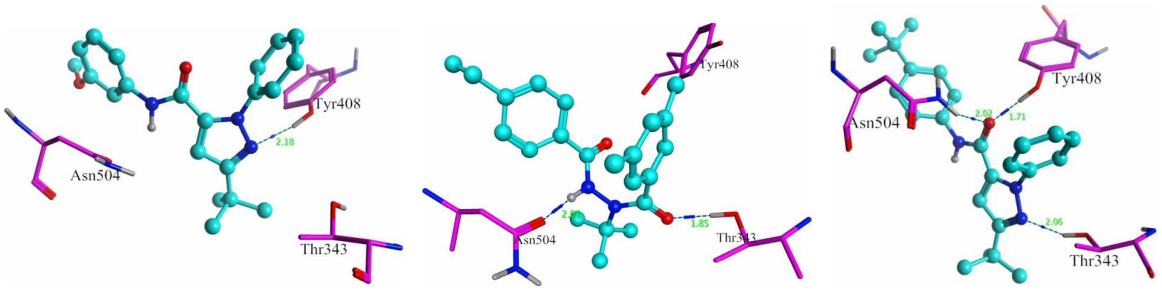


Fig.2 The design strategy of target compound 6 based on the binding conformation of VS008 and BYIO6830 with EcR



Sceme 1. Scheme displaying the synthetic route for obtaining the pyrazole-based analogues **6**. Reagents and conditions: (a) sodium, ethanol, 0 °C, r.t. (overnight); (b) phenylhydrazine (1 equiv.), methanol, 0 °C (30 min), r.t. (1 h), reflux (2 h); (c) (i) NaOH, 80 °C (2 h), (ii) HCl; (d) (i) $SOCl_2$, (ii) substitutedaniline (1.0 equiv.),TEA (2.0 equiv.), DCM, 0 °C (10 min), r.t. (overnight).



（a） (b) (c)

Fig. 3 Hydrogen bonding interactions between the binding pocket in the target EcR subunit and the compounds (a) tebufenozide, (b) **6e**, and (c) **6i.** Carbons in **6e, 6i,** and tebufenozide are represented by light blue spheres, oxygens as red spheres and nitrogens as dark blue spheres. H-bonds are indicated by blue dotted lines.