Final report

Project title:

Protecting pollinators from pesticides: Developing safer, selective pesticides targeting *Varroa* mite and small hive beetle hormone receptors

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Public summary

Australia's honeybee industries face growing threats from pests such as Varroa mite and small hive beetle (SHB). These pests can damage bee health, reduce pollination capacity and threaten the productivity of horticultural industries that rely on managed pollination. Existing pesticides used to control bee pests can also harm honeybees themselves, creating a need for safer and more selective control methods.

This project investigated a new approach to pesticide discovery based on differences in a critical hormone receptor protein known as the ecdysone receptor (ECR). The aim was to identify compounds that interact selectively with the ECR proteins of Varroa mite or SHB while having minimal activity against the equivalent receptor in honeybees. The project brought together expertise in protein structure, honeybee biology, chemistry and high-throughput chemical screening to establish a platform for selective insecticide discovery.

During the project, ECR proteins from Varroa mite, SHB and honeybee were successfully produced and characterised, and their ability to interact with ecdysone and ecdysone analogues validated in the laboratory. Screening assays were developed to test thousands of commercially available compounds for selective receptor activity. The project screened more than 4,000 compounds and identified multiple hit compounds showing selective activity toward pest receptors relative to the honeybee receptor. Follow-up chemistry studies then began modifying these hit compounds to explore whether small chemical changes could further improve pest selectivity and honeybee safety.

The project additionally developed preliminary live-organism assays for both Varroa mites and honeybees. Selected hit compounds identified through receptor screening were tested in live mites and bees, providing an important early step toward assessing whether receptor-level selectivity can translate into whole-organism effects and improved honeybee safety. While further optimisation is still required, the project successfully demonstrated proof-of-principle that selective targeting of pest hormone receptors can provide a viable pathway toward development of safer insecticides for pollinator protection.

Key outputs from the project included validated receptor production and screening methods, large-scale chemical screening datasets, preliminary live-organism testing systems, early structure-activity relationship information, and industry publications. The project established an integrated discovery workflow that can support future optimisation and development of selective pest-control compounds.

The project demonstrated that selective targeting of bee pests is achievable, and established a strong foundation for future development of safer pest-control tools that better protect honeybee health. Future work should include additional lead compound optimisation, hive-based testing and continued engagement with regulatory and commercial partners to support translation of promising lead compounds into practical pest management tools for industry.

Keywords

Varroa mite; small hive beetle; honeybee health; selective insecticides; pollination; ecdysone receptor; pesticide discovery; pollinator protection; chemical screening; safer pest control

Introduction

Pollination by honeybees underpins a substantial proportion of horticultural production, yet this contribution is increasingly threatened by pests such as the Varroa mite (*Varroa destructor*) and small hive beetle (*Aethina tumida*). These pests compromise honeybee colony health, threaten crop productivity, and create significant risks for growers and the broader agricultural economy. Existing chemical controls can be limited by resistance and lack of selectivity, highlighting the need for safer alternatives that effectively control pests while minimising impacts on beneficial insects.

This project was established to address this challenge through a novel target-based approach to insecticide discovery. Rather than relying on conventional broad-spectrum chemistries, the project focused on the insect hormone ecdysone receptor (ECR) as a selective target for next-generation pest control. Because ECRs differ subtly across arthropod species, they provide an opportunity to develop compounds that disrupt Varroa mite and small hive beetle biology while avoiding harm to honeybees and other non-target organisms. As vertebrates lack these receptors, this strategy also offers a potentially safer route for growers, consumers and the environment.

The project brought together expertise in protein structure, honeybee biology and chemical screening to develop innovative tools for discovering safer, more selective pest control products. In doing so, it supported broader Pollination Fund objectives around protecting honeybee health and improving resilience to pest threats through innovative research.

Methodology

Target protein production and validation

A key component of the methodology was the identification, cloning, expression and purification of ecdysone receptor proteins from Varroa mite, small hive beetle and honeybee for use as molecular targets in compound discovery. Protein production methods were refined during the project to incorporate both insect cell and bacterial cell (*E. coli*) expression systems, improving flexibility and efficiency. Purified receptor proteins were then characterised to confirm they formed functional receptor complexes capable of binding hormone ligands, establishing the foundation for downstream screening and structural studies.

Chemical screening and lead compound discovery

Two complementary discovery strategies were pursued in parallel: screening of chemical libraries of approximately 4000 compounds to identify promising hit compounds, and fragment-based discovery, which uses very small molecular fragments to explore broader chemical space and generate novel hit compounds through iterative optimisation. Fluorescence polarisation assays, incorporating a custom ecdysteroid-fluorescein probe and automated liquid handling, were developed as the core screening platform for identifying compounds that bind selectively to pest receptors over the honeybee receptor. Computational analysis and structure-activity relationships were used to interpret screening results and prioritise compounds for follow-up testing and optimisation.

Structural biology for structure-guided design

X-ray crystallography and, later, cryo-electron microscopy were used in efforts to determine three-dimensional structural information about the receptor proteins and how candidate compounds interact with their binding pockets. These approaches were intended to support understanding of selectivity between pest and bee receptors and to inform future optimisation of lead compounds through structure-guided design.

Biological testing and validation

Laboratory bioassays were developed to begin evaluating the activity of candidate compounds in live organisms, namely Varroa mites and honeybees. The unfortunate spread of Varroa mite in Australia also provided access to local live-mite testing, complementing assays being developed for the honeybee. These studies provided an early pathway for assessing biological activity and selectivity beyond the *in vitro* approach.

Together, these complementary approaches established an integrated discovery approach spanning target validation, lead discovery, structure-guided optimisation and early biological testing.

Results and discussion

Production and validation of recombinant ecdysone receptor proteins

A major early objective of the project was the production of recombinant ecdysone receptor (ECR) proteins from Varroa mite, small hive beetle (SHB) and honeybee for use in downstream screening and structural studies. Recombinant receptor proteins from all three species were successfully cloned, expressed and purified, establishing the core molecular platform required for selective ligand discovery.

Protein production strategies evolved over the course of the project in response to differences in protein behaviour between species. Initial expression work focused on insect cell systems; however, *E. coli* expression systems were later incorporated to improve speed, scalability and experimental flexibility. Expression of Varroa and SHB receptor proteins in *E. coli* proved successful and enabled efficient production of material for screening assays. In contrast, the honeybee receptor produced substantially improved yields of correctly folded protein when expressed in insect cells.

Purified receptor proteins were characterised using several complementary approaches to confirm their identity, assembly and biological activity. SDS-PAGE analysis was used to visualise the individual receptor subunits and assess protein purity and expected molecular weight (Figure 1 A), while Western blotting was used to confirm the identity of the expressed proteins (Figure 1 B). Analysis by Multi-Angle Light Scattering (MALS) demonstrated that the purified receptor complexes had molecular weights consistent with formation of the expected heterodimeric assemblies (Figure 1 C). Finally, fluorescence polarisation (FP) assays confirmed that the purified receptor complexes were biologically active and capable of binding ligand molecules (Figure 1 D).

Development and optimisation of fluorescence polarisation (FP) assays

Development of FP screening assays

Fluorescence polarisation (FP) assays were developed as the primary platform for screening large libraries of chemicals to identify compounds capable of binding selectively to pest ECR proteins over the honeybee receptor. The assays were based on competition between candidate compounds and a fluorescently labelled ecdysteroid tracer for binding to the receptor ligand-binding pocket.

Development of the FP platform required optimisation of assay conditions, protein concentrations and tracer performance to ensure reliable detection of receptor-ligand interactions across all three species. Once established, the assay provided a robust and reproducible platform suitable for comparative screening of large numbers of compounds against pest and honeybee receptors.

Throughput optimisation

As the project progressed, the FP assays were progressively optimised to improve throughput and reduce reagent consumption. Assays were successfully miniaturised from 96-well to 384-well plate formats, significantly increasing screening capacity while reducing the amount of recombinant protein and fluorescent tracer required per assay.

Robot-assisted liquid handling workflows were also incorporated to improve reproducibility and facilitate larger-scale screening campaigns. Together, these developments substantially increased the efficiency and scalability of the screening platform and supported the transition from proof-of-concept assays to higher-throughput compound screening.

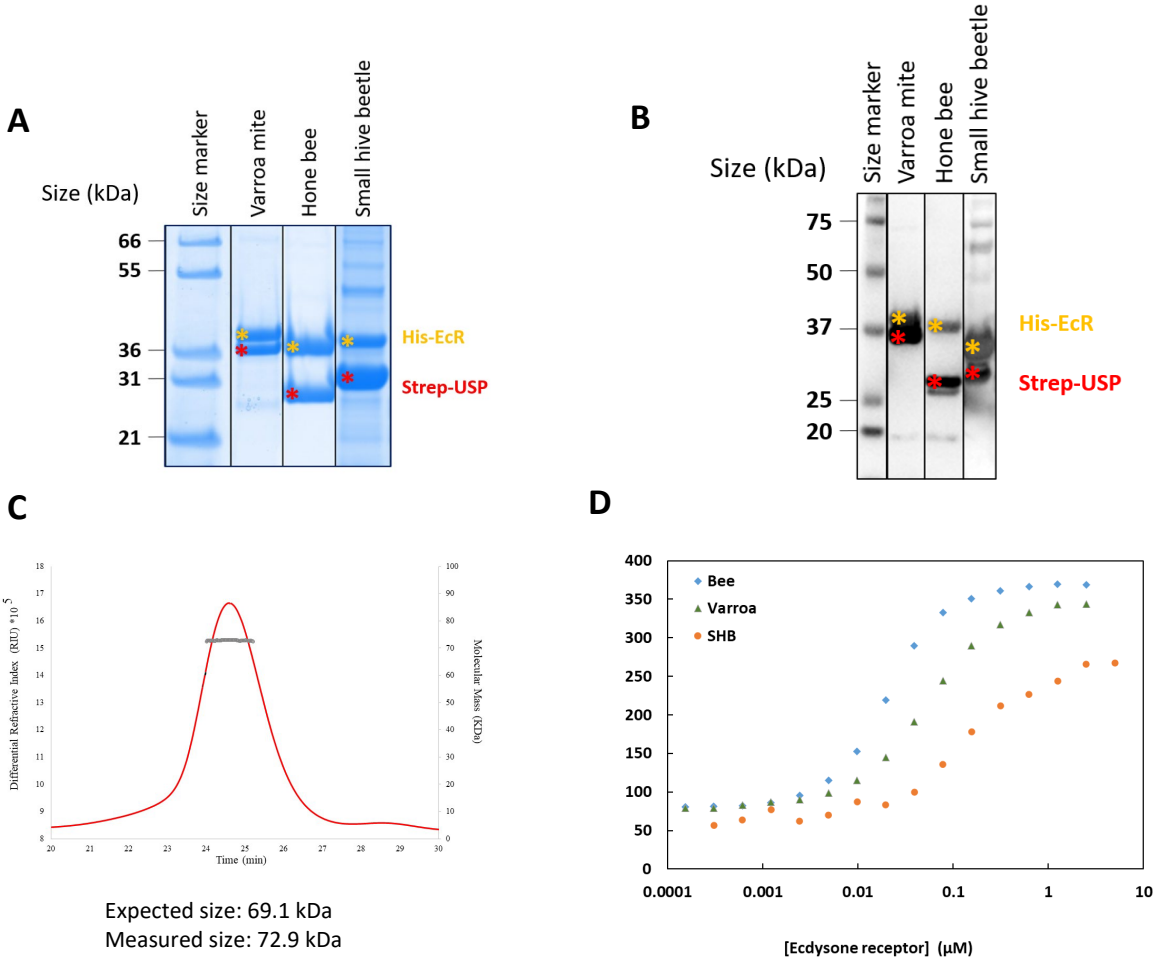

Figure 1. Validation of recombinant ecdysone receptor complexes used for downstream studies. (A) SDS-PAGE analysis of purified Varroa mite, small hive beetle, and honeybee ecdysone receptor (ECR) complexes showing separation of the EcR and USP subunits at the expected molecular weights. (B) Western blot analysis confirming the identity of the purified receptor subunits. (C) Multi-Angle Light Scattering (MALS) analysis of the honeybee ECR complex demonstrating a molecular weight consistent with formation of the expected heterodimer. (D) Fluorescence polarisation (FP) assays showing binding of a fluorescent ecdysteroid tracer to purified Varroa mite, SHB and honeybee ECR complexes, confirming that the receptors are biologically active.

Improved synthesis of fluorescent tracer

The fluorescently labelled ecdysteroid tracer used in the FP assays became an important focus of optimisation during the project. Initial synthesis methods based on published protocols produced complex mixtures with relatively low yields of the desired product, creating a bottleneck for large-scale screening activities.

To address this limitation, the project developed an improved synthesis strategy (Appendix A). This approach substantially improved reaction efficiency and generated cleaner product mixtures, simplifying purification and increasing overall yield of the fluorescent tracer.

The improved synthesis method enabled the establishment of a substantial stockpile of purified fluorescent tracer for ongoing and future screening activities. In addition to improving assay scalability, the higher purity tracer is expected to improve assessment of the quality and biological activity of purified receptor proteins used in screening experiments.

Compound screening and selective lead discovery

Screening strategy and library selection

This project screened molecules from the Compounds Australia Open Access Scaffolds Library containing 34,000 commercially available compounds and 1,200 unique chemical scaffolds, with an average of 28 compounds per scaffold. A rational screening approach focusing on chemotype breadth and novelty was used to identify compounds showing Varroa mite or small hive beetle (SHB) ecdysone receptor activity.

Compound selection aimed to balance broad chemical diversity with inclusion of compounds showing similarities to known ecdysone-active molecules and early fragment-screening hits. Computational analysis and manual chemical curation were used to prioritise compounds and scaffold families for screening and follow-up studies.

Importantly, a counterscreen against the honeybee ecdysone receptor was incorporated throughout the screening workflow to identify compounds with activity against pest receptors while minimising activity against the honeybee receptor.

FP screening and hit identification

Large-scale FP screening campaigns identified a relatively large number of compounds that were active against the pest species receptors, including a smaller number showing activity against each pest receptor but not the honeybee receptor, and two compounds active against both pest receptors while showing little or no activity against the honeybee receptor.

A summary of screening outcomes includes:

- 93 hits active against the Varroa receptor, of which 11 exhibited little or no binding to the honeybee receptor
- 115 hits active against the SHB receptor, of which 6 exhibited little or no binding to the honeybee receptor
- Two hit compounds that bind to both Varroa and SHB receptors while showing little or no binding to the honeybee receptor

These findings provide strong proof-of-principle that selective targeting of pest ecdysone receptors over the honeybee receptor is feasible using a rational receptor-based discovery strategy.

Fragment-based discovery as a complementary screening strategy

Fragment-based discovery was pursued in parallel with larger-scale chemical library screening as a complementary approach for identifying novel lead chemotypes. Unlike focused library screening, which explores a more constrained region of chemical space, fragment-based approaches utilise much smaller molecular fragments that can subsequently be elaborated through iterative optimisation.

Fragment screening identified several low-affinity fragment hits and informed subsequent analogue exploration. However, progression of fragment-derived compounds required substantially more iterative synthesis and testing than the focused library screening approach. As a result, chemical library screening generated biologically testable lead compounds more rapidly during the timeframe of the project.

Despite the slower progression of fragment-derived compounds, fragment-based discovery remains an important long-term strategy for exploration of novel chemical space and development of future lead compounds unconstrained by existing screening libraries.

Selectivity against the honeybee receptor

A major objective of the project was the identification of compounds capable of selectively targeting pest receptors while minimising activity against the honeybee receptor. Counterscreening against the honeybee ECR was therefore incorporated throughout the screening workflow and served as a key criterion for prioritisation of lead compounds.

The discovery of multiple compounds showing differential activity between pest and honeybee receptors demonstrated that selective receptor targeting is feasible despite the structural similarities between these proteins. This represents an important proof-of-principle outcome for the broader strategy of developing safer, more selective insecticides based on receptor-level differences between species.

Emerging structure-activity relationships

Analysis of screening results identified several recurring chemotypes associated with activity against Varroa mite and SHB receptors. Early structure–activity relationships (SAR) also began to emerge within selected scaffold families, providing preliminary insight into the types of chemical modifications that may influence receptor activity and selectivity.

To support early SAR studies, synthetic chemistry efforts were initiated to independently reproduce selected screening hits and generate related analogue compounds for follow-up testing. An initial targeted library of analogue compounds based on one of the lead chemotypes was successfully synthesised using a streamlined multi-component synthetic approach that enabled rapid generation of structurally related molecules. These compounds are currently undergoing validation and screening assays.

This work represented an important transition from initial hit discovery toward iterative lead optimisation and established a foundation for future medicinal chemistry development and SAR exploration. The information generated during the project has defined a clear path for further screening of selected chemotypes against much larger chemical libraries to expand understanding of structure-activity relationships and support future optimisation of hit compounds. In particular, future work will focus on chemical modification of lead compounds to improve pest receptor activity while retaining honeybee safety.

Further details of chemotype analysis and structure-activity relationships are provided in Appendix B. Additional information relating to analogue synthesis and synthetic chemistry methods is provided in Appendix C.

Structural biology and efforts toward receptor structure determination

Crystallisation and X-ray diffraction studies

Structural biology formed an important component of the project, with the long-term aim of obtaining three-dimensional structural information to guide optimisation of selective lead compounds. Extensive crystallisation screening campaigns were undertaken using purified receptor proteins from Varroa mite, SHB and honeybee.

Crystallisation trials produced several receptor crystal hits, including crystals that yielded preliminary X-ray diffraction data suitable for ongoing optimisation efforts (Figure 2). Ongoing optimisation of protein production and crystallisation conditions continues to focus on improving crystal size and quality to support future structural determination efforts.

Expansion into cryo-electron microscopy

To complement crystallography efforts, the project also explored cryo-electron microscopy (cryoEM) as an alternative structural biology approach for studying the receptor complexes. Early cryoEM experiments indicated that the receptor complexes adopt a preferred orientation on the cryoEM grids, preventing collection of the range of views needed to build a 3D model of the receptor complex. Ongoing optimisation is therefore focused on modifying protein-grid interactions, including the use of detergents to promote a broader range of particle orientations and improve the potential for future structural determination.

Implications for future structure-guided optimisation

Although complete structures of the target receptor complexes have not yet been obtained, the structural biology work undertaken during the project established important foundations for future structure-guided optimisation efforts. High-resolution structural information remains a valuable long-term objective because it has the potential to provide detailed insight into ligand binding modes, receptor selectivity and opportunities for rational optimisation of lead compounds.

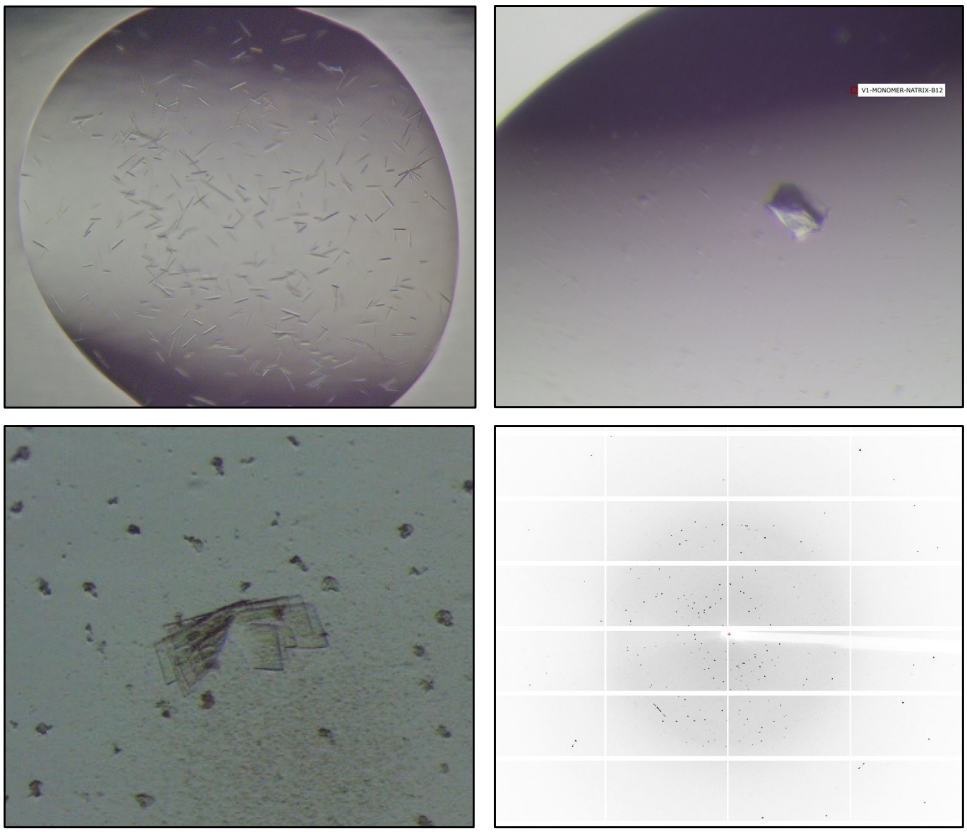

Figure 2. Crystallisation and X-ray diffraction studies of ecdysone receptor proteins. (A) Representative microcrystals obtained for the Varroa mite EcR monomer during crystallisation screening. (B) Magnified image of a Varroa EcR monomer crystal from a condition selected for further optimisation. (C) Crystal obtained for the honeybee ecdysone receptor heterodimer. (D) Representative X-ray diffraction dataset collected from the honeybee heterodimer crystal shown in (C). Although diffraction was observed, the data quality was insufficient for structure determination, highlighting the ongoing optimisation required for structural studies of these receptor complexes.

Development of whole-organism bioassays and early biological validation

Development of Varroa mite bioassays

Initial bioassays were developed to begin evaluating the effects of candidate compounds on live Varroa mites. Compounds were dissolved in acetone and applied to glass vials, which were rotated during solvent evaporation to produce an even coating of compound on the vial walls. Mites were then placed into the coated vials for four hours prior to transfer into feeding capsules containing bee pupae, and survival was monitored over time. Development of these preliminary assays represents an important first step in extending the project from receptor-level screening into live-organism testing, and the resulting data provided an initial framework for future biological evaluation of hit compounds.

Testing of lead compounds against Varroa mites

Eight hit compounds identified through chemical library and analogue screening were selected for initial testing on live Varroa mites. These compounds had previously demonstrated selective binding to the Varroa receptor relative to the honeybee receptor in our biochemical assay.

As expected, the Amitraz positive control caused rapid mite mortality, with complete mortality observed within 24 hours. Diflubenzuron produced comparatively limited mortality over the assay timeframe, consistent with its slower mode of action. Among the screened hit compounds, two candidates produced measurable mite mortality over the seven-day assay period and were prioritised for subsequent honeybee safety testing (Appendix D).

Although the biological activity observed at this stage was modest, these findings provide encouraging evidence that even initial receptor-screening hits can translate into measurable whole-organism effects, which supports continued optimisation of these hit compounds.

Development of honeybee safety assays

Initial honeybee safety assays were established to enable preliminary evaluation of lead compounds identified through receptor screening and Varroa mite testing. The assay system supported monitoring of bee survival over extended time periods under controlled laboratory conditions and provided a framework for comparing candidate compounds against untreated controls as part of an iterative hit optimisation process.

Development of these assays represented an important step toward assessing whether improvements in receptor-level selectivity could translate into reduced toxicity at the whole-organism level.

Preliminary assessment of honeybee safety

The developed bee assay successfully distinguished between untreated controls, highly toxic positive controls and candidate lead compounds. No mortality was observed in untreated (vehicle-only) control bees over the first two weeks of the assay, while treatment with the positive control, a broad spectrum neurotoxin fipronil, resulted in rapid mortality, confirming the sensitivity of the assay system to acute non-specific toxic effects.

The two hit compounds selected from Varroa testing did not produce detectable bee mortality during the early stages of the assay period, but did show some mortality relative to the untreated controls at later time points, particularly at higher dose (Appendix E). These findings indicate that although the compounds showed promising receptor-level selectivity in molecular assays, additional optimisation will be required to further reduce toxicity toward honeybees.

Importantly, the assays established during the project now provide a framework for iterative testing of future hit compounds, enabling direct assessment of whether improvements in biochemical receptor selectivity translate into improved whole-organism safety profiles.

Outputs

Table 1. Output summary

Output	Description	Detail
Fluorescence polarisation screening platform	Development of receptor-based fluorescence assays for identifying selective lead compounds	Screening assays were established for Varroa mite, SHB and honeybee ecdysone receptors and used for chemical library and fragment screening activities. The project also developed an improved synthesis method for the fluorescent tracer compound used in the assays.
Selective lead compounds	Identification of compounds showing selective activity toward pest receptors relative to the honeybee receptor	Multiple hit compounds with selective receptor binding profiles were identified, including compounds active against both Varroa and SHB receptors with limited honeybee receptor binding (Appendix B).
Live-organism bioassays	Development of initial Varroa mite efficacy and honeybee safety assays	Preliminary assays enabled early whole-organism testing of selected hit compounds and established a framework for future optimisation studies.
Analogue synthesis workflows	Development of synthetic chemistry methods for lead optimisation	Initial analogue libraries were synthesised to support early structure-activity relationship studies and future medicinal chemistry optimisation.
Global scan of Varroa control strategies	Review of current and emerging approaches for Varroa management intended for industry and stakeholder audiences	A detailed global scan reviewing chemical and non-chemical Varroa control approaches, resistance issues and future opportunities for selective pesticides was prepared and shared with project stakeholders. Included as Appendix G.
Industry communications	Industry-facing communication materials describing project progress and significance	Project outcomes and progress were communicated through industry publications describing the selective pesticide discovery strategy and early project achievements. An example article published in Australian Grower magazine is included as Appendix H.

Outcomes

Table 2. Outcome summary

Outcome	Alignment to fund outcome, strategy and KPI	Description	Evidence
Proof-of-principle established for selective targeting of pest ecdysone receptors over the honeybee receptor	Aligned with Hort Frontiers Pollination Fund Outcome 1: Improved management of European honeybees for pollination, particularly future-proofing pollination systems against endemic and exotic pests and diseases.	The project demonstrated that receptor-level selectivity between pest species and honeybees can be identified through biochemical screening approaches. Selective lead compounds active against Varroa mite and SHB receptors, with reduced activity toward the honeybee receptor, were successfully identified. Preliminary whole-organism testing further demonstrated that receptor-binding hits could translate into measurable biological effects in live organisms.	• Fluorescence polarisation screening data • Identification of selective hit compounds • Preliminary Varroa mite and honeybee bioassay data • Chemotype and SAR analysis
Establishment of an integrated discovery platform for selective insecticide development	Aligned with Pollination Fund priorities supporting preparedness and innovation for management of pollinator pests and diseases.	The project established an end-to-end discovery workflow integrating receptor production, biochemical screening, structural biology, synthetic chemistry and live-organism testing. This platform now provides a foundation for continued optimisation of selective pest-control compounds targeting Varroa mite and SHB while minimising impacts on honeybees.	• Functional recombinant receptor proteins • Screening assays established • Structural biology workflows • Analogue synthesis activities • Preliminary bioassay systems
Generation of selective hit compounds supporting future IP and commercialisation opportunities	Aligned with Hort Frontiers objectives to support innovation and translation of research outcomes toward industry impact.	The project identified multiple hit compounds and chemotypes displaying selective receptor activity profiles, including compounds active against both Varroa mite and SHB receptors while showing limited binding to the honeybee receptor. These findings established a foundation for future medicinal chemistry optimisation, intellectual	• Lead compound hit • Chemotype analysis • Updated IP register • Discussions regarding future commercialisation pathways

		property development and engagement with potential commercial partners.	
Increased industry awareness of emerging approaches for selective Varroa management	Aligned with Pollination Fund priorities relating to preparedness and resilience of pollination systems.	The project contributed to industry awareness of challenges associated with existing Varroa control methods, including resistance development and off-target impacts on bees, while communicating the potential of selective target-based pesticide discovery approaches. Industry communications and global review documents supported stakeholder engagement and knowledge dissemination.	• Global scan reviewing current methods for Varroa mite control • Industry publications • Stakeholder steering committee meetings • Project reporting activities

Monitoring and evaluation

Table 3. Key Evaluation Questions

Key Evaluation Question	Project performance	Continuous improvement opportunities
Have molecules been developed that interfere with the development of Varroa or SHB, and are these molecules selective and relatively safe for honeybees?	The project successfully identified multiple compounds that bind selectively to Varroa mite and/or SHB ecdysone receptors relative to the honeybee receptor. Preliminary live-organism testing demonstrated that selected hit compounds could produce measurable biological effects in Varroa mites while also highlighting the importance of continued optimisation to further improve honeybee safety profiles. Together, these findings provide encouraging support for the feasibility of selective receptor targeting as a pest-control strategy.	Additional medicinal chemistry optimisation, larger-scale screening and expanded live-organism testing will be required to further improve compound selectivity, efficacy and safety. Future work should also incorporate assessment of sublethal effects (*e.g.* reproductive or developmental effects) in both pests and honeybees.
Has the project demonstrated proof-of-principle for rational selective insecticide design?	The project established proof-of-principle that biochemical differences between pest and beneficial insect receptors can be exploited to identify selective hit compounds using a target-based discovery approach. The integration of receptor biology, fluorescence screening, structural biology, synthetic chemistry and live-organism testing established an effective workflow for selective insecticide discovery.	Further optimisation of structural biology approaches, including crystallography and cryoEM, will support future structure-guided design of more selective compounds. Expansion into larger chemical libraries and iterative analogue synthesis will further strengthen structure-activity relationship analysis and hit optimisation.
Has the project established pathways toward commercialisation and industry engagement?	The project established a clear pathway toward future commercialisation by generating selective hit compounds, chemotype data and proprietary screening capabilities that together provide a strong foundation for future hit optimisation and industry engagement. The project deliberately focused on de-risking the underlying science and advancing the technology platform prior to formal commercial partnering. Potential future commercialisation pathways identified include partner-led development with agrochemical companies, as well as possible spin-out company formation supported by translational funding schemes. Early consideration was also given to	Further medicinal chemistry optimisation, expanded biological validation and progression toward higher Technology Readiness Levels will be required before formal commercial partnerships can be established. Future work should also continue development of the project's intellectual property strategy, including confidential management of hit chemotypes, evaluation of patent opportunities and targeted engagement with suitable commercial partners and regulatory stakeholders.

	future APVMA regulatory requirements to help guide future hit optimisation and reduce downstream development risk.	
To what extent were target engagement and communication objectives achieved?	Regular project updates were provided through milestone reports, stakeholder steering committee meetings, and industry communication articles.	Future engagement with beekeepers and pollination stakeholders will become increasingly important as the project progresses toward hive-based testing and practical implementation considerations.
What efforts were made to improve project efficiency?	The project adopted a flexible and adaptive research strategy throughout its duration. Protein production workflows were expanded to include both *E. coli* and insect cell expression systems depending on receptor performance. Screening priorities evolved toward chemical library screening approaches that generated hit compounds more rapidly, while fragment-based discovery was retained as a complementary longer-term strategy. The arrival of Varroa mite in Australia also enabled local live-mite testing without the need for overseas access.	Future work could further improve efficiency by introducing medium-throughput cell-based assays to bridge the gap between high-throughput biochemical screening and lower-throughput live-organism testing. Such assays would enable more efficient prioritisation of candidate molecules before progression into complex biological assays. Additional dedicated research capacity for analogue synthesis and live-organism testing would also accelerate iterative hit optimisation and biological validation workflows.

Recommendations

1. Continue *in vitro* development of selective ECR-targeting compounds

The project demonstrated proof-of-principle that selective targeting of pest ecdysone receptors is a feasible strategy for development of safer insecticides. Continued investment in medicinal chemistry optimisation, structure-activity relationship studies and expanded screening is recommended to further improve pest selectivity, efficacy and honeybee safety.

2 Develop intermediate cell-based screening capability

Development of medium-throughput cell-based assays is recommended to bridge the gap between biochemical receptor screening and live-organism testing. Such assays would improve efficiency of candidate prioritisation by assessing receptor activity within living cells before progression into more complex biological studies.

3. Expand biological validation and hive-based testing

Further development of live-organism assays is recommended to better assess efficacy, sublethal effects and long-term impacts in Varroa mite, SHB and honeybees. Future work should include expanded hive-based and reproductive assays to evaluate biological performance under conditions more closely relating to those in the hive.

4. Continue structural biology and analogue synthesis efforts

Continued optimisation of crystallography and cryoEM approaches is recommended to support future structure-guided design of selective compounds. Expanded analogue synthesis capability will also be important for accelerating iterative hit optimisation and improving selectivity profiles.

5. Progress commercialisation and regulatory planning

Future project stages should continue development of intellectual property, commercial engagement and regulatory planning activities.

Refereed scientific publications

Journal article

Winkler D.A. and Hill R.J. 2025. Small organic ligands for the ecdysone receptor - agrochemicals, gene switches and beyond. Pesticide Biochem and Physiol. Volume 214, November 2025, 106585.

Whole book

Chapter in a book or paper in conference proceedings

Intellectual property

Project intellectual property generated during the project includes proprietary know-how relating to fluorescence polarisation screening methodologies and identification of selective hit chemotypes arising from chemical library screening activities. An internal Record of Invention (ROI) has been prepared relating to development of a fluorescence polarisation assay platform suitable for high-throughput screening of compounds targeting ecdysone receptors.

At the completion of the project, no patent applications specific to individual hit compounds have been filed. This reflects the current stage of technology development and a deliberate strategy to avoid premature public disclosure prior to further hit optimisation, biological validation and assessment of commercial potential.

The project team considers the confidential hit compound data, associated chemotype information and screening methodologies to represent potentially valuable background for future commercialisation activities. Intellectual property arising from the project is being managed in consultation with the University of Sydney Commercial Development and Industry Partnerships (CDIP) unit and Hort Innovation in accordance with the Research Agreement.

An updated Intellectual Property Register was prepared and submitted to Hort Innovation as part of the final reporting process (Appendix F).

Acknowledgements

The project team gratefully acknowledges the support of the Drug Discovery node of Sydney Analytical for assistance with fragment-based screening activities and associated technical support.

Appendices

Appendix	Title	Confidentiality status
Appendix A	Improved synthesis of the inokosterone-fluorescein adduct using nucleophilic catalysis and implications for the project	Confidential – contains sensitive methodological and assay development information relevant to future commercialisation activities
Appendix B	Chemotype analysis of candidate molecules	Confidential – contains commercially sensitive hit compound and structure-activity relationship information
Appendix C	Analogue synthesis for Structure-Activity Relationship studies	Confidential – contains commercially sensitive synthetic chemistry methods and analogue development information
Appendix D	Preliminary Varroa mite bioassay results for selected hit compounds	Confidential – contains sensitive hit compound efficacy data
Appendix E	Preliminary honeybee safety assessment of selected hit compounds	Confidential – contains sensitive hit compound safety and toxicity data
Appendix F	Updated Intellectual Property Register	Confidential – contains commercially sensitive information relating to emerging technologies, future intellectual property opportunities and commercialisation strategy
Appendix G	Global scan of Varroa management strategies	Non-confidential
Appendix H	Industry communication article – Australian Grower	Non-confidential
Appendix I	Refereed scientific publication entitled ‘Small organic ligands for the ecdysone receptor – agrochemicals, gene switches and beyond’.	Non-confidential
Appendix J	Technology Exploitation Plan	Confidential – commercially sensitive commercialisation and intellectual property planning document to be provided separately to Hort Innovation

Confidential appendices have been provided separately to Hort Innovation due to the inclusion of commercially sensitive material.

Current and future methods for *Varroa* mite control

KEY MESSAGES

In this global scan we review the current methods for Varroa mite control used throughout the world, covering the different types of control methods available, with a focus on chemical controls. We outline the pros and cons of different treatment methods, examine why different methods have varying levels of success, and provide recommendations for a best practice for Varroa control based on global experiences with Varroa. We also address the development of acaracide resistance that has occurred to currently used chemicals in global mite populations, and examine opportunities for discovery of new safe, selective methods to control the Varroa mite with a focus on maintaining pollinator and environmental health.

AIM

Review current Varroa control methods used around the world with a focus on chemical control, the development of resistance to current methods, and opportunities for discovery of novel methods of control.

1. INTRODUCTION

Varroa destructor (hereafter Varroa) is the most significant pest of the commercial honey bee industry worldwide (1). During the last century, Varroa has spread rapidly across the globe, severely impacting commercial honey bee colonies if left untreated. In June 2022, Varroa mites were first detected in Newcastle. It is important to consider appropriate methods for Varroa control in an Australian context if the unfortunate scenario of a failed eradication eventuates, and in preparation for potential future incursions.

2. VARROA CONTROL METHODS

Throughout the world, a variety of treatment and management strategies have been implemented to aid in controlling Varroa mites. These strategies can each be considered under an **Integrated Pest Management** framework.

Integrated pest management (IPM) is a multifactorial approach to pest management that implements sustainable and ecologically-friendly methods to reduce the impact of a pest. IPM strategies operate on the premise below a certain threshold, pests are tolerable and do not require eradication. Thus, IPM aims to establish the threshold at which a particular pest will cause economic damage to an industry, and to identify the various thresholds at which increasingly drastic control strategies are justified. As IPM aims at minimising ecological damage, most IPM strategies advocate for synthetic chemicals to be used only as a last resort.

IPM strategies follow a tiered approach to pest control which aims to reduce environmental risk, and includes **prevention, cultural, mechanical, biological** and **chemical** approaches (Figure 1). Varroa control methods can be viewed in the IPM framework, however the majority of Varroa management programs around the world have previously failed to implement IPM strategies in Varroa control (1). Devising standardised IPM strategies for varroa control is difficult, as domestic honey bee colonies are kept in a broad variety of habitats and climates, which can have significant effects on both bee and mite life histories. Additionally, the variation in accuracy of infestation estimates and in efficacy of treatments means that accurately establishing the threshold for the use of a particular treatment is not straightforward. Australia provides a ‘blank canvas’ to put into practice an effective IPM strategy for Varroa control.

Prevention is a key part of an integrated pest management strategy, and involves removing conditions that allow pests to establish or build populations. In Australia, this might mean maintaining active biosecurity measures to prevent additional Varroa incursions, ongoing surveillance to prevent spread from the existing incursion zone, or actively eradicating the current Varroa incursion to prevent widespread establishment. Throughout the rest of the world where Varroa is endemic, it might mean regulating the movement of bees, minimising swarming and hive placement to reduce drifting of bees within apiaries (1).

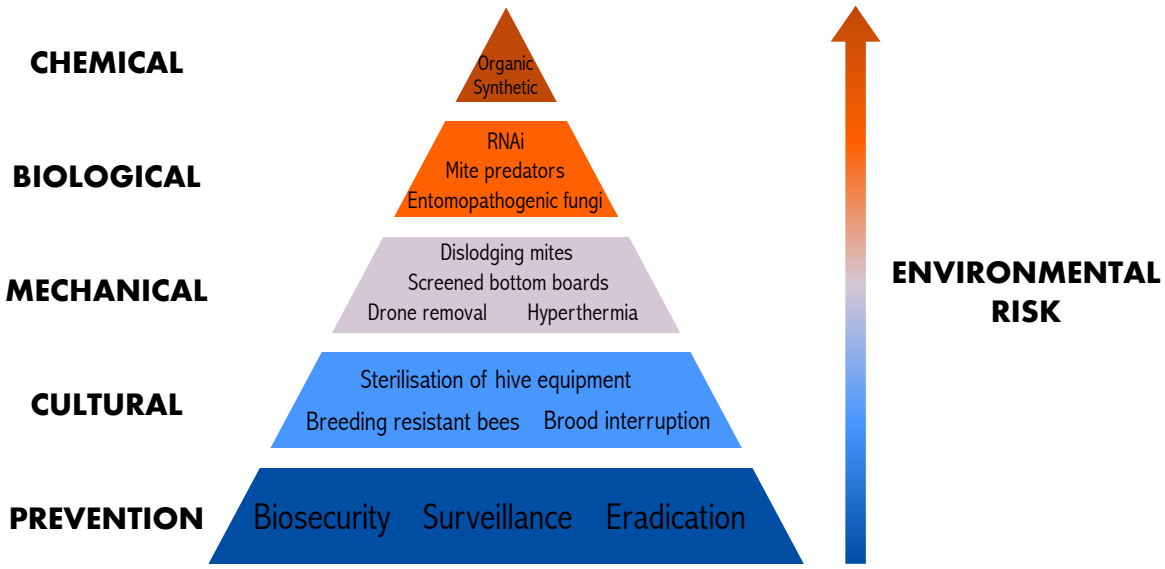

Figure 1: An integrated pest management framework for the control of Varroa destructor (adapted from 1).

Cultural control involves changing conditions to be less favourable for pests. In the case of Varroa, beekeepers can create a hive environment that is less suitable for mites, while limiting the impact on bees. Some examples include breeding Varroa-resistant or hygienic honey bee stocks which remove mite-infested brood from hives, or placing queens in cages to generate a 'brood break'. Inhibiting brood production for short periods disrupts the Varroa reproductive cycle (1). Cultural methods are often used together with chemical methods to improve treatment efficacy.

Mechanical control involves physical methods or mechanical devices, such as barriers and traps, to physically aid in the removal of pests, or prevent pests from entering a location. In Varroa management, mechanical controls include placing sticky or screened bottom boards in hives, which either trap fallen mites or allow fallen mites to fall out of the hive rather than return to bees. Mites preferentially invade drone brood, so during periods of high male production, beekeepers can remove drone brood to reduce mite loads. In addition hyperthermia, involving heat-treating hives at ≥40ºC, is lethal to Varroa while only mildly agitating to bees if short in duration. The efficacies of such methods are yet to be widely determined (1).

Biological control relies on manipulating a living agent to reduce pest success. There are a number of natural enemies of Varroa that may be useful as biological controls, however distinguishing the risk to honey bees is important. Some biological methods under investigation include the use of entomopathogenic fungi which infect Varroa and cause lethality, however efficacies in field tests have been low, and there is evidence of bee brood infection (1). Natural mite predators, such as pseudoscorpians have also been investigated along with some bacteria, however these currently lack compelling evidence of effectiveness in field hives (1).

Chemical control, as the name suggests, relies on the use of chemicals applied to a pest to kill, disable or prevent its reproduction. In the case of Varroa, it involves applying a chemical to the hive environment to reduce the incidence of mites. Chemical treatments for Varroa fall into two broad categories - synthetic chemicals and organic chemicals. There are advantages and disadvantages to both categories.

Synthetic chemicals are named as such because they are created via chemical reactions in laboratories and factories, rather than extracted from a natural source. Several synthetic chemicals are used throughout the world to treat *Varroa* (Table 1). Each of these chemicals works by interfering with some aspect of the mite's nervous system and cellular functioning.

Organic chemicals are derived from natural sources and include organic acids and plant essential oils. The most commonly used are oxalic acid, formic acid and the plant essential oil thymol (extracted from thyme) (Table 2). As with synthetic acaricides, they are commercially available as the active ingredients in several different products. Organic chemicals work in a non-specific manner by impacting multiple biological systems in mites.

3. CHEMICAL CONTROL

Chemical treatments for Varroa should be viewed as one component of an effective pest management strategy, reserved for situations where other methods are impractical or ineffective. Rather than used in isolation, the effectiveness of most chemical control methods are linked to other factors in a honey bee hive, such as total mite load, specific stage of the brood cycle, or seasonal and environmental conditions.

Synthetic chemicals

Current synthetic chemical treatments for Varroa typically have one of four common active ingredients: **amitraz**, **coumaphos**, **flumethrin** and ***tau*-fluvalinate** (2; Table 1). They are usually administered by placing plastic strips containing the chemicals into the brood nest. Bees contact the strips as they move around the hive, exposing the mites to the pesticides (1).

Amitraz is the active ingredient in the commercially available products Apivar and Apitraz. It is a formamidine that works by blocking octopamine receptors in the mite nervous system (3). Amitraz is an effective acaracide that can cause high mite mortality, is easy to administer and it does not persist in honey or wax (1, 2). Prolonged use of amitraz leads to lower efficacy and resistant mite populations, although mites have remained susceptible to it for longer than other synthetic acaracides (1). Additionally, high doses of amitraz can negatively impact brood survival and bee health (4, 5), and amitraz products are not considered affordable by many beekeepers (1, 2).

Coumaphos is an organophosphate and neurotoxin that interferes with neuromuscular transmission by inhibiting the enzyme acetylcholinesterate, which acts upon the neurotransmitter acetylcholine (3). Coumaphos is commercially available in the product CheckMite (2). These products are highly effective in unexposed Varroa populations, however have been largely abandoned by beekeepers worldwide due to the prevalence of increasingly resistant populations (6). Additionally, coumaphos can have many negative effects on bee health, including reduced brood and queen survival, negative impacts on learning and memory (1), and is associated with high winter colony losses in the United States (6). Coumaphos was once registered in Australia for veterinary use, however has been cancelled due to lack of safety verification (AVPMA, http://www.apvma.gov.au/chemrev/coumaphos.shtml), thus it is unlikely to be registered in Australia for Varroa use.

Flumethrin and ***tau*-fluvalinate** are pyrethroids that disrupt neurotransmission via interaction with sodium receptors (3). Flumethrin is sold as the products Bayvarol and PolyVar Yellow, and *tau*-fluvalinate is marketed as Apistan (2). Pyrethroids are favourable as they are toxic to mites in low doses, and are administered to hives in doses low enough to have low toxicity for honey bees. Rather than kill mites outright, they lead to paralysis and convulsions that cause mites to fall of their host, unable to re-attach. Flumethrin has remained relatively effective, with mite mortality rates >90% reported, however, resistance to flumethrin is becoming more common (1). Resistance develops quickly to *tau*-fluvalinate, killing 96% of mites in the 1st year of use but dropping to 76% by year 3.

Table 1: Summary of synthetic chemicals used in Varroa control

Active ingredient ***(Chemical class)***	**Commercial products**	**Mode of Action**	**Pros**	**Cons**
Amitraz *(Formamine)*	Apivar Apitraz	Octopamine receptor	Highly effective Easy to administer Mites remain susceptible for longer than other compounds Does not persist in honey or wax	Expensive High doses can negatively impact bee brood survival Mite resistance is growing
Coumaphos *(Organophos-phorus)*	CheckMite	Acetylcholin-esterase	Highly effective in unexposed populations Easy to administer	May be harmful to larvae or adult bees Mite populations are largely resistant
Flumethrin *(Pyrethroid)*	Bayvarol PolyVar Yellow	Sodium receptor	Highly effective in unexposed populations Easy to administer Low impact on larvae and adult bees	Effectiveness varies due to increasing mite resistance
tau-Fluvalinate *(Pyrethroid)*	Apistan	Sodium receptor	Highly effective in unexposed populations Easy to administer	Resistance develops quickly: cannot be used for longer than 3 yrs Must be used in rotation with other chemicals Cannot be administered until after honey is removed Can negatively affect adult bee learning and memory

Organic chemicals

The most widely used organic treatments for *Varroa* are organic acids such as **formic acid**, **oxalic acid** and **hop beta acids**, and the essential oil **thymol** (derived from thyme). Organic Varroa treatments are an increasingly popular alternative due to their perception as being less harmful to bees and the environment (1). It is recommended that beekeepers use organic treatments where possible so that *Varroa* populations do not become resistant to more reliable synthetic compounds (2).

The major advantage of organic acaricides is that they have multiple cellular targets, so mite populations are extremely unlikely to become resistant to them (2). Additionally, most organic chemicals do not leave residues in honey or wax and so can be used more freely (ref). However, due to the large range of formulations available and variety of application methods, the efficacy of organic chemicals is highly variable in comparison to synthetic chemicals (1). Some also pose risks to beekeepers and require personal protective equipment prior to application. Organic chemicals are usually used in conjunction with brood interruptions to keep mite populations in a colony low.

Formic acid has been used to treat Varroa since the 1980s. The mode of action is not well characterised, however it is thought to inhibit electron transport in the mitochondria by interacting with the mitochondrial protein cytochrome C oxidase (7). Formic acid formulations are commercially available in various forms, including gels (MAQS), tablets (Varterminator) or liquids (Nassenheider Professional), and show activity on phoretic mites attached to adult bees for transport, and reproductive mites sealed in brood cells (1). However, formic acid can cause lethality in to brood and queen bees if ambient temperatures are too warm. It is also an irritant to humans and requires appropriate personal protective equipment (2). Despite this, it is a commonly used Varroa treatment throughout Europe and North America (1)

Oxalic acid is an organic acid found in many plant species. It has been in use for Varroa control for decades with no reports of mite resistance. The mode of action of oxalic acid is unknown, though it kills mites on contact, and encourages increased grooming by bees which additionally works to dislodge mites. Oxalic acid is administered in liquid form via sugar syrup or sublimated via heating solid oxalic acid to 160°C, where it vapourises and spreads throughout the hive. It is often used in conjunction with broodless periods achieving up to 100% efficacy, as it is unable to kill mites enclosed in brood cells (1). Some negative effects have been observed on honey bee brood development (1) and it can be lethal to open brood (2). Oxalic acid is a corrosive substance that can be dangerous to humans, with potential to cause chemical burns and permanent eye damage if exposed, and thus requires correct personal protective equipment (ref).

Hop beta acids are comprised of compounds called lupulones, derived from hop plants. They have an unknown mode of action but are used as a repellent for other mite pests. Hop beta acids as a Varroa treatment are commercially sold as cardboard strips (HopGuard), and have the advantages of being active at high temperature and are safe for humans and honey bees, though some studies have shown limited mite efficacies (1).

Thymol is an essential oil and may act via binding to octopamine or GABA receptors in the mite nervous system (1), or by disrupting proteins and cellular membranes (2). Thymol is a volatile that can be applied as slow release gels, tablets or wafers which release volatiles throughout the hive. Efficacy is optimal at temperatures between 20-30°C, killing 50-80% of mites, with limited activity below 15°C. Thymol is potentially harmful to honey bee brood and queens during high ambient temperatures.

Table 2: Summary of organic chemicals used in Varroa control

Active ingredient	Commercial products	Administration	Pros	Cons
Formic acid	MAQS FormicPro Nassenheider Professional Varterminator	Gel strips added to the brood nest (MAQS, FormicPro)	Can be applied to hives before honey is extracted* Can kill both phoretic mites (mites on adult bees) and mites within brood cells	Is an irritant to humans and must be handled with gloves Causes some adult bee mortality
Oxalic acid	Oxivar Oxybee Api-Bioxal Dany's Bienenwohl powder	Trickling: dry oxalic acid is dissolved in 50% sugar syrup and trickled between frames Sublimation: dry oxalic acid heated to > 160°C, forming gas Requires specialist equipment and PPE	Can kill over 90% of mites No evidence of mites becoming resistant ideal winter treatment - sublimation does not require hives to be opened	Lethal to brood, should not be applied to colonies with larvae Toxic to humans, PPE must be worn when handling Requires costly specialist equipment
Hop beta acids	HopGuard	Strips hung between frames	Can kill up to 90% of mites Can be applied in summer when temperatures are high Non-toxic to humans and bees	Effectiveness varies - studies report a wide range of mite mortality
Thymol	Apiguard Apilife Var Thymovar	Either via strips between frames, gel packets, cellulose wafers Thymol will become a vapour and spread throughout the hive - requires air above the combs where the treatment is placed.	Highly effective; approximately 80% of mites killed if used correctly Mites unlikely to become resistant	Irritant, must be handled with gloves Honey must be harvested before treatment May cause brood mortality if applied at high temperatures

* MAQS only; honey should not be extracted from colonies treated with other products containing formic acid

Other organic chemicals

Many other plant-based essential oils have been trialled against Varroa, including garlic, clove, menthol, and origanum. The majority of these essential oils include monoterpenes and act as fumigants. There are some promising laboratory studies that indicate a level of acaracide activity against Varroa, however reliable delivery methods and formulations are yet to be developed and field efficacies are highly variable. In addition, some beekeepers attempt off-label formulation of their own acaracides from available essential oils, which should be discouraged due to variable efficacies and potential risks to bees and humans with unregulated or incorrect use (1)

4. RESISTANCE TO CHEMICAL CONTROLS

Synthetic acaricides are highly effective in unexposed populations of Varroa, with mite mortality typically greater than 90% in treated colonies. However, initial over-reliance on chemicals and off-label misuse has led to mite populations quickly developing resistance to synthetic acaracides, decreasing their effectiveness. Resistance can arise rapidly due to genetic mutations in key genes involved in the acaricide mode of action. Most synthetic acaracides interact with mites via a single, specific target (Table 1), meaning that resistance could develop easily due to a single mutation. In some cases, mite populations remain resistant for years after the chemical is no longer in use, rendering the chemical useless within a couple of seasons of treatment.

Resistance is well documented around the world (1; Table 3). Mite populations resistant to the four most commonly used synthetic acaricides have been reported since the early 1990s (1, 8-10).

Table 3: Summary of documented resistance to synthetic chemicals (from (1):

Country	**Amitraz** (formamidine)	**Coumaphos** (organophosphate)	**Fluvalinate** (pyrethroid)	**Flumethrin** (pyrethroid)
Americas				
Argentina	■	■		
Canada		■	■	
Mexico	■		■	■
United States	■	■	■	
Uruguay				■
Europe				
Austria			■	
Belgium			■	
Cyprus			■	
Czech Republic	■		■	■
France	■		■	
Germany				■
Greece		■	■	
Ireland				■
Italy		■	■	
Poland			■	■
Slovenia		■	■	
Spain			■	
Switzerland			■	
United Kingdom			■	■
Middle East				
Israel			■	
Asia/Pacific				
New Zealand	■	■	■	■

Note: Chemicals listed in this table are not registered for use in all countries and/or there may be no available data in some locations

5. OTHER CONCERNS FOR VARROA CONTROL

Climate: The efficacy of some Varroa chemical treatments can vary due to temperature and humidity, and thus may only be used effectively during particular seasons or in limited locations. Australia has a wide range of climactic conditions and some of the currently available products would not be suitable in warmer climates.

Beekeeper safety: Certain methods of application require specialised personal protective equipment as chemicals used in Varroa control may have harmful or irritant effects on humans. Beekeepers need to be aware of and consider risks and benefits of available treatments.

Bee health: Although sold and marketed as acaracides, the majority of chemicals used to treat mites are not mite-specific, and can have harmful effects to bees if used incorrectly. Of particular risk are the reproductive queen and the developing brood.

Residues in hive products: Chemical treatments can accumulate in honey and wax with prolonged use. As many acaricides are lipid-soluble, they can readily accumulate in comb wax, and persist for months or years after treatment (1, 11). This accumulation can lead to chronic exposure to the acaricide, both for bees and for Varroa mites, causing negative impacts on bee health. Additionally, chronic exposure to sublethal doses of acaricide via wax may contribute to the development of mite resistance (12).

6. FUTURE OF VARROA CONTROL

Due to the development of resistance to key synthetic chemicals, there is a growing need to discover novel control methods for Varroa, as part of an integrated pest management strategy.

Identifying a selective pesticide that is harmful to *Varroa* but safe for honey bees would provide a valuable addition to our arsenal of strategies to combat mites. **Target-based drug discovery** involves identifying a protein target within Varroa which contains differences in structure to the equivalent honey bee protein. An ideal target is one that performs an essential biological function, but is subtly different between the beneficial insect (honey bee) and the pest (Varroa), providing potential for selective insecticide design. Once a target is in hand, high throughput screening of an extensive library of chemicals is performed to identify candidate molecules that bind to and inhibit the Varroa protein but not the honey bee protein. These molecules are then optimised to increase affinity and selectivity.

To prolong the effectiveness of any new selective insecticides identified, beekeepers should follow an IPM framework that decreases reliance on chemicals, reserves chemical treatments for situations where all other options have been exhausted, and strict adherence to recommended doses and treatment regimes.

7. SUMMARY

a. Take home messages
    i. After decades of research, we are yet to find long-term solutions for *Varroa* control. Discovery and development of new *Varroa* control methods should remain a priority (1).
    ii. No single control method is 100% effective, and there is a risk that *Varroa* may develop resistance to currently effective methods.

iii. Best practice for sustained *Varroa* control requires implementation of an Integrated Pest Management framework, that incorporates appropriate seasonal use and rotation of chemical methods, with beekeeping practices and hive management strategies

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Development of ecofriendly selective pesticides to safeguard honeybees

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Progress is being made on a pesticide that selectively targets Varroa mite without harming bees.

Of the 100 crop varieties that provide 90 percent of the world's food, 71 are pollinated by bees[1]. However, the recent invasion of *Varroa destructor*, a mite that parasitises honeybees, has led to thousands of hives to being burned, seriously impacting the livelihood of beekeepers and threatening the productivity of horticultural industries[2].

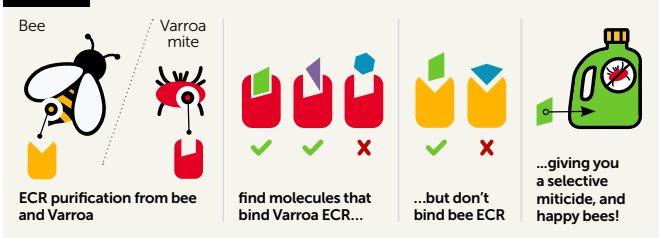
As part of an effective strategy to deal with this incursion, more specific and effective pesticides would be a valuable weapon. However, most pesticides currently used in agricultural applications work non-selectively against both pests and beneficial arthropods. A selective pesticide that is harmful to *Varroa* but safe for honeybees would therefore provide a valuable weapon in our arsenal and might also be a starting point for the development of pesticides that target other harmful mites.

In order to design pesticides that display significant selectivity, a target-based approach can be employed. This strategy leverages protein biochemistry, genomics, structural biology, toxicology and chemistry to identify molecules that interact with specific biological targets. In this context, a hormone binding protein in the *Varroa* mite known as the ecdysone receptor protein is a promising target for pesticide development.

This protein interacts with the pest hormone ecdysone to regulate mite development, reproduction and behaviour[3]. Disruption of this interaction by designed chemicals would significantly impair mite development and reproduction,[4] meaning that such chemicals could be potent pesticide candidates. Also, because this receptor is absent from vertebrates and is also subtly different between insects, chemicals targeting this receptor would be safe for farm workers, consumers and also beneficial insects such as honeybees. Such chemicals can also be applied in conjunction with current insecticides to improve potency and reduce the development of resistance.

Above L-R. The project team: Jen Suh, Shahnaz Sultana, Professor Joel Mackay, Dr Milad Ghafoori, Zahra Falahati and Dr Ingrid Macindoe.

Figure 1



Our research at The University of Sydney, led by Prof Joel Mackay, Prof Ron Hill and Dr Emily Remnant and funded by Hort Innovation and a generous philanthropic donation, aims to develop such a selective insecticide. Already, we have made considerable progress on this quest.

We have identified and purified significant quantities of the *Varroa* ecdysone receptor protein and also the corresponding protein from the honeybee through a laboratory-based protein production process. We have used an array of experimental methods to demonstrate that these lab-generated receptor proteins can interact with the ecdysone hormone and can therefore be used as targets for pesticide development.

With our target in hand, we have recently commenced the search for chemicals that can hit that target. Our strategy is to search collections of thousands of commercially available chemicals to find that needle in the haystack – a chemical that potently interferes with the *Varroa* ecdysone receptor but does not interfere with the equivalent target in the honeybee (and so is safe for our beloved pollinators *(Figure 1)*. Excitingly, we have already discovered several 'hits' – chemicals that are starting points on that journey. Time will tell whether these starting points can be honed to yield our holy grail: a pesticide that can be deployed against *Varroa* without harming honeybees.

Concurrently, we are using this strategy to target another pollinator pest – the small hive beetle. To date, we have already purified and characterised the hormone receptor protein from the beetle and are on track to follow in the footsteps of our *Varroa* work. We hope that this new approach to pesticide design offers a competitive route to the identification of safer, more selective and environmentally friendly agents for the control of insect and arachnid pests, thereby helping to protect global agricultural systems.

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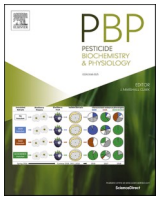

Review

Small organic ligands for the ecdysone receptor – agrochemicals, gene switches, and beyond

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ABSTRACT

While pesticides are essential for the world to meet its increasing demand for food, off-target toxicity in humans and other species is an ongoing environmental issue. There is a strong motivation for developing more selective pesticides that can target pest insects, for example, while being benign for beneficial insects such as bees, and other nontarget species more generally. The ecdysone receptor is absent in vertebrates so constitutes a very useful target for green insecticides. It has also been found to be an extremely useful gene switch in molecular biology. While the natural ecdysone ligands are complex steroidal compounds, a wide range of simpler synthetic agonists and antagonists have been developed. Here we review the diversity of chemotypes that have been shown to bind productively to the ecdysone receptor and have found application as insecticides and gene switches. We discuss the similarities and differences in these chemotypes, the origin of which is the remarkable flexibility of the ligand binding domain in the receptor. We provide a perspective on the discovery of further useful chemotypes with potentially higher binding and selectivity between pest and beneficial insects.

1. Introduction

The first demonstration that steroids act directly on insect gene transcription to cause subsequent cellular changes was reported by Becker, and Clever and Carlson more than 60 years ago (Becker, 1959; Clever and Karlson, 1960). They observed 'puffs' on the polytene chromosomes of fly larval salivary glands incubated with ecdysone. Despite the importance of these observations, the molecular mechanisms driving regulation of insect development took many years to be revealed due to experimental difficulties in isolating and identifying the relevant regulatory factors. Recombinant DNA and polymerase chain reaction (PCR) technologies have greatly accelerated research on this front. Recent progress and the earlier history of experiments on the molecular basis of hormonal action in insects has been reviewed by Henrich and Brown (Henrich and Brown, 1995) and more recently by Hill et al. (Hill et al., 2013), based primarily on nuclear hormone receptors in *Drosophila melanogaster*. More recently, reviews on insect nuclear receptors more generally were published by Fahrbach et al. in 2012 and Jarvela and Pick in 2017 (Fahrbach et al., 2012; Jarvela and Pick, 2017). The ecdysteroid receptor (ECR) comprises two members of this large family.

In Mecopterida (higher insects, including Diptera and Lepidoptera), the ECR functions as a noncovalent heterodimer of the EcR protein and ultraspiracle protein (USP). EcR and USP are the insect orthologs of the mammalian farnesoid X receptor (FXR) and retinoid X receptor proteins (RXR) receptively (Rani et al., 2018). The ecdysteroid-binding pocket is located in the ligand binding domain (LBD) of the EcR subunit. However, in the *Mecoperida* EcR must bind to USP (or RXR) before high-affinity ligand binding is possible. Agonist ligand binding causes a conformational change in the C-terminal region, including helix H12 of the LBD, that initiates transcriptional activation of genes (Fig. 1).

EcR homologues have been identified in many arthropods, However, in lower insects and the class Arachnida, typified by the scorpion *Liocheles australasiae,* EcR can function to control ecdysteroid-dependant transcription as a homodimer. For evolutionary perspectives see for example Hill et al. (Hill et al., 2013).

The presence of EcR in arthropods, but not in vertebrates, provides a pathway for development of potentially safe 'green' insecticides.

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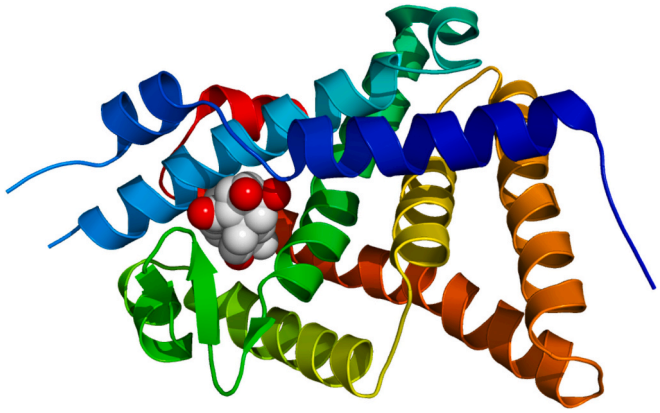


Fig. 1. X-ray structure of the LBD of EcR (N-terminus = blue, C-terminus = red) from *Heliothis virescens* complexed with ponasterone A (space-filling model). GNU General Public License. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Readers are referred to the cited reviews for more detailed information on the discovery of modulators of ecdysone gene expression and the effects on insect growth and development.

The primary aim of this review is to survey the relatively large number of chemotypes that can bind to the EcR and produce agonist or antagonist responses. Some examples of these diverse chemical classes are provided in Fig. 2. These chemotypes have yielded very important insecticidal compounds that have been used commercially for several decades. By understanding the molecular details of the interactions of these EcR ligands, the structure-activity relationships within and between chemotypes, and their differential activities across insect species, we will be better equipped to develop new, potent, selective, and mammalian vertebrate-safe insecticides with improved performance and selectivity for pest versus beneficial insects.

2. The importance and ubiquity of ecdysone receptors

The ecdysone steroid hormones play a key role in coordinating moulting, metamorphosis, and reproduction in insects (Liu et al., 2007). These hormones, principally 20-hydroxyecdysone (20E), bind to the ecdysone receptor component of the heterodimer complex with the ultraspiracle protein. The ligand-bound EcR-USP heterodimer then binds to a DNA ecdysone response element (EcRE) on the promoter region of several ecdysteroid-responsive genes, triggering a cascade of gene expression required to regulate key developmental events in arthropods. Consequently, along with the absence of its homolog in mammals, the EcR is an important target for the design of new, green insecticides against pest species. As the LBDs of EcRs from insects belonging to different orders vary, it appears possible to discover compounds selective for pests and relatively harmless to other arthropods, especially beneficial ones like bees. The structural and functional characteristics of EcRs, the molecular mode of action of ecdysteroid ponasterone A (PonA) and a diacylhydrazine (BYI06830) with EcR/USP, and EcR-based screening assays and gene switches have been reviewed by Liu et al. (Liu et al., 2007).

2.1. Gene switches

Chemically-inducible gene switches that regulate expression of endogenous and exogenous genes have many applications in basic gene expression and gene therapy. The negligible toxicity of ecdysteroids and nonsteroidal agonists to vertebrate cells make them attractive tools for medicine (Spindler et al., 2009). As the focus of this review is insecticides and nematicides exploiting the ecdysone pathway, we provide only a brief summary of the history of ecdysone gene switches. Reviews of the use of ecdysone gene switches have been published recently for readers interested in this aspect e.g., (Lee et al., 2016).

During a collaborative study with Stanford University into ecdysone regulated gene networks in *Drosophila*, Hill in CSIRO raised the possibility of transferring an elementary ecdysone gene control system from arthropods to mammals for the regulation of transgenes. A plasmid encoding the *Drosophila* ecdysone receptor was provided by Stanford for this purpose. The hypothesis became the basis of a successful project funded by the Australian Wool Research and Development Organisation from 1989 to1991, the results of which were subsequently published by Yang et al. (Yang et al., 1995).

Early work on the potential of ecdysone switches to control gene expression was also reported by Gossen and colleagues and Delort and Capecchi in the mid-1990s (Delort and Capecchi, 1996; Gossen et al., 1994). Gossen et al. reported the use of regulatory proteins from species distinct from mammals to establish highly specific control systems in eukaryotic cells. Delort and Capecchi constructed a switch composed of three components: an inducible promoter that is absent in mammalian genomes; a receptor that activates transcription from the inducible promoter when bound to a drug; and a drug such as RH486, whose pharmacological properties in humans and other mammalian species have been well studied. Their molecular switch functions transiently; increased induction levels over 100-fold and could regulate a mouse transgene using a gene therapy paradigm.

3. Structural biology of ecdysone receptors

Some of the earliest work on EcR structure was reported by Wurtz and colleagues (Wurtz et al., 2000). They constructed two homology models of the *Chironomus tentans* EcR LBD using the known crystal structures of the retinoic acid and vitamin D receptors as templates. They docked 20E and dibenzoylhydrazines into the receptor ligand

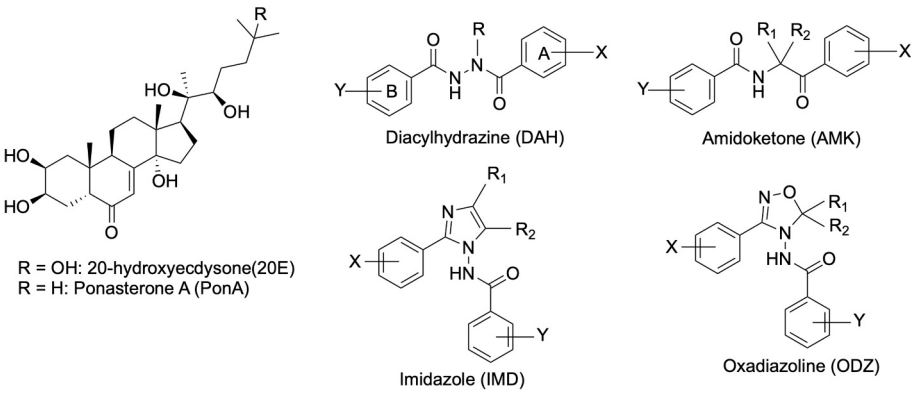


Fig. 2. Representative EcR ligands.

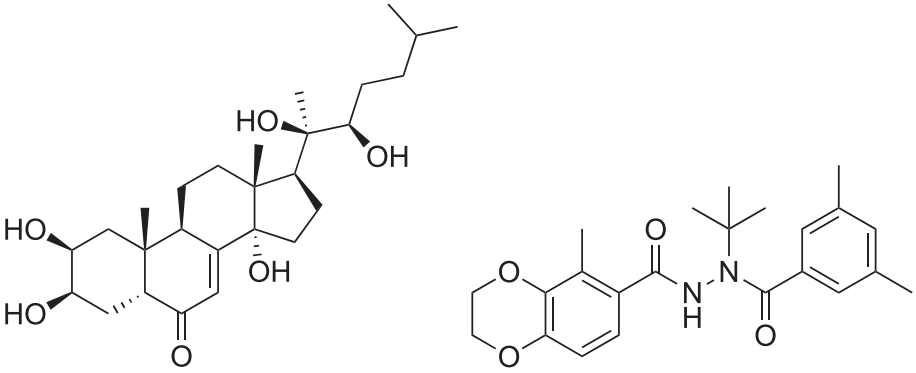


Fig. 3. Structures of PonA and BY106830.

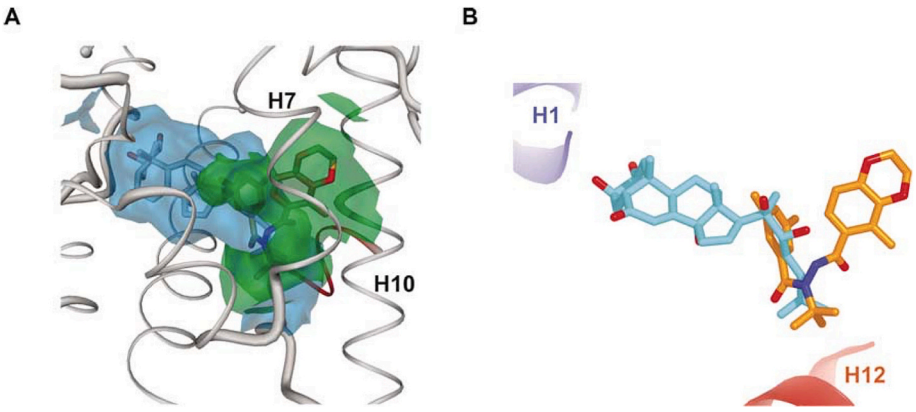


Fig. 4. The LBDs of EcR complexed to steroidal and nonsteroidal ligands. (A) Ligand-binding cavities for PonA (blue) and BYI06830 (green). (B) Overlap of the steroidal and nonsteroidal EcR ligands in the LBD. Atom coloring: red for oxygen; blue for nitrogen; cyan (PonA) or orange (BYI06830) for carbon. Used with permission from Billas and Moras (Billas and Moras, 2005). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

binding domain to elucidate the basis for activity of these diverse chemotypes. They reported that the *N*-t-butyl substituent of the dibenzoylhydrazines overlapped significantly with the 20E molecular volume. They suggested the homology models provide new insights that can be exploited in the rational design of new environmentally safe insecticides.

Billas et al. were first to publish the crystal structures of the ligand-binding domains of the moth *Heliothis virescens* EcR-USP heterodimer in complex with the ecdysteroid PonA and with a non-steroidal, lepidopteran-specific agonist BYI06830 (Fig. 3) (Billas et al., 2003). Their structures underscored the universality of EcR-USP heterodimerization in both vertebrates and invertebrates. Conspicuously, the complexed EcR structures showed how radically different and only partially overlapping the ligand-binding pockets were, explaining the lack of success in predicting ligand interactions by molecular modelling and docking studies.

Billas and Moras subsequently reported a more detailed analysis of the structure of the LBDs of the EcR-USP heterodimer complexed to PonA and BY106830 (Billas and Moras, 2005). This afforded fine atomic level detail for these important functional complexes. Their seminal structural biology study showed that protein structures with different steroidal and nonsteroidal ligands had LBDs with quite different sizes and shapes (Fig. 4). The EcR appears to adapt to different ligand chemotypes, generating radically different, partially overlapping ligand-binding pockets with different residues involved in ligand recognition.

Carmichael and coworkers from CSIRO also reported a structural biology study of the heterodimer of EcR and its USP partner protein of the hemipteran *Bemisia tabaci* (Bt, sweet potato whitefly) in complex with PonA (Carmichael et al., 2005). They showed that most of the selectivity of the bisacylhydrazine insecticides can be understood in terms of their binding to EcR LBD heterodimers. Comparing the structure of the EcR LBD heterodimer of Bt with that of the lepidopteran *Heliothis virescens* (Hv) showed that the PonA binding was very similar. However, pockets were structurally different in regions not in contact with PonA. They also suggested the selectivity of bisacylhydrazine insecticides may be due to differences in the LBD pocket topographies. More recently, Ren et al. obtained the first structure of an apo-form of an EcR LBD (Ren et al., 2014).

Three of the four commercially available ecdysone agonists, tebufenozide, methoxyfenozide and chromafenozide are highly lepidopteran specific while halofenozide is used to control both coleopteran and lepidopteran insects. However, unlike the very high binding affinity of DAH analogues for lepidopteran EcRs, halofenozide has a low binding affinity for coleopteran EcRs. Soin and colleagues reported the development and evaluation of two coleopteran-specific reporter-based screening systems to discover and evaluate ecdysone agonists and generated an initial in silico 3D model of its ligand-binding pocket docked with PonA and tebufenozide (Soin et al., 2009).

Recently, Wang and Hu reviewed the structural characteristics of the EcR: the transactivation domain, DNA-binding domain, hinge region, ligand binding domain and F region. They discussed the structure and function of EcR and USP, the mechanism of interaction with moulting hormone and use of EcR in agricultural pest control (Wang and Hu, 2017).

4. Species specificity of ecdysone mimetic agents

Clearly, one of the most attractive attributes of ecdysones and their

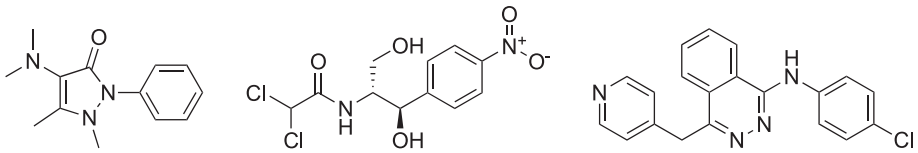


Fig. 5. Aminopyrine, chloramphenicol, and vatalanib.

mimics is the lack of a vertebrate receptor, rendering them intrinsically safe for these animals. While the EcR is absent in vertebrates, the USP, with which it forms a heterodimer, is the insect homolog of the vertebrate retinoid X receptor. Ecdysteroids differ greatly from vertebrate steroid hormones in polarity, size, and shape so would not be expected to interact with the steroid-hormone receptors or steroid-metabolising enzymes in mammals (Dinan and Lafont, 2006). However, selectivity for specific insect species is also very important to minimize their impacts on beneficial insect species.

4.1. Insect species

Pesticide poisoning is potentially devastating for humans and harmless or beneficial species, such as bees. Worldwide human deaths have been estimated to be 200,000 per annum in 2017 (Elver, 2017). Consequently, there is considerable pressure to discover selective insecticides that are benign for mammals – 'green' insecticides (Spaggiari et al., 2021). Pesticides acting as endocrine disruptors alter production of endogenous hormones mainly via nuclear receptors. These promise high degrees of selective toxicity towards insects and other arthropods. Of these, mimics of juvenile hormone and ecdysteroids have progressed furthest down the insecticide development pathway. In particular, ecdysone agonist compounds have been shown to be safe to mammals and beneficial organisms such as fish and insect natural enemies (Sun et al., 2003). Spaggiari et al. studied the possible negative effects of pyriproxyfen and its metabolite on human and bee health. They probed the mechanism of binding with the USP-EcR heterodimer of *A. mellifera* and the farnesoid X receptor/retinoid X receptor alpha (FXR-RXR alpha) heterodimer of *H. sapiens* using molecular dynamic simulations. They found that that both molecules stabilized these heterodimers and bound more strongly than the natural ligands. This provided a potential mechanism for endocrine interference by these pesticides and prompted further in vitro studies of the biological effects of pyriproxyfen and its metabolite as potential leads for the EcR-USP complex.

Ruivo and colleagues reviewed the evidence for the wider occurrence of EcR orthologues across metazoan lineages, with unknown physiological consequences (Ruivo et al., 2022). They stressed the importance of a much better sampling of physiological responses across a representative taxonomic scope, especially nuclear receptors, given their use as targets for environmental chemicals. The EcR receptor has been extensively targeted by pesticides that seem largely seemingly innocuous to non-target organisms. They reviewed the state-of-the-art in the phylogenetic distribution and functional characterization of metazoan EcRs and provided a critical analysis of the potential for disruption of these receptor pathways by pesticides.

Mellor and colleagues used computational methods to develop a series of rule sets, derived from the key structural and physicochemical features in ecdysone receptor ligands, that identified compounds likely to bind to the EcR LBD (Mellor et al., 2020). Data on activities of 555 distinct chemicals were aggregated from a variety of assays in the literature, for 9 insect species and three non-insects. The chemicals consisted of four main classes: steroids; diacylhydrazines (also known as bisacylhydrazines (BAHs) or dibenzoylhydrazines); methylene-γ-lactams; and tetrahydroquinolines. The species tested were the insects *Bombyx mori* (silk moth), *Calliphora erythrocephala* (blow fly), *Chilo suppressalis* (rice stem borer), *Chironomus tentans* (non-biting midges), *Choristoneura fumiferana* (eastern spruce budworm), *Drosophila melanogaster* (fruit fly), *Lucilia cuprina* (Australian sheep blowfly), *Musca domestica* (housefly), *Spodoptera littoralis* (cotton leafworm), the arachnid *Agelena sylvatica* (grass spider), the crustacean *Callinectes sapidus* (blue crab), and the myriapod *Lithobius peregrinus* (centipede). The derived rulesets (akin to pharmacophores) for potential binding activity at the EcR were used to screen 8795 compounds from the US EPA ToxCast initiative that included pharmaceuticals, food additives, cosmetic ingredients, and synthetic precursors. This identified 34 potentially active EcR binders – 29 nonsteroidal and 5 steroidal. The rule set screen identified pesticides such as the diacylhydrazines halofenozide, methoxyfenozide, and tebufenozide present in the training set but the predicted binders were mainly pharmaceuticals. Several pyrazolone nonsteroidal anti-inflammatory drugs (aminopyrine, phenazone, and propyphenazone), structurally similar to the methylene-γ-lactams were identified. Larger compounds containing similar structural motifs were also flagged, including doxapam, eltrombopag, and azo dyes. The diacylhydrazine rule set identified some amphenicol antibiotics (chloramphenicol, florfenicol, and thiamphenicol). Vatalanib, a vascular endothelial growth factor receptor inhibitor was also selected because of its embedded tetrahydroquinoline moiety (Fig. 5).

4.2. Other potentially valuable target species

Charrois and colleagues reported that ecdysteroids cause salivary gland degeneration in the ixodid (hard or scale) tick *Amblyomma hebraeum* Koch (Charrois et al., 1996). They studied three brassinosteroids (putative ecdysone antagonists) – HHCS (22S,23S-homocastasterone), SSBR (22S,23S-homobrassinolide), STGM (2 alpha,3 alpha(OH)(2)-Delta(22)-stigmasten-6-one) – and two nonsteroidal mimics of ecdysone, RH 5849, and RH 5992, in a variety of insects. They concluded that the brassinosteroids and the RH compounds affect the tick salivary gland ecdysteroid system differently to insect systems. At a similar time, Guo et al. studied the effects of ecdysteroids on a related ixodid tick *Amblyomma americanum* (L, *Aam*) (Guo et al., 1997). To determine the possible role ecdysteroids play they isolated cDNAs encoding three ecdysteroid receptor isoforms (AamEcRA1, AamEcRA2, and AamEcRA3) that have common DNA and ligand binding domains. Their analysis showed that processing of the AamEcR gene is complex, producing multiple transcripts and splice variants with incomplete open reading frames. Their results in whole animals and isolated tissues are consistent with complex regulation of AamEcR expression and demonstrate that the AamEcR gene encodes a functional ecdysteroid receptor.

Similarly, Li et al. recently reported a study of 14 ecdysone response genes in red citrus mite *Panonychus citri* (Acari: *Stigmaeidae*), a widespread pest of citrus crops (Li et al., 2017). They investigated ecdysteroid biosynthesis, signalling, and expression over the five developmental stages (egg, larva, protonymph, deutonymph and adult) of the mite,

A homologue of the ecdysone receptor has been identified in human filarial parasites (nematode roundworms) and Mhashilkar and colleagues reported it is a potential chemotherapeutic target (Mhashilkar et al., 2016). Administration of 20E to gerbils infected with *Brugia malayi* larvae disrupted their development to adult stage parasites. Using a stable HEK293 mammalian cell line incorporating the *B. malayi* ecdysone receptor ligand-binding domain, its heterodimer partner, and a secreted luciferase reporter, they screened ecdysone ligands and identified seven sub-μM agonists. They found an excellent correlation between the results from virtual screening and the filarial screening

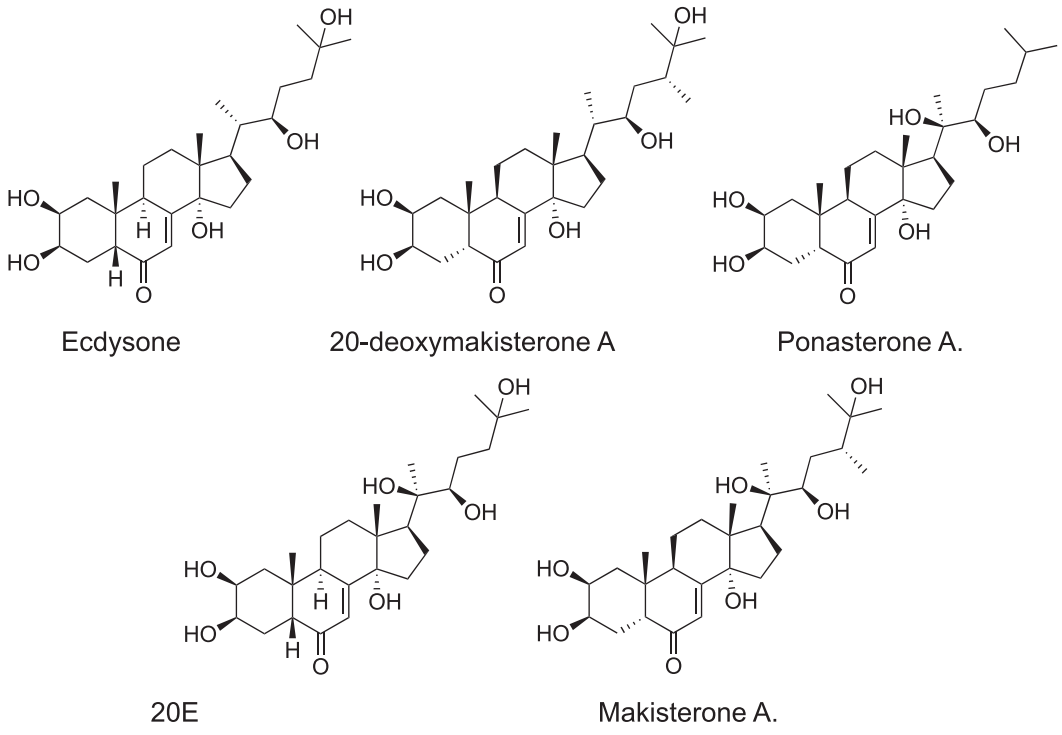


Fig. 6. Structure of representative ecdysteroids.

assay. Thus, steroidal ecdysone agonists and the diacylhydrazines are safe potential lead drugs for filarial infections.

The Hill group at CSIRO also reported that mM concentrations of 20E inhibit the development of the eggs of the nematode *Haemonchus contortus* (Hc, barber's pole worm) to the L3 larval stage (Graham et al., 2010). They conducted a phylogenetic study of an EcR ortholog (HcEcR) from *H. contortus* mRNA expressed during L3, and putative EcR from *Brugia malayi* that forms a separate branch between arthropod EcRs and liver X receptors. They found that 35 % of the highly conserved ecdysteroid-contacting residues in insect EcRs are also conserved in the HcEcR LBD, but there are unusual residue choices at other ligand-contacting positions.

Hormonal regulation of moulting and development in spiders is poorly understood. Yang et al. used the sequenced genome and transcriptomes in the pond wolf spider (*Pardosa pseudoannulata*) to characterize Halloween genes and detect active forms of ecdysteroid (Yang

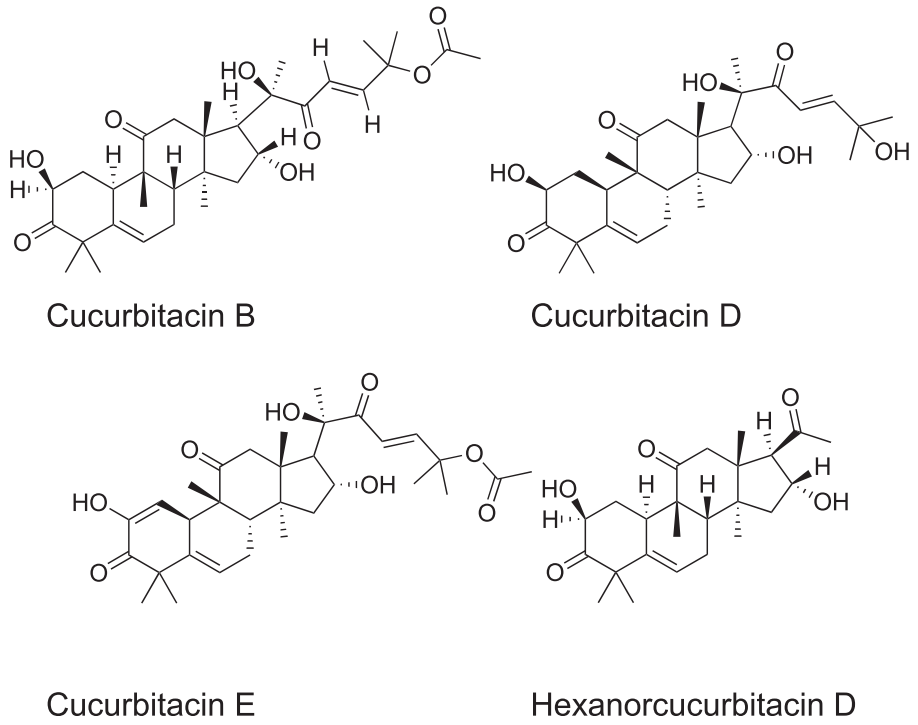


Fig. 7. Cucurbitacins with EcR activity from studies of Dinan et al. and Toyofuku et al. (Dinan et al., 1997; Toyofuku et al., 2021).

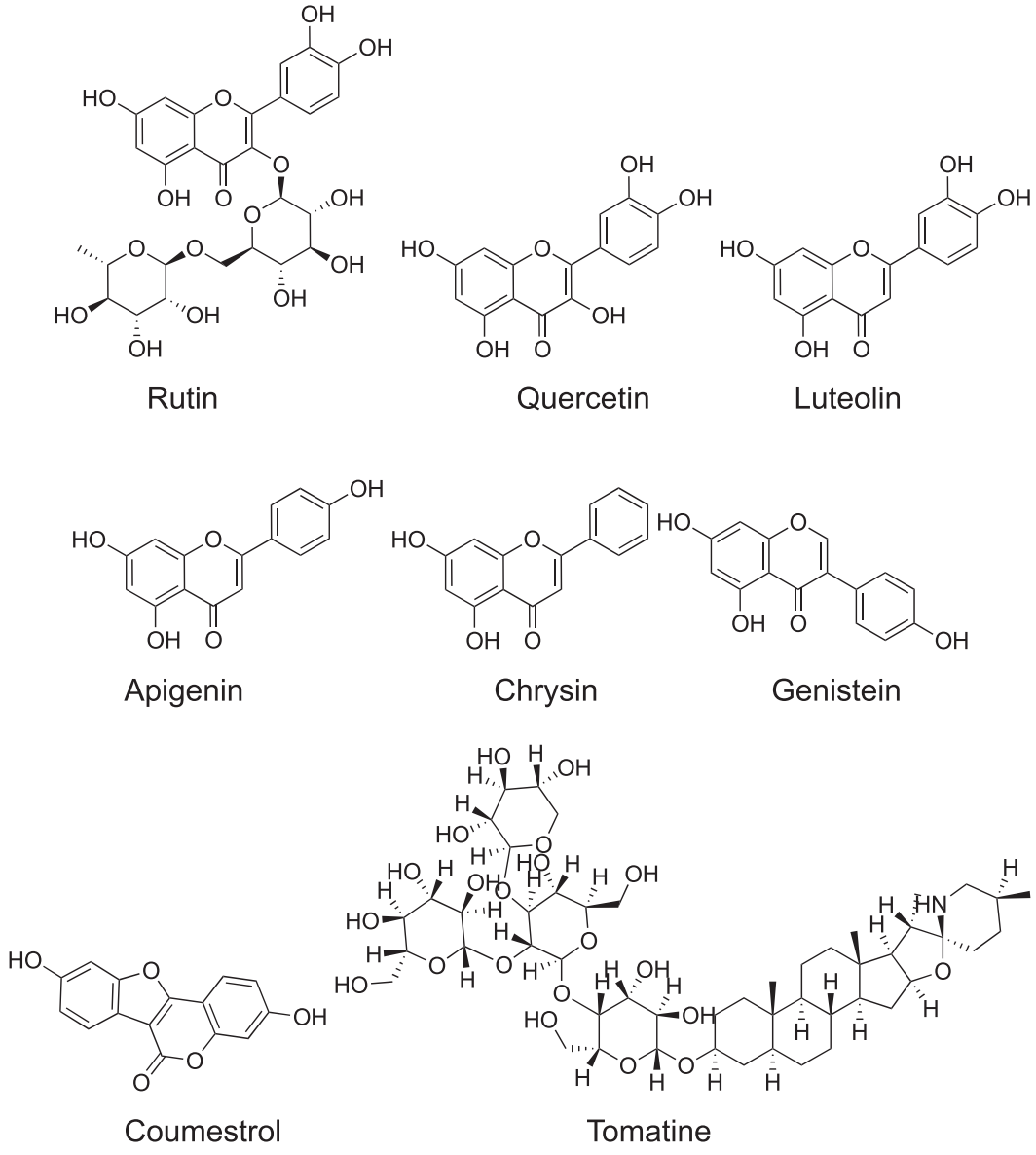


Fig. 8. Flavins and related non-flavin agonists and antagonists of EcRl (Oberdörster et al., 2001).

et al., 2021). They identified the presence of PonA, but not 20E, by LC–MS/MS analysis and showed it accelerated moulting. Their study revealed differences in the ecdysteroid biosynthesis pathways in spiders and insects.

Developing and using pesticides with minimal non-target effects on pest natural enemies is very important for conserving biological control. Schmidt-Jeffris and colleagues published a very interesting study of the effects of the major pesticides (herbicides, fungicides, insecticides) on phytoseiid mites, the most well-studied natural enemies in pesticide selectivity research (Schmidt-Jeffris et al., 2021). Their meta-analysis revealed that insecticides in mode of action groups 9 (chordotonal organ modulators), 17 (dipteran moulting disruptors), 18 (ecdysone receptor agonists), and 28 (ryanodine receptor modulators) did not increase adult phytoseiid mortality. All other insecticide classes (and multiple pesticide classes) had a significant effect size (standardized difference between two group means or log odds ratio for binary data).

Curiously, 20E has been shown to reduce tonic convulsion and absence-like seizures in spontaneously epileptic rats (Hanaya et al., 1997). Pretreatment with convulsant bicuculline antagonized the inhibitory effects of 20E but absence-like seizures were not affected. This suggests that 20E ameliorates tonic convulsions by modulating $GABA_A$ receptors.

5. Structural classes (chemotypes) of organic ecdysone ligands

A comprehensive review of the activity of steroidal and non-steroidal synthetic ecdysone agonists was published by Dinan et al more than a decade ago (Dinan et al., 2012). In the intervening years, there has been continual development of small molecule EcR ligands for crop protection and gene switching applications. The following concise review provides an update to this prior review, disclosing additional chemotypes and structure-activity relationships.

5.1. Steroids, hormones, and other natural products

Over 300 different ecdysteroid derivatives are produced by animals and plants (Dinan, 2009; Lafont and Koolman, 2009). Representatives of some of the more prominent chemical classes are provided in Figs. 2 and 6.

Research by Nakanishi at Tohoku University over 30 years ago identified PonA, the first phytoecdysteroid, shortly after the determination of the structure ecdysone and 20E (Nakanishi, 1992). Shortly

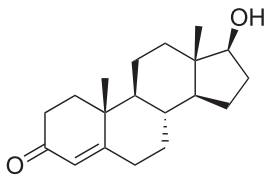


Fig. 9. Structure of the EcR antagonist testosterone.

after, Dinan and coworkers isolated two triterpenoids, cucurbitacin B and D (Fig. 7), from seeds of *Iberis umbellata* (Cruciferae) that antagonized 20E-induced morphological changes in the *Drosophila melanogaster* B-II permanent cell line (Dinan et al., 1997). Both cucurbitacins displaced bound radiolabelled PonA from EcRs. This was the first literature report of EcR antagonists. The δδ (23)-22-oxo side chain functional group was important for antagonistic activity as hexanorcucurbitacin D, which lacks C-22 to C-27, was a weak EcR agonist.

Toyofuku et al. also investigated the effects of the plant-derived tetracyclic triterpenoid compounds found cucumbers, pumpkins, squash, and melons, cucurbitacin B and E (CucB and CucE, Fig. 7) on the development of *Drosophila melanogaster* reared on low sterol food (LSF) with, or without cucurbitacins (Toyofuku et al., 2021). Most larvae without supplementation or with CucE died at the second or third larval instar stages, while CucB-administered larvae died without moulting. Ecdysone supplementation could partially rescue the developmental effects suggesting that CucB acts, not only as an antagonist of the ecdysone receptor, but also as an inhibitor of ecdysone biosynthesis.

Dinan, Hormann, and Fujimoto assessed the EcR agonist activity of 71 ecdysteroids in a *Drosophila melanogaster* B-II tumorous blood cell line assay (Dinan et al., 1999). The ED_{50} values for these steroids spanned almost 6 orders of magnitude. These data were used to train a 3D quantitative structure-activity relationships (QSAR) model that predicted the training set EcR activities with an $r^2 = 0.90$ and a cross-validated q^2 of 0.70. The model suggested that ecdysteroid binding is principally controlled by localized interactions from approximately six specific loci.

Zou et al. screened 126 steroid-like compounds, including cucurbitacins, as possible EcR antagonists using *Drosophila melanogaster* Kc and *Bombyx mori* Bm12 cells (Zou et al., 2018). Of these, three cucurbitacins (CucB, D and E, Fig. 7), were again confirmed as antagonists in both Kc and Bm12 cells, with CucB causing significant moulting defects and mortality in larvae of both insects. It was also a strong antifeedant for *Helicoverpa armigera*.

Based on the observation that many plant compounds can modulate growth and reproduction of herbivores by directly interacting with steroid hormone systems. Oberdörster et al. (Oberdörster et al., 2001) investigated whether flavins rutin, luteolin, and quercetin (metabolites of rutin), apigenin and chrysin, and three non-flavones, coumestrol and genistein (both estrogenic) and tomatine could act as agonists or antagonists of the EcR using a reporter-gene assay and a cell differentiation assay of an ecdysone-responsive cell line, C1.8+ (Fig. 8). None were agonists but the flavones exhibited significant antagonist activities. The phytoestrogens quercetin and coumestrol were mixed agonists/antagonists, while genistein (isoflavone), tomatine (alkaloid), and apigenin (flavone) synergized the cell growth reduction induced by ecdysteroids. They suggested that rutin modulates insect moulting via its metabolites, luteolin or quercetin, while tomatine does not bind to the EcR.

Testosterone (Fig. 9) is known to cause developmental arrest of embryonic *Daphnia magna*. Mu and Leblanc investigated whether this was due to antagonism of ecdysteroidal activity by testosterone (Mu and LeBlanc, 2002). They found that testosterone did indeed antagonized the action of 20E in an ecdysone-responsive cell line at relatively high doses, but had negligible effect on endogenous ecdysone levels in daphnids. As endocrine disruptors interfere with hormone signalling via multiple mechanisms, Mu and LeBlanc subsequently described the

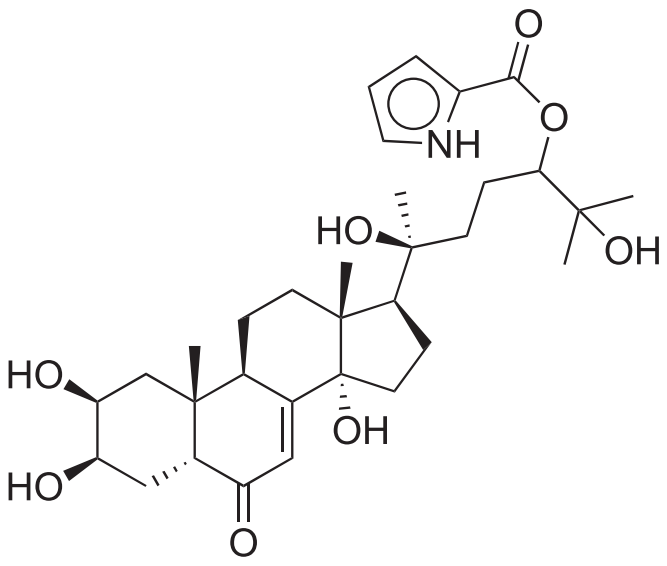


Fig. 10. Canescensterone.

synergistic effects of the ecdysteroid-synthesis inhibitor fenarimol and the ecdysteroid receptor antagonist testosterone on ecdysteroid-regulated development in the *Daphnia magna* (Mu and LeBlanc, 2004).

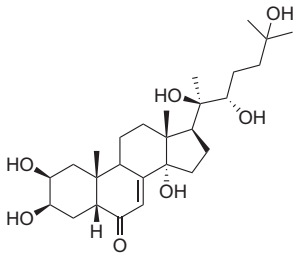
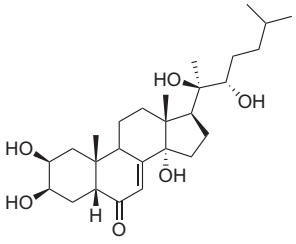
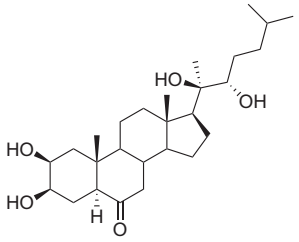
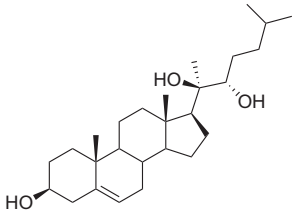
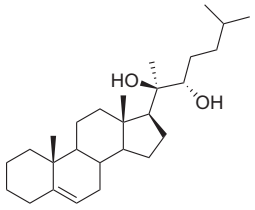
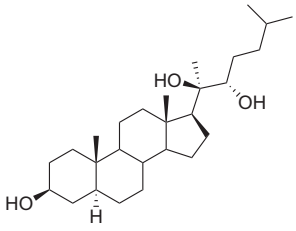
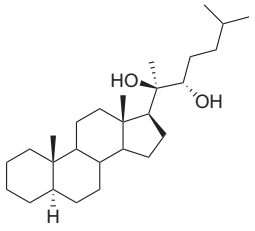
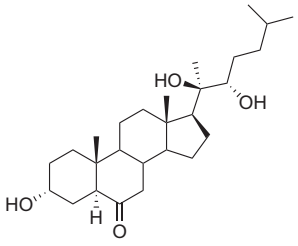
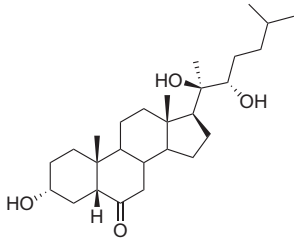
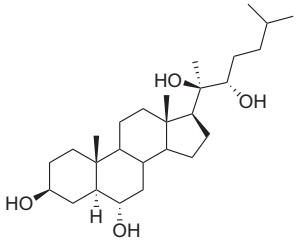
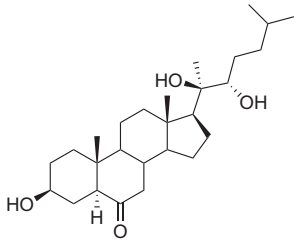
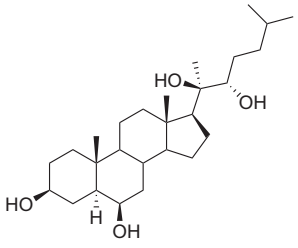
The uncommon phytoecdysteroid, canescensterone (Fig. 10), is a potent activator of EcR gene-expression systems that displays remarkable selectivity towards non-lepidopteran over lepidopteran EcRs, making it a valuable insecticide lead. It possesses an unusual pyrrole-2-carboxylate group attached to C-24 that is absent in all other natural ecdysteroids. It absolute stereochemistry was meticulously determined by Sun et al. (Sun et al., 2010)

PonA is one of the most potent ecdysteroids, approximately 20-fold higher affinity for EcR in some species. Given this exceptional affinity, Arai and colleagues synthesized a series of PonA derivatives to assess their binding activity to the EcR of *Drosophila* Kc cells (Table 1) (Arai et al., 2008). Compounds with no hydrogen bond donors or acceptors like OH and C=O groups on the steroid skeleton were inactive. Some compounds containing the A/B-trans ring fusion, which is different from that of ecdysteroids (A/B-cis) were also active. The oxidation of CH_2 at 6-position to C=O, enhanced the activity 19 times, but the activity was erased by the reduction of the ketone to OH group at 6-position. The activity was enhanced about 250 times by the conversion of A/B ring configuration from trans [(20R,22R)-2 beta,3 beta,20,22-tetrahydroxy-5 alpha-cholestan-6-one: $pIC_{50} = 4.84$] to cis [(20R,22R)-2 beta,3 beta,20,22-tetrahydroxy-5 beta-cholestan-6-one: $pIC_{50} = 7.23$. The latter cis-type compound, the most potent compound synthesized in this study, was equipotent to the natural moulting hormone, 20E, although it has only 2 % of the activity of PonA.

Harada and coworkers subsequently studied the QSAR in these compounds, plus 20E and PonA (Harada et al., 2009). Hydrogen bonding (HB) is one of the most important physicochemical properties for ligand binding to the ecdysteroid receptor, so they estimated the number of interactions between the ecdysteroids and the x-ray structure of the LBD of EcR using docking. They found a statistically significant correlation between the number of hydrogen bond interactions and the pI_{50} values of the steroids ($r^2 = 0.63$). To better understand the contributions of steric and electrostatic effects on the ligand-receptor binding, they employed a 3D molecular field QSAR method, comparative molecular field analysis (CoMFA). Addition of the 3D fields slightly improved the model and added visual interpretation of the structure-activity relationship in these compounds

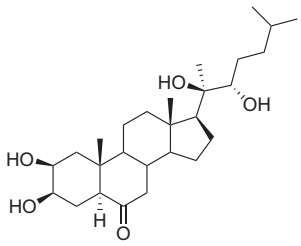
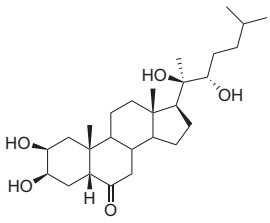
Zotti and colleagues developed a luciferase reporter gene cell assay to screen the natural ecdysteroids castasterone, cyasterone, inokosterone, 20E, PonA and the non-steroidal compounds tebufenozide and EPH

Table 1
Binding activity of ecdysteroids against ecdysone receptor of Kc cells (Arai et al., 2008).

Compound		pIC_{50} (M)	Compound		pIC_{50} (M)
No.	Structure		No.	Structure	
1		7.3	20		6.1 ± 0.2
2		8.9	21		4.1 ± 0.3
3		6.5	24		<3.61 (42 %)
4		4.4 ± 0.3	26		5.0 ± 0.5
8		<3.61 (23 %)	28		<3.61 (19 %)
9		4.4 ± 0.2	32		4.8 ± 0.3

(*continued on next page*)

Table 1 (*continued*)

Compound No.	Structure	pIC_{50} (M)	Compound No.	Structure	pIC_{50} (M)
12		<3.61 (8 %)	34		7.2 ± 0.1

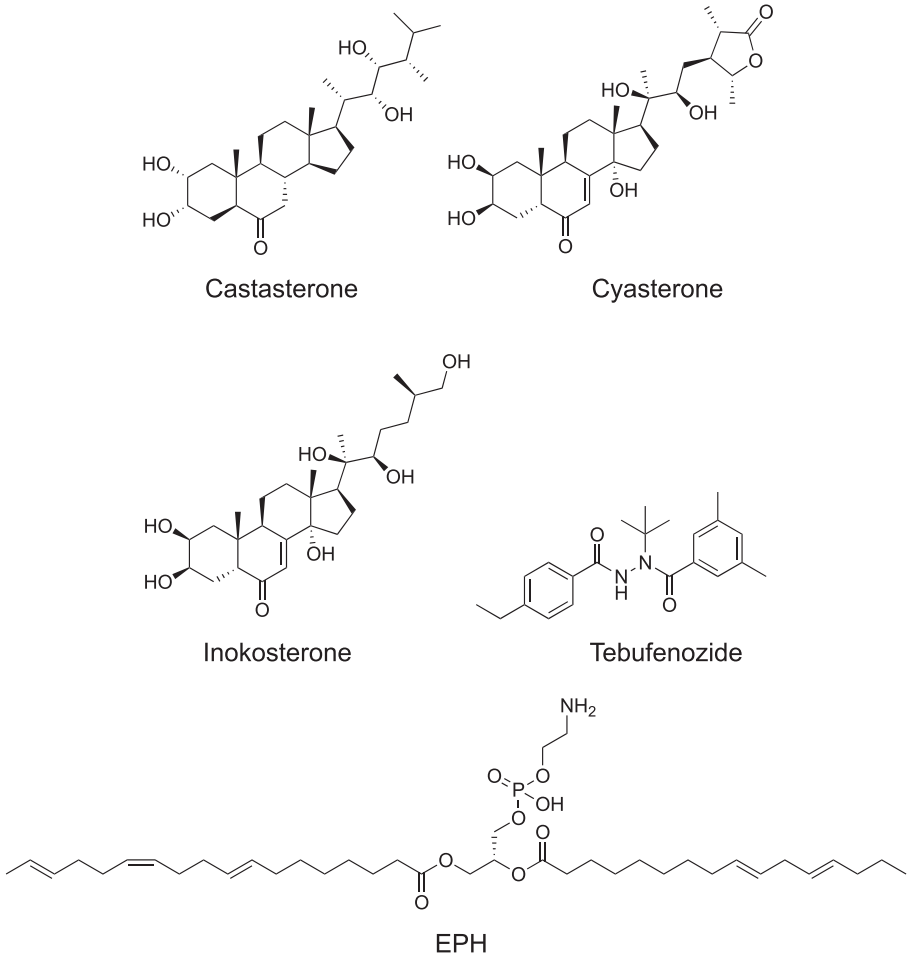


Fig. 11. Natural ecdysteroids and tebufenozide assayed and modelled by Zotti et al. (Zotti et al., 2013). Used with permission.

(l-alpha-phosphatidyl-beta-oleoyl-gamma-palmitoyl-phosphatidylethanolamine) in dipteran Schneider S2 cells of *Drosophila* melanogaster and the lepidopteran Bm5 cells of *Bombyx mori*, important pest insects in medicine and agriculture (Fig. 11) (Zotti et al., 2013).

Castasterone and inokosterone were inactive in agonist assays in both in S2 and Bm5 cells. The commercial dibenzoylhydrazine-based insecticide tebufenozide exhibited 1000-fold selectivity for the lepidopteran over dipteran insects (EC_{50} of 7 nM and 9 pM, respectively). Interestingly, castasterone was a much stronger selective antagonist in S2 cells (IC_{50} of 40 nM) than Bm5 cells (IC_{50} = 18 μM), a 500-fold selectivity ratio for dipterans. To gain more insight in the steroid-EcR interactions, molecular models of the LBD of the EcRs from the dipteran *D. melanogaster* and lepidopteran *B. mori* were generated using the homology modelling tool, MODELLER. Subsequent docking and normal mode analysis calculations suggested that the antagonist activity of castasterone may be due to direct binding to the receptor in a way that alters protein flexibility.

At library of 25 non-azadirachtin neem limonoids were computationally screened for natural plant-based agonist activity by Ydadav and colleagues (Yadav et al., 2015). They used the commercially available insecticide, tebufenozide, as a positive control. Their docking studies for the EcR-ligand binding domain in *Helicoverpa armigera* identified 6 compounds, nimbolide, azadirone, nimolinone, meliacinol, nimbocinol, azadiradione (Fig. 12), as strong binders to the active site of *H. armigera* EcR-LBD. These data could help in the application of natural compounds as alternatives to synthetic agrochemicals in pest management.

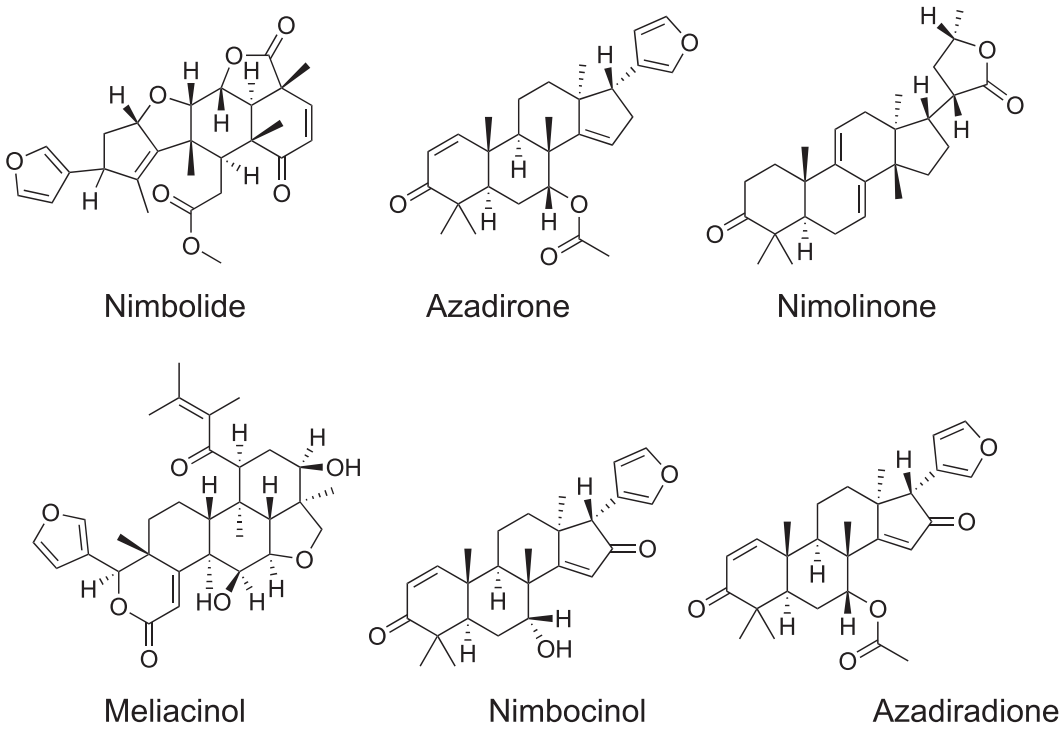


Fig. 12. Six most active neem limonoid agonists for *Helicoverpa armigera* EcR from the study by Ydadav et al. (Yadav et al., 2015).

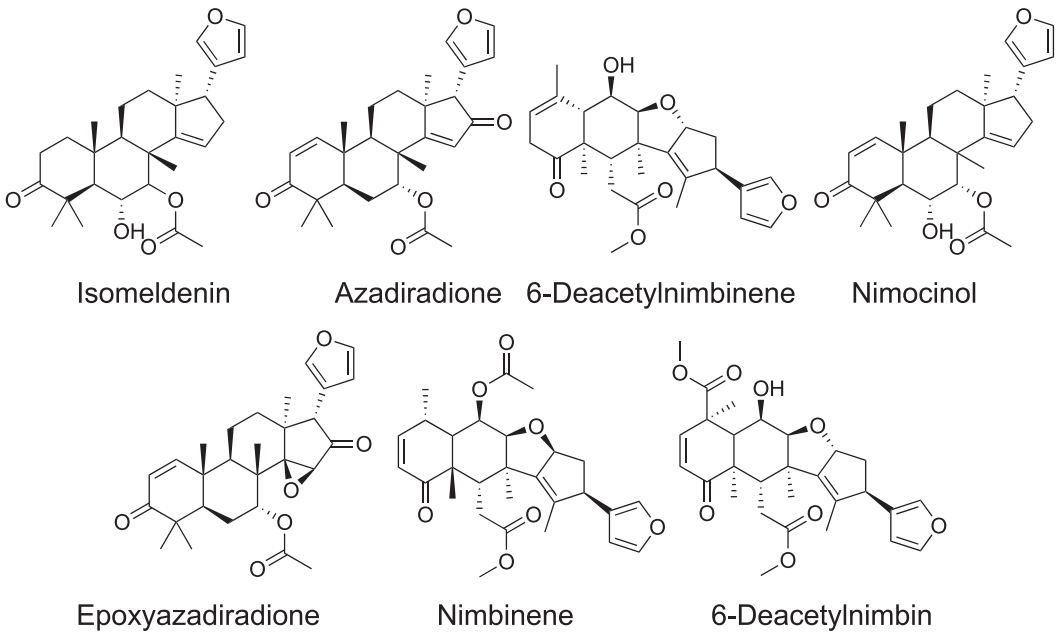


Fig. 13. Phytochemicals from Neem tree showing EcR activity in study by Khanna et al. (Khanna et al., 2023).

Very recently, Khanna et al. reported an in silico docking study of 20 triterpenoids from *Azadiracthta indica* (neem, Fig. 13) with the EcR of the lepidopteran pests *Helicoverpa armigera* (HaEcR) and *Plutella xylostella* (PxEcR) (Khanna et al., 2023). Using AutoDock Vina, 6 – 9 triterpenoids exhibited stronger binding than the DAH insecticide, tebufenozide used as a reference ligand. Four of these triterpenoids – isomeldenin, azadiradione, 6-deacetylnimbinene, and nimocinol – were predicted to bind strongly to the EcR of both insects. Epoxyazadiradione and nimbocinol bound more strongly to the PxEcR while nimbinene and 6-deacetylnimbin docked more effectively with HaEcR. They identified two key amino acid residues, Asn504 of HaEcR and Ser504 of PxEcR, that were critical for forming hydrogen bonds with most lead triterpenoids tested. Most triterpenoids obeyed the Tice rule of five for a successful pesticide (Tice, 2002) except tirucallol, 3-tigloylazadirachtol, and azadirone, despite having stronger EcR binding than the standard. The authors concluded that many of these neem triterpenoids constitute possible ecdysone agonist, or antagonist leads for effective insect control.

Bruceine D is a quassinoid, a type of triterpenoid lactone from *Brucea javanica*, with insecticidal activity. Although it was known to inhibit growth and metamorphosis of *Plutella xylostella* and *Drosophila melanogaster*, its molecular target and mechanism of insecticidal activity was

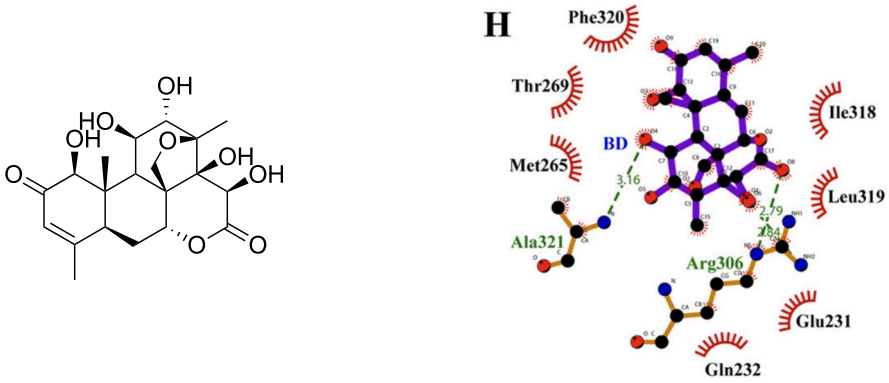


Fig. 14. Structure of bruceine D (left) and its interaction with DmEcR. Adapted with permission from Chen et al. (Chen et al., 2023). Copyright {2023} American Chemical Society.

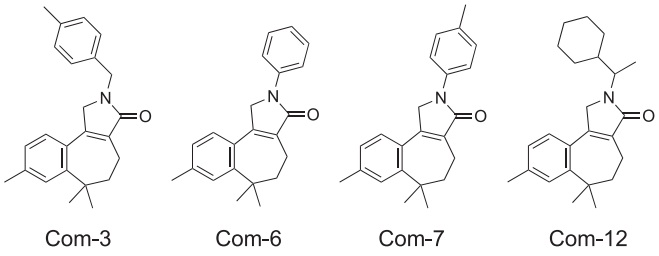


Fig. 15. Structures of modified benzosuberenes based on a cedarwood essential oil natural product lead (Singh et al., 2021).

unknown. Recently, Chen and colleagues studied a range of proteins with high affinity for bruceine (Fig. 14) using surface plasmon resonance and HPLC coupled with MALDI ToF mass spectrometry, identifying the EcR as its main target. In vivo, bruceine D inhibits the expression of 20E response genes and in vitro it suppresses transcriptional activation of EcR by blocking the ecdysone response element-triggered transcriptional cascade. This indicates that it inhibits formation of the 20E-EcR-USP-EcRE complex. Moreover, molecular docking demonstrated that BD bound well to EcR of *P. xylostella* and *D. melanogaster*. Unravelling the mechanism for the insecticidal mechanism of BD will aid development of green pesticides.

In a recent computational study, various synthetic derivatives of natural products containing pyrrolone-fused benzosuberene (PBS) scaffolds (Fig. 15) were screened for potential interactions with Hemiptera and Coleoptera EcRs (Singh et al., 2021). The essential oils from the wood of *Cedrus deodara* (cedarwood) contains himachalenes, on which the analogues were based. Molecular docking results suggested that some derivatives may exhibit strong binding to these EcRs. Subsequent molecular dynamics simulations validated the docking results. Com-7 and Com-3 are potential EcR agonists against Hemiptera and Coleoptera, respectively and Com-8 and Com-12 are putative lead compounds against Coleoptera.

Whitefly (*Bemisia tabaci* Gennadius) is an economically important hemipteran sucking insect cotton pest (Mangat et al., 2022). The BtEcR was the target of a large computational screening study reported by Mangat and coworkers. They retrieved 98,072 natural compounds from the ZINC database and used docking-based virtual screening (AutoDock Vina) to identify compounds that bound strongly to BtEcR. The top lead compounds (ZINC08952607 and ZINC04264850, Fig. 16) were subsequently subjected to molecular dynamics simulation (GROMACS) to generate better estimates of the binding affinities and to analyse the binding free energy of BtEcR-ligand complexes. These natural compounds constitute leads for development of environmentally safe insecticides against the whitefly.

5.2. Early small molecule leads

In 1969, Kenaga et al. reported the effect of L-alanosine (Fig. 17) on ecdysis in insects (Kenaga, 1969). A subsequent study by Matsumoto in1984 confirmed the effect of this simple compound on the common armyworm *Leucania separata*, cabbage armyworm, *Mamestra brassicae* and silkworm, *Bombyx mori*, with IC_{50} values for inhibition of ecdysis of 5–10 ppm (Matsumoto et al., 1984). However, more recent studies have

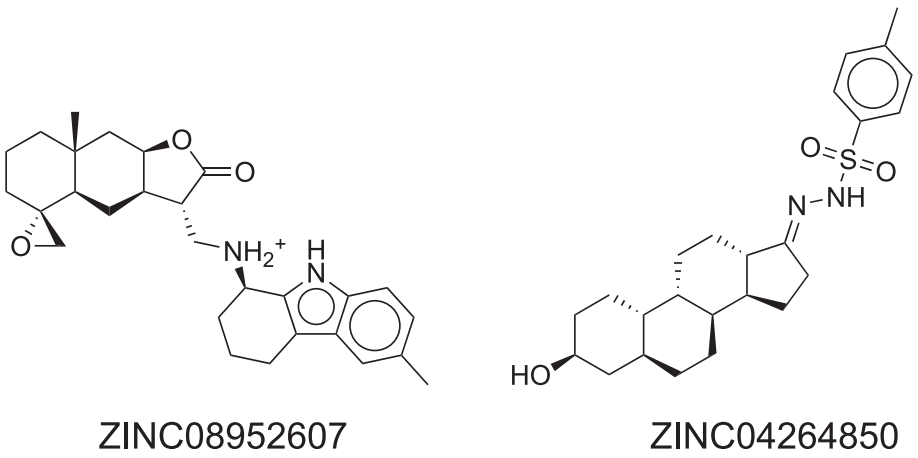


Fig. 16. Structures of top lead compounds from ZINC database screen.

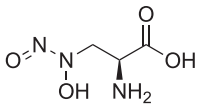


Fig. 17. Structure of L-alanosine.

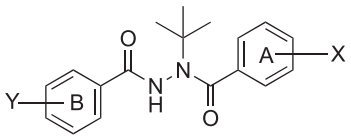


Fig. 18. Generic structure of DAH compounds and ring numbering (A/B). RH-5849: X=Y=H; tebufenozide: X=3,5-dimethyl, Y=4-Et.

suggested that L-alanosine probably inhibits RNA-adenine synthesis and hence the synthesis of the cuticular protein required for sclerotization (Deshpande et al., 1988).

One of the prominent ecdysone research teams, the Hill group at CSIRO, developed a very useful high throughput fluorescence polarization chemical library screen that has led to the discovery of at least one new class of ecdysone receptor ligands, the methylene lactams (vide infra) (Hill et al., 2012). This screening tool is ideally matched to the provision of relatively large datasets of the binding or in vivo effects of chemical libraries, some of which are reported below.

5.3. Bisacylhydrazines/diacylhydrazines (BAH/DAH)

This class of ecdysone receptor ligands (Fig. 18), is by far the most intensively studied, resulting in several commercial insecticides. The first to be commercialised was tebufenozide, discovered in the 1980s at Rohm & Haas. The company later commercialised methoxyfenozide, and halofenozide. Later, other companies commercialised chromafenozide and fufenozide. Tebufenozide is largely effective against lepidopteran larvae, while methoxyfenozide is more potent and effective against a wider range of lepidopteran pests. Chromafenozide is also primarily toxic to lepidopteran larvae and alofenozide is effective against both lepidopteran and coleopteran larvae, especially in soil. DAHs were estimated to account for around 1 % of the US$18.4 billion 2018 global pesticide market.

In the 1990s, Rohm & Haas developed RH-2485, a second-generation ecdysone agonist insecticide. It selectively controlled a range of lepidopterous larvae at low use rates (crop application rates 20 – 300 grams of active ingredient per hectare (ai/ha)) by ingestion, contact, ovicidal, and root-systemic activity). As described above, ecdysone agonists like these synthetic analogues, cause premature moulting that is lethal within days.

The structures of key commercial ecdysone insecticides and candidates in this chemical class are summarized in Fig. 19.

In a very early study, Salgado, 1992 reported that RH- 5849 and other DAHs blocked K+ channels in neurons and muscle, causing neurotoxic activity when injected (Salgado, 1992). RH-5992 (tebufenozide) applied to larvae of the spruce budworm, *Choristoneura fumiferana* (Clem.), induced a lethal precocious incomplete moult (Retnakaran et al., 1997).

A conference paper at the British Crop Protection Conference in 1996 by Le et al. first reported methoxyfenozide, RH-2485 (N'-tert-butyl-N'-(3,5-dimethylbenzoyl)-3-methoxy-2-methylbenzohydrazide), as a second-generation ecdysone agonist insecticide (Le et al., 1996). It exhibited primarily broad control of lepidopterous larvae at low rates. RH-2485 binds rapidly to the EcR, inhibiting larval feeding and causing premature lethal moult. It is also selective towards pollinators, arthropod predators, and insect parasites, and is non-phytotoxic.

Subsequently, Carlson et al. reported the very high affinity binding of methoxyfenozide [N-tert-butyl-N'-(3-methoxy-o-toluoyl)-3,5-xylohyrazine RH-2485] to the EcR of lepidoptera (K_d = 500 pM) (Carlson et al., 2001). Like 20E (20E), it exhibited potent agonist properties across a wide range of pest insects but an excellent safety margin for non-target and beneficial insects.

In one of the earliest series of SAR studies, Nakagawa and colleagues probed the effects of various substituents in the benzoyl (A-ring) moiety closer to the tert-butyl group (the other benzoyl (B-ring) moiety was unsubstituted) against the beet armyworm, *Spodoptera exigua* (Hubner) (see Fig. 18) (Nakagawa et al., 2002). They employed classical QSAR methods to elucidate the physicochemical effects of the A-ring

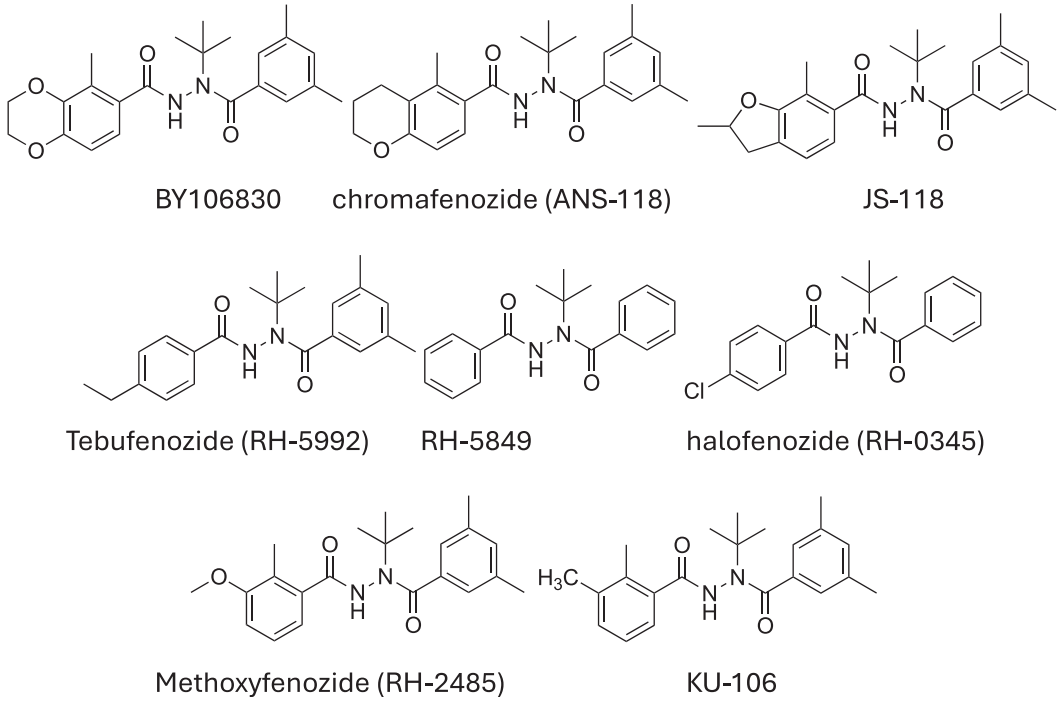


Fig. 19. Structures of diacylhydrazone commercial and candidate insecticides.

substituents on EcR activity (LD_{50} for the insect). Small, hydrophobic substituents at any position improved activity, as did electron-withdrawing substituents at ortho positions. They reported that the effect of substituents in the A-ring moiety on the larvicidal activity for *Spodoptera exigua* was similar that for the rice stem borer, *Chilo suppressalis* (Walker).

In a subsequent study, Minakuchi and colleagues reported QSAR studies on DAHs in which both the A and B rings were substituted by chemical functionality with differing physicochemical properties (Minakuchi et al., 2003). They used the EcR B1 and the ultraspiracle of the lepidopteran, *Chilo suppressalis*, to determine the ligand-binding affinity of the analogues. The seven nonsteroidal ecdysone agonists they studied exhibited a range of pI_{50} values. Those with dimethyl substituents on the A ring (as in tebufenozide and methoxyfenozide) had pI_{50} values equalling or exceeding that for PonA (pI_{50}~9) when the B rings contained alkyl or alkoxy groups. With unsubstituted or chloro substituted A rings, the activity fell by 2–3 orders of magnitude, being close to that of the ecdysteroids 20E, cyasterone, inokosterone, and makisterone A (pI_{50} ~ 6–6.5). An analogue with a 4-CN substituent on the B-ring had the lowest binding affinity. They found an almost perfect correlation between the receptor binding of the ecdysteroids and small molecule agonists and their moulting activity in insects.

Retnakaran et al. reported detailed mechanistic studies of four potent DAH insecticides, RH-5992 (tebufenozide), RH-0345 (halofenozide), RH-2485 (methoxyfenozide), and RH-5849 (Retnakaran et al., 2003). These DAHs bound strongly to EcRs and down-regulated genes necessary for cuticle elaboration, sclerotization, and ecdysis resulting in lethal precocious incomplete moult. Tebufenozide and methoxyfenozide, were lepidopteran-specific while halofenozide acts mainly on coleopteran larvae. Again, these compounds had negligible effect on non-target species.

Beckage and coworkers reported the effects of three DAH insecticides against the mosquitoes *Aedes aegypti, Culex quinquefasciatus* and *Anopheles gambiae* (Beckage et al., 2004). *A. gambiae* was the most susceptible species and *Ae. aegypti* the most resistant. The DAH potency was in the order RH-2485 > RH-5992 > RH-5849 for all species.

Minakuchi and colleagues published two subsequent reports on the insect moulting effects of DAH analogues (Minakuchi, 2005; Minakuchi et al., 2005). For the lepidopteran *Chilo suppressalis*, they found a linear correlation between the EcR-binding activity of the compounds and in vitro moulting and insecticidal activity. The receptor binding activity was essentially unchanged when the USP was exchanged between *C. suppressalis* and *D. melanogaster*. These data supported the hypothesis that the selective toxicity of DAH analogues toward Lepidoptera is due to differences in the EcR structure not that of USP. A related study provided quantitative confirmation that the EcR-binding activity of non-steroidal ecdysone agonists is the main driver of their moulting and insecticidal properties (Minakuchi et al., 2005).

Ogura, and colleagues investigated the effect of substituted DAHs on Sf-9 fall armyworm (*Spodoptera frugiperda)* ovary cells (Ogura et al., 2005). These cells had been previously shown to be a suitable substitute for the purified recombinant EcR protein. The 18 analogues had chlorine at the ortho-position on the benzene ring closest to the t-butyl group (A ring) and the other benzene ring (B ring) contained a range of para substituents. These substituents spanned a range of lipophilicities, electron withdrawing and donating properties, and sizes allowing QSAR models to be generated to predict their biological responses. Lipophilicity alone accounted for >70 % of the variance in the model and this increased to 90 % when electronic effects were included using the Hammett σ constants. More lipophilic substituents favoured activity, as did electron donating substituents (negative s constants)

A very comprehensive study of 172 DAH analogues was reported by Wheelock et al. (Wheelock et al., 2006). These compounds were assessed for their activation of an ecdysone-dependent reporter gene in a silkworm (*Bombyx mori*) cell-based HTS assay. The EC_{50} values were used to train a CoMFA (comparative molecular field analysis, a type of 3-D QSAR) model of structure-activity relationships in the large DAH dataset. Both the A- and B-rings were substituted with functional groups possessing a range of sizes, electronic effects, and lipophilicities. Twenty compounds also explored the effects of replacing the A- or B-rings with other cyclic or acyclic substituents, and 18 probed the effect of various substituents on either or both hydrazine nitrogen atoms. 14 DAH compounds were inactive so were excluded from the model. The resulting equation accounted for 74 % of the activity for 158 compounds (r^2 = 0.74 and q^2 = 0.45), with electrostatic effects accounting for 42 % of the interactions and steric effects 58 %. CoMFA fields were used to visualize the steric and electrostatic potential fields that were favourable and unfavourable for biological activity. Of particular interest was the observation that the hydrophobic parameter (log P) was not necessary for describing the observed activities, although previous studies have cited the importance of hydrophobicity in both classical and 3-D QSAR analyses of these compounds. Modeling studies of the *B. mori* EcR supported the observed physicochemical parameters required for activity reported by the CoMFA models. Comparison of this analysis with those performed using other lepidopteran assay systems identified a high degree of correlation (r^2 = 0.81 for a Sf-9 cell-based assay and r^2 = 0.89 for a *Chilo suppressalis* integument-based assay, suggesting that it is valid to compare the results of the *B. mori* cell-based system to those from previous lepidopteran assays. This novel assay system is amendable to a high-throughput screening format and was reported to be very useful for discovering new EcR agonists.

Bordas et al. reported an interesting hybrid method for interpreting computational receptor docking scores of EcR ligands in terms of 3D molecular field interaction contributions (Bordas et al., 2007). Their method required no experimental biological data (other than the x-ray structure) to map the receptor-binding site. Structures in a virtual library of known DAH ecdysteroid agonist analogues were first docked into the ligand-binding pocket of the crystal structure of the moth *Heliothis virescens* EcR using the FlexX software. The superimposed molecular alignment from the docking experiments were used to train a 3D molecular field-based model using CoMFA and the related technique, CoMSIA (Comparative Molecular Similarity Indices Analysis). The CoMFA and CoMSIA models could accurately recapitulate the docking scores of the ligands, elucidating the contributions of steric, electrostatic, hydrophobic, and hydrogen bond donor and acceptor molecular interactions. This study mapped hitherto unexplored regions of the receptor ligand binding cavity and identified important receptor-ligand interactions useful for design of new, more potent DAH-type EcR ligands.

Classical statistical QSAR methods used to analyse structure-activity data for DAH analogues were reviewed by Fujita and Nakagawa (Fujita and Nakagawa, 2007). Fujita, with Hansch, was one of the originators of the QSAR method. They synthesized a suite of analogues substituted in one or both benzene rings and measured their larvicidal activity in *Chilo suppressalis*. EcR binding affinity was also assessed in Sf-9 cell lines and using receptor proteins expressed in a cell-free preparation of integumentary tissue of *C. suppressalis*. Their QSAR models found correlations between these two activities and physicochemical molecular and substituent parameters. They compared the QSAR models with EcR X-ray structures to understand the importance of physicochemical parameters in terms of molecular mechanism of the analogues.

Zhao et al. designed several chromanone and chromone analogues of DAH derivatives substituted with methyl groups or halogens on the chromone or benzene rings (Fig. 20) (Zhao et al., 2007). They reported their insecticidal activity against *Aphis medicagini (A. medicagini), Nilaparvata legen (N. legen), Mythima separata (M. separata), and Tetranychus cinnabarinus (T. cinnabarinus) at 500 or 250* ppm. *M. separata* was the most susceptible, with most analogues showing high kill percentages. For *T. cinnabarinus,* only the dimethyl substituted chromanone analogues (1) with small ortho substituents exhibited useful insecticidal activity, with the other two insect species displaying negligible susceptibility.

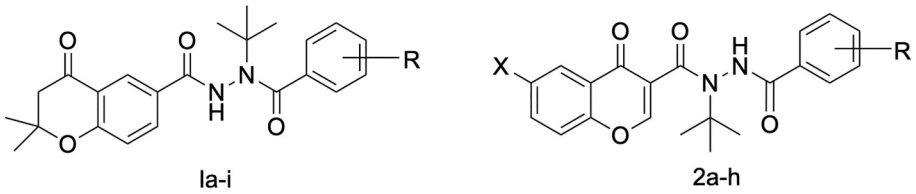


Fig. 20. Structures of chromanone(1) and chromone (2) DAH analogues (Zhao et al., 2007).

CoMFA was also used to model the $logLD_{50}$ (pLD_{50}) values of larvae of the Beet Armyworm (*Spodoptera exigua*), the rice stem borer (*Chilo suppressalis*), and the Colorado Potato Beetle (*Leptinotarsa decemlineata*) treated with 77 DAH analogues (Hormann et al., 2008). Hormann and colleagues trained 3D QSAR models for each type of insect toxicity. The dataset was divided into 67 compounds used to train the models and 10 molecules in a test set used to assess the model predictivities. The best models exhibited training set r^2 values of 0.77 – 0.88 and test set r^2 values of 0.54 - 0.72. They attempted to rationalize the structure-activity relationships for each type of insect toxicity in terms of EcR LBD sequence alignments and residue contacts based on the known *H. virescens* EcR LBD holoreceptor structure. Fujita and Nakagawa subsequently reported QSAR studies on DAH analogues with substituted benzene rings (Fujita and Nakagawa, 2009) using larvicidal activity against *C. suppressalis* as the activity measure. EcR binding affinity was assessed in intact Sf-9 cell lines using receptor cloned from a cell-free preparation of integumentary tissue of *C. suppressalis*. Models mapping the in vivo and in vitro biological activities to physicochemical molecular and substituent parameters were developed using classical QSAR methods. As with the previous study by Hormann et al., they also used 3D QSAR methods (CoMFA) to quantitatively model the receptor response using cultured cells of *Bombyx mori*. By comparing the molecular property field maps from the CoMFA study with X-ray co-crystal structure of EcR bound to ligands, they could interpret the physicochemical meaning of the relevant features. This helped elucidate the molecular mechanism of moulting hormonal action.

Amor and coworkers explored the effects of DAH derivatives on beneficial insects such as *Orius laevigatus* (Fieber), a predator for thrips, aphids, mites, whiteflies, moths and insect eggs (Amor et al., 2012). Their in vivo toxicity assays showed that several DAH-based insecticides had negligible effect against *O. laevigatus*. Using a homology protein model, they studied the LBD of *O. laevigatus* EcR. Docking studies confirmed that 20E could bind to the LBD but a steric clash due to a restricted ligand-binding cavity of the EcR prevented binding of DAH analogues.

Bengochea, and colleagues investigated the use of EcR ligands as control agents for the economically important pest, olive fruit fly, *Bactrocera oleae* (Rossi) (Bengochea et al., 2013). They measured the effects of contact and oral exposure of *B. oleae* adults to the DAH compounds methoxyfenozide, tebufenozide and RH-5849. Only RH-5849 was active, killing 98–100 % of the treated insects 15 days after treatment. The researchers generated a homology model of the EcR LBD of this pest using MODELLER. Using molecular docking to this structure, they reported that RH-5849 could bind well to the LBD, but the restricted binding cavity meant that methoxyfenozide and tebufenozide had severe steric clashes with part of the binding pocket.

In an analogous study by Morou and colleagues, the toxicities and binding strengths of three known and one novel DAH insecticides against larvae of *Anopheles gambiae were determined* (Morou et al., 2013). This insect is the major vector for human malaria. The three commercial DAHs studied were tebufenozide, methoxyfenozide, and halofenozide, and the experimental DAH was KU-106. As with the previous study, they generated a homology model of the *A. gambiae* EcR LBD using MODELLER and used molecule docking to study how ligands interacted with the receptor. The structurally related KU-106 exhibited similar activity against the larvae similar as the commercial compounds.

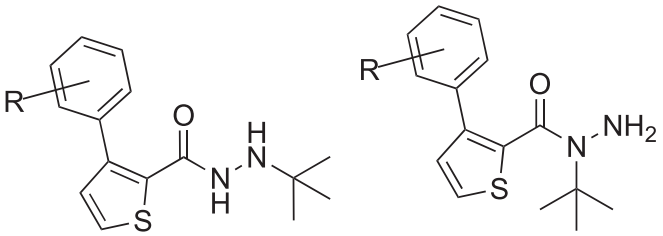


Fig. 21. General structures of novel thenoylhydrazide compounds synthesized by Song et al. (Song et al., 2016).

Song et al. synthesized pairs of chemical isomeric N-tert-butylphenyl thenoylhydrazide compounds (Fig. 21). Some of the chemotypes had excellent larvicidal activity against *Culex pipiens pallens*. Two of these compounds were found to be ecdysteroid agonists in an *Spodoptera frugiperda* (Sf9) reporter gene cell line assay. Molecular docking of the most potent compound elucidated the structure-activity relationships in this class of molecules and provided a rationale for development of new agents for the control of mosquitoes.

Hu, et al. reported a comprehensive computational study of a series of previously-reported DAH and ecdysteroid ligands (Hu et al., 2017). This study employed homology models, molecular docking, molecular dynamics (MD) simulation, binding free energy calculation, and per-residue binding free energy decomposition in an attempt to understand the different binding mechanisms of two types of ecdysone agonists. They reported that docking scores were sufficient to accurately rank the potencies of the DAHs, but MM/PBSA (molecular mechanics Poisson-Boltzmann surface area) calculations were required to rank the steroidal ecdysone agonists. To also reported the relative importance of key residues involved in ecdysone agonist binding. Hu and coworkers also reported a comprehensive steered molecular dynamics study of the entry and exit of ligands to the LBD of the EcR (Hu et al., 2018a). Using the selective ecdysone agonist, BYI06830, they showed that the ligand dissociated from the LBD by four different pathways. Using the potential of mean force (PMF) for the four pathways, path 2 appeared to be the most likely exit path for BYI06830, elucidating the unbinding mechanism of ecdysone agonists from EcR LBD.

Guo et al reported a series of 25 novel 4,5,6,7-tetrahydro-2H-indazole hydrazide derivatives as insecticides (Fig. 22) (Guo et al., 2022). A docking study revealed that all target compounds could potentially bind well to the EcR with many compounds adopting a similar binding pose with to that of tebufenozide. Some compounds displayed

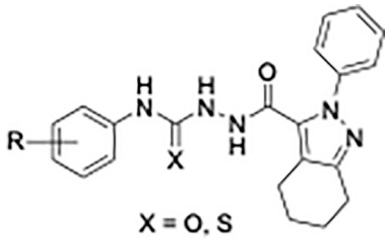


Fig. 22. General structure of tetrahydro-2H-indazole hydrazide derivatives (Guo et al., 2022).

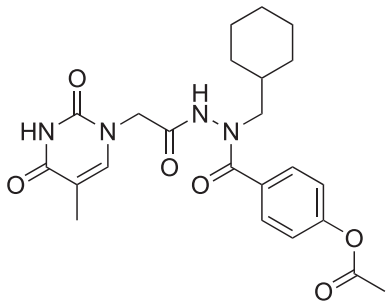


Fig. 23. Structures of most active novel thymine DAH analogue 2h synthesized by Liu et al. (Liu et al., 2013).

insecticidal activity against *Plutella xylostella* at 500 ppm, but activities were inferior to tebufenozide and the analogous cyclohepta[c]pyrazole hydrazide.

Thymine was recently reported to be an ecdysteroid agonist, prompting Liu et al. to synthesize a series of 1,4-disubstituted DAH derivatives with a thymine moiety (Liu et al., 2013). The activities of these compounds against *Spodoptera litura* (Fabricius) were evaluated. The cyclohexylmethyl DAH 2h (Fig. 23) was the most potent agonist, with a median lethal concentration of 23 ppm. It was further demonstrated to be an ecdysteroid agonist using the Sf9 reporter gene assay. A molecular docking study indicated that hydrophobic interactions and the formation of hydrogen bonds between the compounds and the ecdysone receptor play critical roles in promoting the binding affinity of the compound. The authors suggested that compound 2h may serve as a useful template for development of new EcR agonists containing the thymine moiety.

5.4. Arylamides and isosteres

One of the first chemical screening studies was reported by Mikitani, (Mikitani, 1996) who assessed a number of chemicals for their abilities to compete with EcR binding of ^{3}H-PonA. The most effective compound, the EcR agonist 3, 5-di-tert-butyl-4-hydroxy-N-isobutyl-benzamide (DTBHIB, Fig. 24), was discovered using an automated EcR binding assay and an ecdysteroid responsive gene expression assay. Its EC_{50} in the gene expression assay was 2 to 3-fold higher than that of a-ecdysone. DTBHIB showed similar EC_{50} values to RH5849 in molecular and cellular ecdysteroid assays. Interesting the N-isopropyl analogues of DTBHIB did bind the receptor or induce the responsive gene.

Recently, another virtual screening study for identifying novel ligands that bind to EcR was reported by Hu and colleagues (Hu et al., 2018b). Combining pharmacophore models and molecular docking reduced the initial compound candidate pool to the most likely active compounds. The computational screens identified 9 hits that were confirmed by an *in vitro* reporter gene assay. The hit compounds were largely bisarylamides (or isosteres) (Fig. 25). None exhibited agonist activity at 100 μM but two, VS-006 and VS-009, inhibited 20E signalling in an antagonist assay. VS-009 was a novel type of DAH that lacked the traditional hydrazone *N*-t-Bu or similar substituent.

5.5. Methylene lactams

Using high-throughput screening, researchers at CSIRO Australia discovered and patented a new class of compounds, the γ-methylene γ-lactams (Fig. 26), which bind to insect EcRs with high affinity (Birru et al., 2010). They found that the size and shape of the binding pocket in the EcR subunit changes markedly depending on the nature of the ligand bound to it, making computational methods like docking challenging. However, it was demonstrated that it is possible to generate excellent 3D QSAR models for a given type of ligand, suggesting the existence of a relatively restricted number of binding site configurations and shapes.

These compounds represent a new class of high-affinity ligands for ecdysone receptors. A library of 113 methylene lactams probed the effects of a diverse range of substituents on the rings. Most data were obtained for *Bovicola ovis* (Phthiraptera) EcR, where multiple analogues exhibited low μM IC_{50} activity, the best being 2 μM, comparable with PonA and 10x more potent than 20E. Data for *Lucilia cuprina* (Diptera) binding activity were less numerous. One analogue exhibited a whole insect LD_{50} value of 26 ppm for *B. Ovis*. A subsequent X-ray crystallographic study of PonA and the methylene lactams showed that the ligand-binding domains of both subunits of the EcR exhibit significant conformational flexibility when binding to different ligand chemotypes (Ren et al., 2014).

5.6. Tetrahydroquinolines

Smith and colleagues reported the ability of a series of 1-aroyl-4-(arylamino)-1,2,3,4-tetrahydroquinolines (THQ) derivatives act as EcR agonists,(Smith et al., 2003) leveraging data from earlier unpublished conference papers. A 35-compound library was screened at 33 μM in a cell line expressing EcR from *Aedes aegypti* and a luciferase gene under the control of an ecdysone response element. They employed Me and F substituents on the aniline and tetrahydroquinoline rings, with the

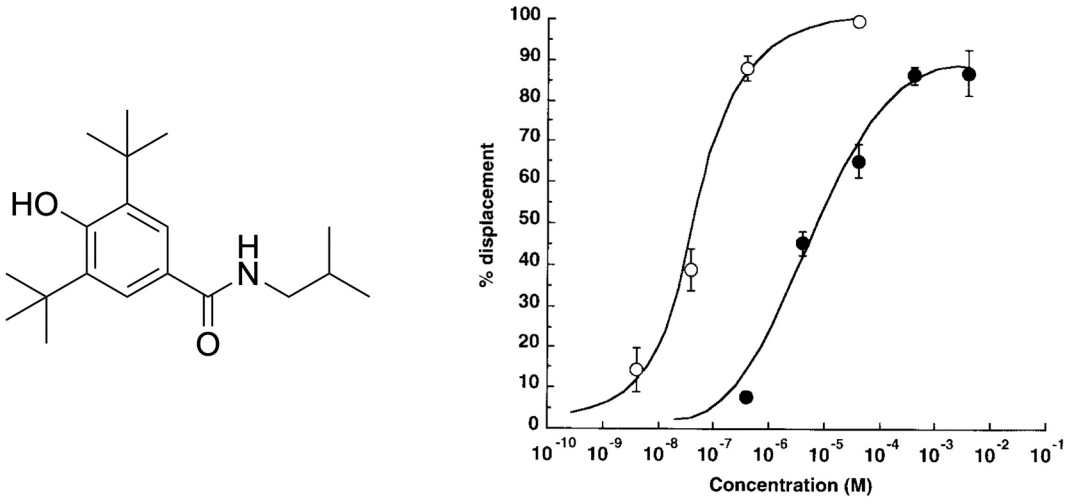


Fig. 24. Structure of DTBHIB (left) and displacement of ^{3}H-PonA binding to Kc cell cytosol by DTBHIB (black) and 20E (white).Used with permission from Mikitani (Mikitani, 1996).

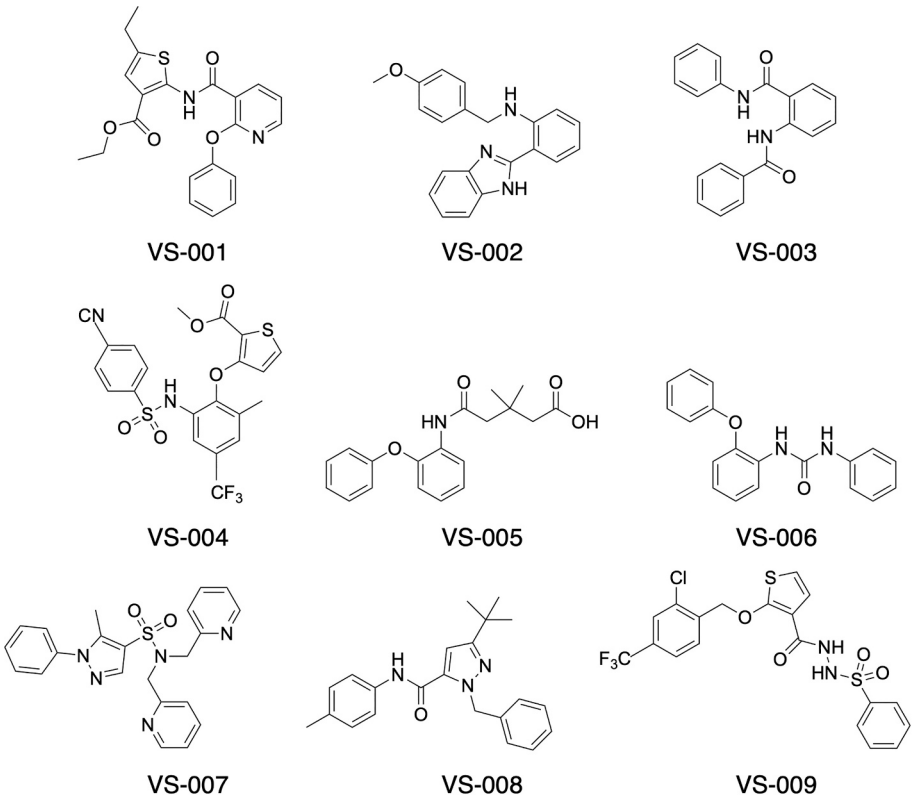


Fig. 25. Arylamide ecdysone agonists (Hu et al., 2018b).

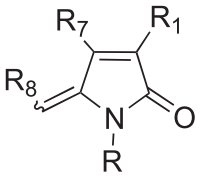


Fig. 26. General structure of the methylene lactams. The most potent compounds had R=substituted phenyl, R^1=CN, R^7=small alkyl, and R^8=H.

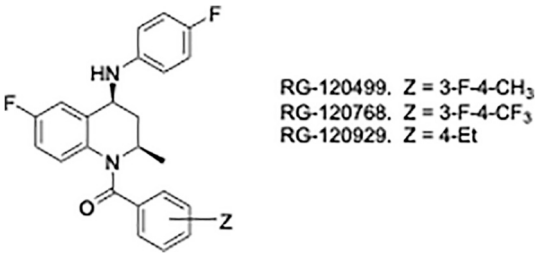


Fig. 27. Structures of THQ EcR agonists (Kumar et al., 2004).

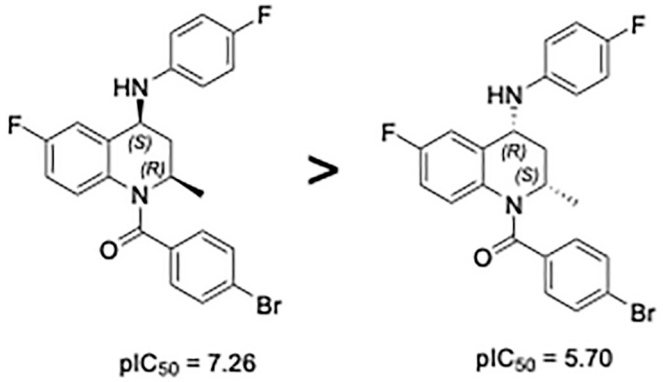


Fig. 28. Stereospecific activity of the THQ EcR agonists and pIC_{50} values for in a Asian tiger mosquito (*Aedes albopictus*) cell line (Yokoi et al., 2019).

difluoro analogues exhibiting the best performance (Fig. 27). A larger variety of substituents on the carboxamide phenyl ring were synthesized, the best of which exhibited EC_{50} values for transactivation of a luciferase gene under the control of an ecdysone response element of ≤ 1μM compared with 0.44 μM for DAHs. Kumar, et al. subsequently studied these THQs as ecdysone agonists. They determined their efficacy relative to the DAHs in several mutants of *Choristoneura fumiferana,* using a reporter assay. Introducing a second A110P mutation, the cause of ecdysteroid insensitivity, in a C57BL/6 mouse model coactivator interaction assay and in insect cells, showed that the mutant EcR was activated by THQ but not ecdysones or DAHs. They also reported data on the flexibility of the EcR binding site, with a single amino acid being sufficient to control the discrimination for two ligand chemotypes.

The absolute configurations of the lead THQ compounds had not been resolved in the earlier studies. Consequently, Kitamura and colleagues resolved the four enantiomers and tested them against mosquito larvae and in a competitive EcR binding assay. The (2R,4S) enantiomer exhibited 55 times higher activity against mosquito larvae and was 36-fold more active in the binding assay compared to the (2S,4R) enantiomer (Fig. 28). Similar results were subsequently reported by Yokoi, Nakagawa, and Miyagawa, who also generated a library of 40 THQ enantiopure analogues having the most potent stereochemical configurations around the 2- and 4-carbons (Fig. 28) (Yokoi et al., 2019). They also generated a QSAR model for the agonists that helped elucidate the physicochemical requirements for potent EcR agonist activity (Fig. 29). The most potent THQ derivative exhibited twice that of the moulting hormone 20E, and between 5–10 times the activity of the DAH

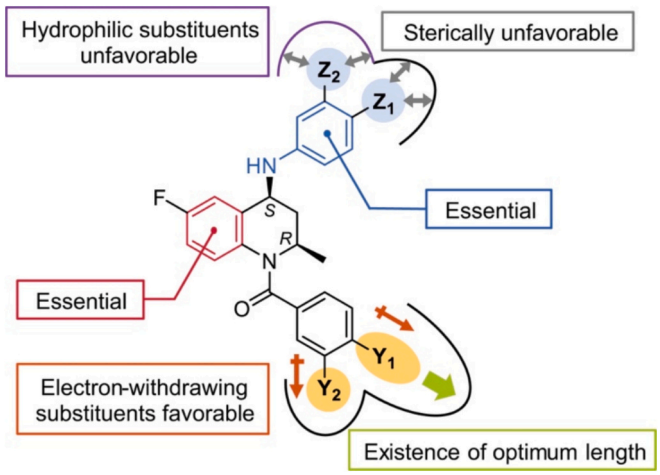


Fig. 29. Structural requirements for potent THQ binding to the *Aedes albopictus* EcR. Used with permission from Yokoi et al. (Yokoi et al., 2019).

compounds methoxyfenozide and tebufenozide respectively.

Recently, Savchenko et al. reported a study on conformationally constrained enantiopure 4-substituted-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolines **7–10** (Fig. 30) (Savchenko et al., 2023). Assays against the initial stage of spring ontogeny in *Musca domestica* showed an acceleration of reproductive maturation. Computational docking of these ligands into the binding site of the *Heliothis virescens* EcR showed good predicted binding affinities and docking poses coincident with that of PonA.

5.7. Acylamino ketones

This chemotype is essentially an isostere of the DAH compounds, with a substituted methylene carbon atom replacing one of the hydrazine nitrogen atoms. Tice et al generated libraries of novel α-acylaminoketones as potential gene switches based on potent DAH ecdysone agonists (Fig. 31) (Tice et al., 2003a). The compounds were assessed in mammalian cells expressing the EcR from *Bombyx mori* for their ability to promote expression of a reporter gene downstream of an ecdysone response element. The most potent α-acylaminoketones were comparable to the DAH, GS-E in this assay.

In a subsequent study, these researchers synthesized a more focused library of 15 novel α-acylaminoketones to optimize the potency of these leads as EcR agonists (Tice et al., 2003b). The compounds were again assayed in mammalian cells expressing the ecdysone receptors from *Bombyx mori* and also *Choristoneura fumiferana* for their ability to cause expression of a reporter gene downstream of an ecdysone response element. The most potent α-acylaminoketone (Fig. 32) with a spiro cyclohexyl ring had an activity comparable to DAHs in the *C. fumiferana* assay, with larger and smaller cycloalkyl rings showing lower activity.

5.8. Imidazo- and pyrazole carboxamides and pyrazole amides

Deng and colleagues were among the first to report the EcR activities of this class of compounds (Deng et al., 2016b). By analysing conformational preferences of two EcR ligands, VS008 (a pyrazole carboxamide identified in an earlier virtual screen) and the DAH BYI06830 bound to the active site of the EcR from crystallographic studies, they designed new pyrazole amide derivatives. A bioassay revealed that two derivatives, (R=3-OMe) and (R=4-t-Bu) (Fig. 33), exhibited good activity against *Helicoverpa armigera* at low concentration, with similar activity against *Mythimna separata* and *Pyrausta nubilalis*. These compounds exhibited the same poisoning symptoms as tebufenozide. They also used molecular docking and MD to study the binding conformation of the two pyrazole amide compounds and tebufenozide in the active site pocket of the EcR subunit. The binding modes of the pyrazole amides were similar to that of tebufenozide. The effects of different substituents on the pyrazole ring were also reported by the same group (Deng et al., 2016a).

Hu et al. subsequently reported an *in silico/in vitro* screening procedure for identifying new EcR ligands (Hu et al., 2021). This procedure

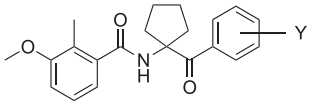


Fig. 31. (a) The active gene expression promoter acylaminoketones synthesized by Tice et al. (Tice et al., 2003a).

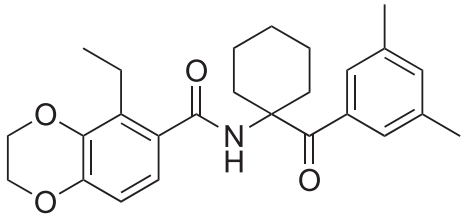


Fig. 32. The most potent acylaminoketone in the EcR reporter assays described by Tice et al. (Tice et al., 2003b).

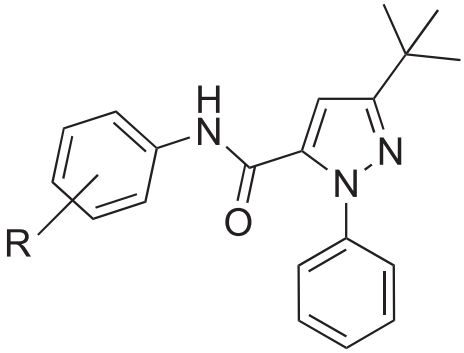


Fig. 33. Structures of pyrazole carboxamides synthesised by Deng et al. (Deng et al., 2016b).

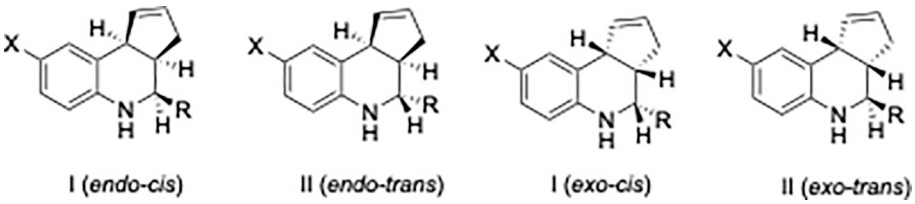


Fig. 30. Structures of the most potent polycyclic THQ analogues reported by Savchenko et al. R=CO_2Et, X=F (**7**); R= 4-CF_3 phenyl, X=F (**8**); R=3-Cl phenyl, X=F (**9**); R=2-F phenyl, X=H (**10**) (Savchenko et al., 2023).

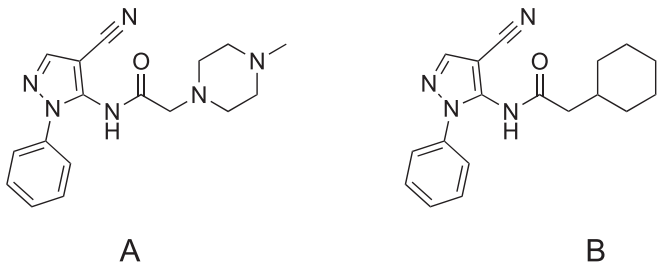


Fig. 34. Structure of lead pyrazole amide (A) and most active analogue (B) reported by Hu et al. (Hu et al., 2021).

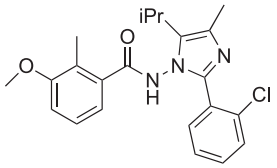


Fig. 35. Most active, novel (A) imidazole- and (B) thiadiazoloimidazole-type ligands reported by Holmwood and Schindler (Holmwood and Schindler, 2009).

uses a 3D pharmacophore model, molecular docking, and a MM/PBSA rescoring method to virtually screen a library of >200,000 chemicals. They identified a novel piperidine-substituted pyrazole amide hit with good binding activity against the *Plutella xylostella* EcR (Fig. 34A). Docking and MD simulations elucidated the binding interactions of the pyrazole amide moiety and identified a hydrophobic binding pocket accommodation the piperazine ring. Subsequent synthesis and testing of analogues with other hydrophobic functionality in place of the piperazine identified compounds with IC_{50} values against *Plutella xylostella* EcR ranging from 0.64 to 23.21 μM (Hu et al., 2021). The cyclohexyl analogues (Fig. 34B) had the lowest IC_{50}, similar to that of RH-5992.

The success of 2D and 3D QSAR methods in generating predictive, quantitative models of EcR ligand activity prompted Holmwood and Schindler to review structural biology and molecular field-based quantitative models of the relationships between small molecule EcR ligands and biological responses they invoke (Holmwood and Schindler, 2009). The review attempted to provide potential pathways for overcoming the impact of the extreme EcR binding site plasticity, which complicates studies using x-ray structures, by augmenting this information with ligand-based information. By studying how EcR LBD changes as different small molecule or steroidal ligands bind to it, they exploited this information to generate new ligand chemotypes. Given the broad binding capabilities of the steroidal ligands and the relatively narrow species specificities of small organic ligands, they aimed to design new molecules that occupied the binding site in a similar way to the steroidal ligands. The insights gained from this approach resulted in the development novel imidazole-type ligands with high binding affinities and moderate insecticidal activities. The best imidazole (Fig. 35) was equipotent with 20E (100 nM) in an EcR induction assay.

These analogues provided complete control of fall armyworm, *Spodoptera frugiperda*, and *Phaedon cochleariae* at at 1000 ppm, with actual LD_{50} values likely much lower. Likewise, two-spotted spider mite, *Tetranychus urticae*, was completely controlled at 100 ppm and mosquito, *A. aegypti*, larvae at 17 ppm, suggestive of a somewhat broader spectrum of activity than solely lepidopterans.

Guo and coworkers recently reported elaboration of the pyrazolamide chemotype into dual-target insecticide leads that interact with both the EcR and chitinases, another enzyme family important for the moulting stage of insect pests (Guo et al., 2023). As the OfChtI, OfChtII, and OfChi-h isoforms of the *Ostrinia furnacalis* chitinase (OfChtI) has similar substrate-binding clefts and highly conserved catalytic residues, they designed 24 hexacyclic pyrazolamide derivatives containing small flexible linkers, (similar to those previously discovered) to target both the EcR and the OfChtIs (Fig. 36). Several compounds showed activities against *Plutella xylostella*, *Spodoptera frugiperda*, and *Ostrinia furnacalis* larvae. Their studies showed that insertion of an additional methylene spacer between the substituted phenyl ring and the amide bond improved insecticidal activity. The most potent compound, I-17 (R=4-CF_3) exhibited very good insecticidal activities against *P. xylostella* (LC_{50}, 93 mg/L), *O. furnacalis* (LC_{50}, 115 mg/L), and *S. frugiperda* (86 % mortality at 500 mg/L), better than that of 6j but 4–6 times weaker than the single DAH target insecticide tebufenozide.

Guo et al. very recently followed up this work on dual-target insecticides, Again, based on the lead compound 6j (Fig. 36), they designed and synthesized 19 novel derivatives (Guo et al., 2024). Here, an acetamido moiety was incorporated into the amide bridge based on the flexibility of the binding cavities EcR and three chitinases. Insecticidal activities against three lepidopteran pests *P. xylostella*, *O. furnacalis*, and *S. frugiperda* were assessed, showing that most derivatives had moderate to good larvicidal activities. Compound I-17

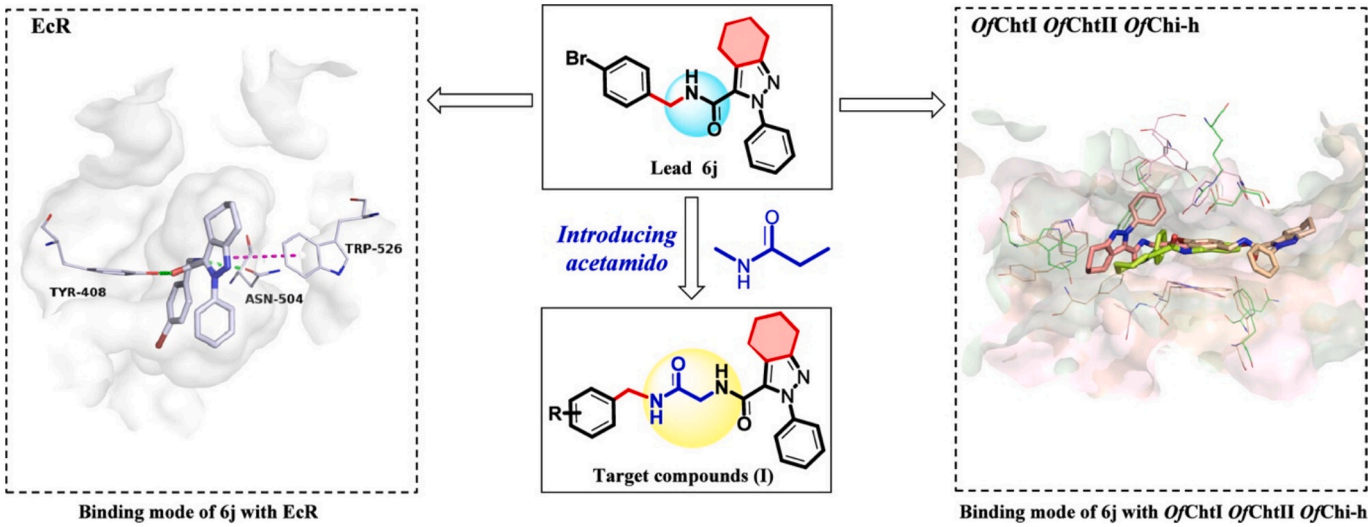


Fig. 36. Binding mode of multitarget lead 6j to EcR and OfChtI, OfChtII, and OfChi-h chitinases active sites, and strategy for designing novel multitarget ligands. Reprinted with permission from Guo et al. (Guo et al., 2023). Copyright 2023 American Chemical Society.

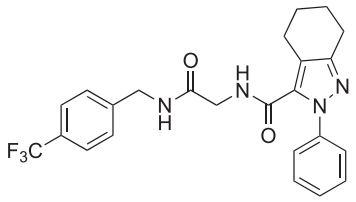


Fig. 37. Structure of most active lead from Guo et al. (Guo et al., 2024).

(Fig. 37) displayed excellent insecticidal activities against *P. xylostella* (LC_{50}, 93 mg/L), *O. furnacalis* (LC_{50}, 115 mg/L), and *S. frugiperda* (86 % mortality at 500 mg/L), significantly better than the lead compound 6j. Subsequent protein validation and molecular docking showed that it bound to EcR (18 % binding at 8 mg/L), OfChtI (69 % binding at 50 μM), OfChtII (72 % binding at 50 μM), and OfChi-h (74 % binding at 50 μM), disclosing its potential as a multi-target insecticide lead.

Similarly, Jiang and co-workers used computational methods to design 27 new analogues of the lead compound N-(4-(tert-butyl) phenyl)-2-phenyl-2,4,5,6,7,8-hexahydrocyclohepta[c]pyrazole-5-carboxamide via scaffold hopping strategy (Fig. 38) (Jiang et al., 2020a). Their bioassay showed that III-27 (N-(2-methylphenethyl)-1-phenyl-1,4,5,6,7,8-hexahydrocyclohepta[c]pyrazole-5-carboxamide, Fig. 37B) had excellent activity against *Plutella xylostella*. Subsequent experiments and docking studies confirmed that it acted on both the ecdysone receptor (EcR) and *Ostrinia furnacalis* chitinase. These researchers subsequently reported studies of the most potent hits from the prior work, focusing on pyrazole carboxamides with no hydrocarbon linker between the amide nitrogen atom and the phenyl ring (n=0 In Fig. 37A) (Jiang et al., 2020b). As in their previous study, they leveraged the structure-activity relationship and molecular docking results of the lead compound 6i reported by Deng et al (Fig. 33). By replacing the t-butyl group on the pyrazole ring with a cycloheptane they enhanced the hydrophobicity in this region, as suggested by the docking studies. They synthesized another set of heptacyclic pyrazolamide derivatives with different, largely lipophilic substituents on the phenyl ring. The bioassay results showed that most analogues exhibited weak insecticidal activity. The 4-t-Bu analogue (Fig. 37C) displayed best activities against *Plutella xylostella* (LC_{50}, 52 ppm) and *Mythimna separata* (100 % mortality at 2.5 ppm). As with their prior work, this analogue works on both EcR and the *Ostrinia furnacalis* chitinase as suggested by molecular docking and MD studies on the lead compound's binding mechanisms.

5.9. Oxadiazolines and N-aminoimidazoles

These classes of EcR ligands were designed to exploit the tendency of the substituted amide in tebufenozide to adopt a cis conformation with respect to t-butyl group and the neighbouring carbonyl group. By conformationally constraining these atoms by ring formation, the resulting novel isostere may retain EcR affinity. In chemical terms, the oxadiazole C=N is isosteric for one of the DAH carbonyl groups, while the tertiary carbon on the oxadiazole ring is an isostere of the t-Bu group present in the most potent DAHs.

A series of patents in the early 2000s disclosed the efficacy of oxadazolines as modulators of the EcR (Fig. 39) (Robert Eugene Hormann et al., 2007; Hormann et al., 2004). The most potent oxadiazolines exhibited low nM EC_{50} values against *C. fumiferana* EcR and LC_{50} values as low as 5 ppm across a range of insects such as beet armyworm and cabbage looper control and ~150 ppm for two-spotted spider mite.

5.10. Imidazothiadiazoles

As well as designing aminoimidazole EcR ligands as discussed in section 5.7, Holmwood and Schindler also developed several imidazothiadiazoles (ITDs) as EcR ligands (Fig. 40) with high binding affinities and moderate insecticidal activities. Although their best imidazole (Fig. 35A) was only equipotent with 20E (100 nM), the most active imidazothiadiazoles (Fig. 35B) was 50-fold more potent than 20E in their EcR induction assay.

Yokoi et al. reported several studies of imidazothiadiazoles as ecdysone receptor agonist insecticides. in which they generated QSAR models of larvicidal activity against various insects and cell lines (Yokoi et al., 2015; Yokoi et al., 2017). Their first study used competitive binding assays in lepidopteran Sf-9 cells to determine the affinity of compounds (IC_{50} for inhibition of the binding of [^{3}H] PonA). They also assessed the larvicidal activity of the ITDs against the Oriental leafworm, *Spodoptera litura*. This initial study involved only 8 analogues, so the quality and predictive range of the QSAR model was very limited. As

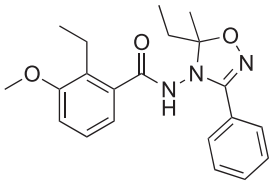


Fig. 39. Structure of lead oxadiazoline from US7,304,162B2.

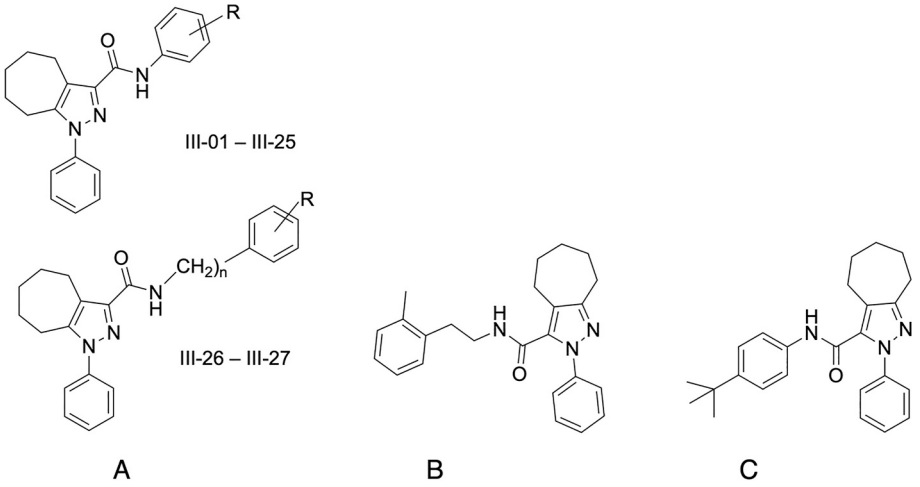


Fig. 38. Structures of pyrazole carboxamides EcR ligands (A) and the most potent lead analogue (B) and the optimized compound with highest activity (C) (Jiang et al., 2020a, 2020b).

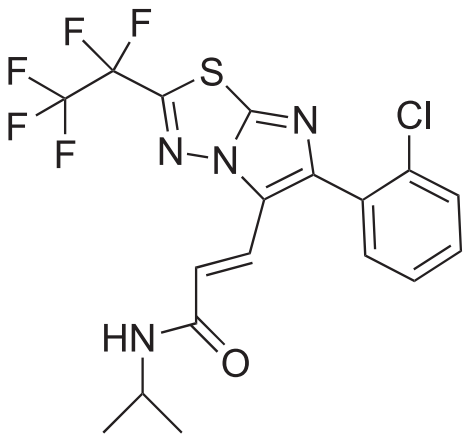


Fig. 40. Most active, novel imidazothiadiazole-type ligand reported by Holmwood and Schindler (Holmwood and Schindler, 2009).

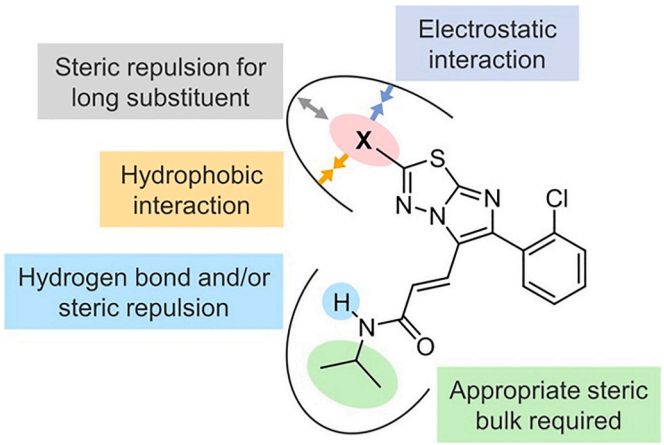


Fig. 41. Putative binding model for the ITDs against lepidopteran Sf-9 cells. Used with permission from Yokoi et al. (Yokoi et al., 2017).

with DAHs, ITDs were highly specific to lepidopteran EcRs.

They subsequently increased the number of analogues with 3-substituents to fifteen and developed a more robust QSAR model. The analogues had pIC_{50} values affinities between 4.76 and 8.36 (M) and pLD_{50} for larvicidal activities of <4 to 5.2 (mmol/larva). The poor solubility of some of the analogues and likely metabolic and pharmacokinetic effects precluded the development of a QSAR model for larvicidal activity. However, they generated a robust, linear QSAR model with lipophilic, electronic, and steric parameters for the Sf-9 cell binding. The model could predict binding of the training set compounds with an R^2 of 0.97 and a standard error of 0.35 logs. No test set was used to validate the predictivity of the model, somewhat compromising the validity of the model. The models showed that the lipophilicity of the 3-substituent was a major driver of binding affinity, with electron withdrawing and steric effects playing a smaller, but significant role. They reported a putative binding model for the ITDs (Fig. 41).

They synthesized 18 additional imidazothiadiazole compounds and determined their binding affinities to the lepidopteran (Sf-9), coleopteran (BCRL-Lepd-SL1), and dipteran (NIAS-AeAl2) EcRs (Fig. 42) (Yokoi et al., 2015). They found that 6-(2-chlorophenyl)- 2-(trifluoromethyl)imidazo[2,1-b] [1,3,4]-thiadiazol-5-yl)acrylamide analogues with secondary amides were very potent against Sf-9 cells, tertiary amides much less active, and primary amides inactive. Saturation of olefin region of the acrylamide moiety decreased activity by 150-fold. Substitution of the imidazothiadiazole ring in the 2-position by the highly lipophilic perfluoropropyl moiety increased activity by 7500-fold compared to the unsubstituted compound in Sf-9 cells. They further showed that hydrophobic and electron-withdrawing groups improved receptor binding, some of which exhibited good larvicidal activity against *Spodoptera litura* but not diptera and coleoptera.

5.11. Amino acid analogues

Horoiwa and colleagues used the MM/PBSA method to predict the binding activity of small organic EcR ligands (Horoiwa et al., 2019). They validated their computational method using 40 known EcR ligands, largely DAHs and chemical isosteres. The correlation between the MM/PBSA binding free energies and the known binding affinities was only satisfactory when ligand conformational entropy was accounted for in the calculations (Winkler, 2020). This computational approach method was used to select 12 candidate compounds, all polycyclic amides, from a database of 3.8 million compounds. Five of the 12 compounds were active in a cell-based competitive binding assay and a substantial number of the candidates were proline analogues. The most potent compound **42** was also a proline derivative (Fig. 43) that exhibited low micromolar binding activity. Synthesis of its pure enantiomers showed that the (S,S)- and (R,S)-**42** has twice the potency of the racemic mixture, while the (S,R)- and (R,R)-42 were >10x weaker.

5.12. Diverse chemotypes from virtual screening

Harada et al. used a computational shape-based method, based on PonA, to virtually screen a large chemical database (> 2 million compounds) to discover new nonsteroidal insecticides (Harada et al., 2011). A range of different chemotypes (Fig. 44) provided μM hits against two strains of insect cells. Docking poses of the most potent hits against the EcR structure showed that the predicted binding mode of the ligands was like that of PonA.

6. Conclusions and perspective

This review shows the relatively large, small molecule diversity exhibited by synthetic EcR ligands. Structural biology studies have been very useful for identifying how steroidal and synthetic small molecules bind to the LBD of EcRs. They have also elucidated LBD features that could be exploited to generate small molecule leads with strong selectivities towards pest species while having negligible activity against useful insects or vertebrates. Selectivity at this level is important in many ecological and agricultural systems involving pest and beneficial arthropods including, for example, *Varroa* mites preying on honeybees. There are also other mites such as *Phytoseiidae* that are employed in the control of agricultural pests further emphasising the importance of

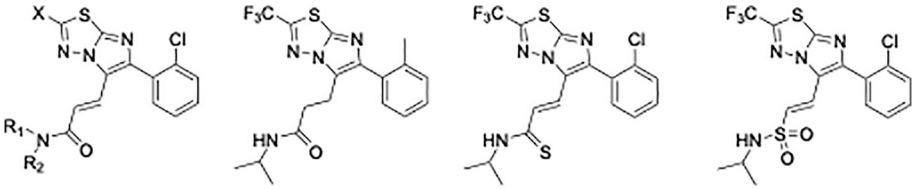


Fig. 42. Imidazothiadiazole analogues synthesized and tested by Yokoi et al. (Yokoi et al., 2015).

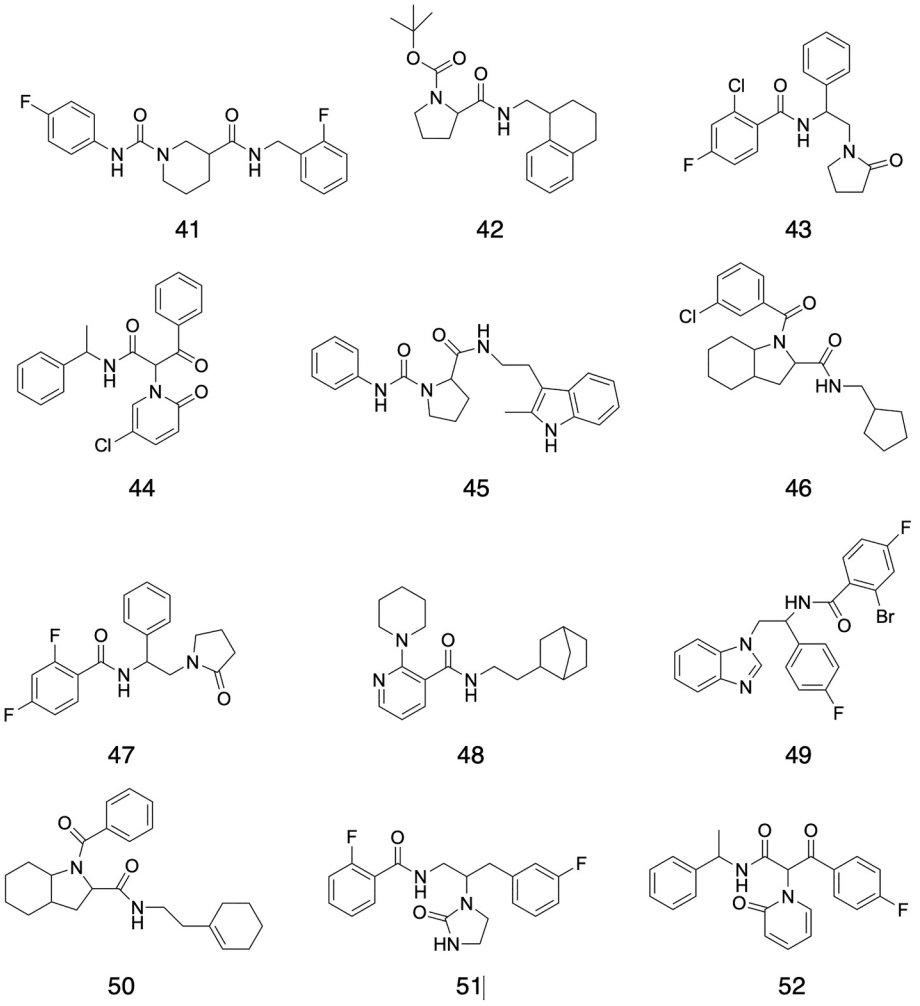


Fig. 43. Chemical structures of compounds selected by virtual screening from Horoiwa et al. (Horoiwa et al., 2019). Proline analogue **42** displayed the highest activity against the EcR.

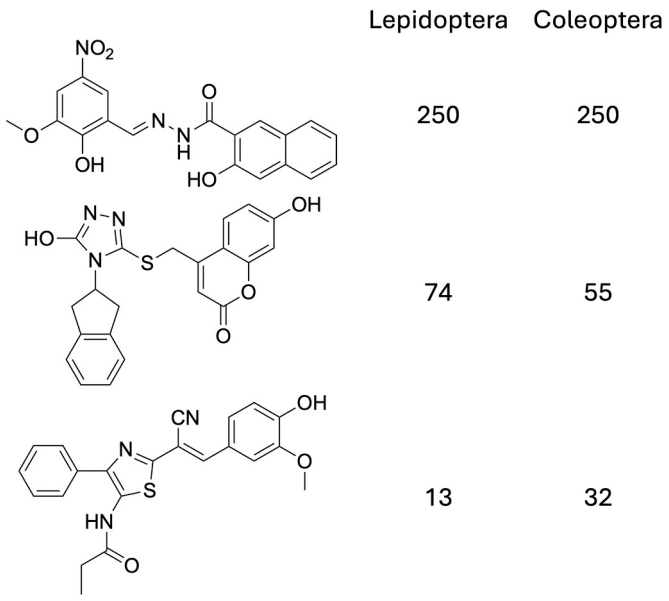


Fig. 44. Activity against lepidoptera and coleoptera (IC_{50} in μM) of a selection of diverse chemotypes exhibiting the most potent EcR agonist activity in the shape-based virtual screen from Harada et al. (Harada et al., 2011).

developing selective chemistries exploiting the evolutionary variation exhibited by ecdysone receptors.

The structural biology studies have further identified the dynamic nature of the EcR LBD, showing that the volumes, shapes, and LBD residues van vary markedly depending on the location on the pocket wall and the types of ligands bound to them. A 2.7 Å X-ray structure of an apo-form of the *B. ovis* ECR did not resolve residues contributing to the EcR binding pocket in the liganded form of the protein suggesting that they may be in a mobile state (Ren et al., 2014). Binding of a ligand would be expected to stabilise the position of these residues in the structure very likely along with a concerted stabilisation of the whole protein complex as is well established experimentally.

Perusing the different chemical structural types that have shown useful binding to the EcR, some broad similarities emerge. Browning et al. used structural biology to show that the DAH, imidazole, and oxazole chemotypes bound in very similar poses and conformations (Fig. 45A) and interacted with the same or similar residues in the EcR LBD (Fig. 45B) (Browning et al., 2021). Other structural similarities that are suggestive of similar binding models and interaction with LBD residues also exist, notably in what might be broadly defined as bisamides and isosteres. Supplementary Fig. S1 highlights the structural isosteres in the "DAH-like" and "bisamide-like" chemotypes

The high mobility of the LBD of EcR results in very different shapes and volumes for the binding site, depending on the chemotype of the ligand binding to it. Thus, there are considerable challenges in using structural biology-derived 3D structures for docking and related types of

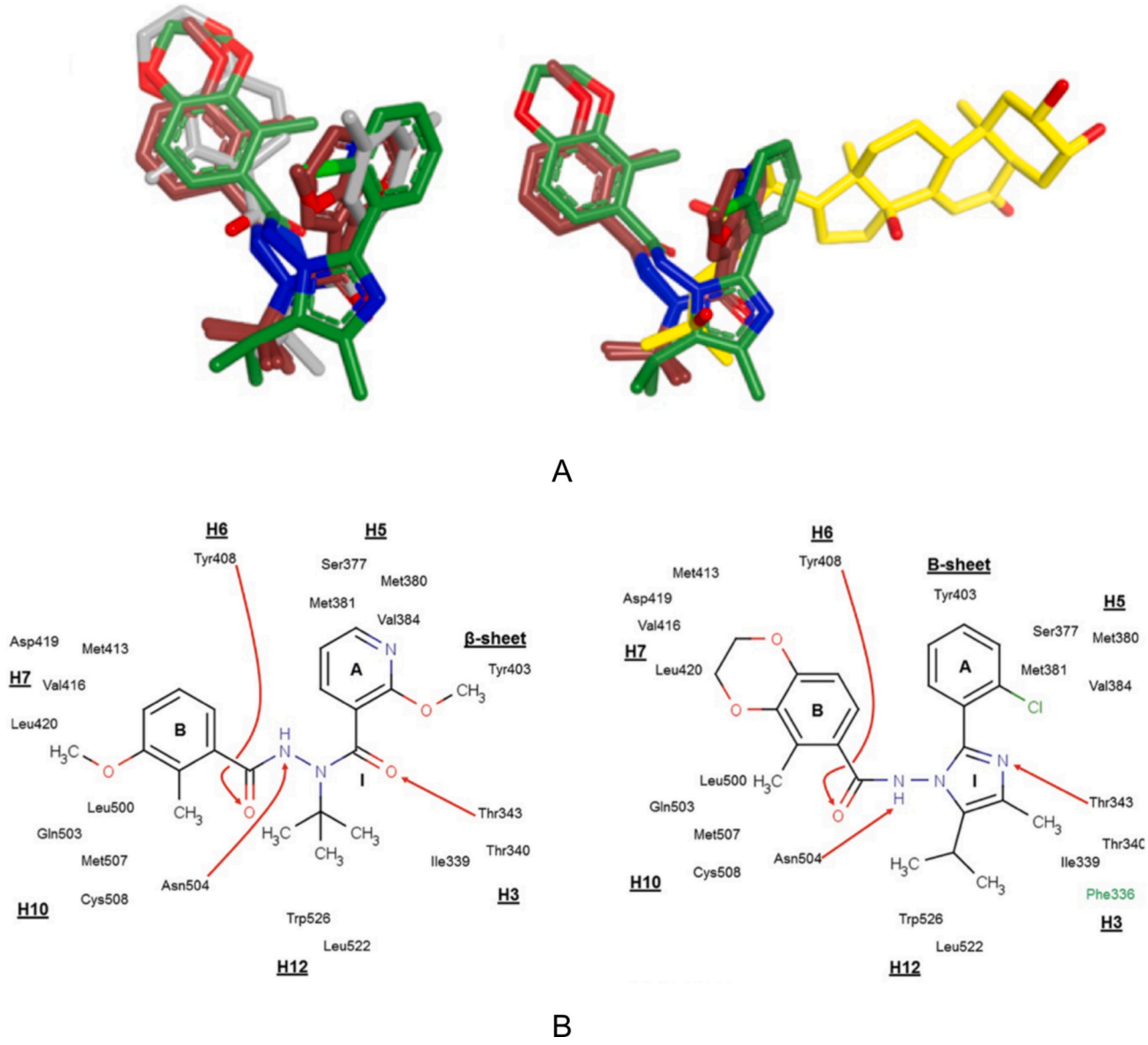


Fig. 45. (A) Superimposition of DAH, imidazole, and oxazole ligands in the ligand binding pocket of EcR (left) and superimposition of DAH and imidazole ligands and ecdysteroid 20E in the ligand binding pocket of HvEcR (right). (B) Interactions of DAH and imidazole ligands with residues of the EcR LBD, red arrows indicate hydrogen bonds. Creative Commons [Attribution-NonCommercial-NoDerivatives 4.0 International] license. from Browning et al. (Browning et al., 2021).

conformation-dependant screens *in silico*. However, the multiple robust QSAR models developed for the diverse chemotypes summarized in this review and in our earlier publications reinforce our hypothesis that the LBDs are not continuously deformable but rather may adopt a finite number of stable binding configurations (Birru et al., 2010; Hill et al., 2012). Although, as this review reveals, many EcR ligands have been discovered, in a range of different chemotypes, it is likely that additional stable states of the LBD may be found that bind strongly to chemotypes not yet discovered. This bodes well for high-throughput physical screening of chemical libraries in concert with rational design of selective EcR ligands. Optimisation of small-molecule ligands by medicinal chemistry guided by an understanding of receptor structure including the location related variability of pocket wall residues also has a role to play.

Given the vastness of small organic molecule drug-like space ($\sim 10^{60}$), there are likely to be an immense number of novel selective EcR ligands yet to be discovered, if we can find them. Increased use of high throughput wet-laboratory screens and improved computational modelling methods exploiting dramatic developments in AI and machine learning constitute a paradigm shift for discovery of new bioactive agents for control of pest insects and members of other arthropod classes such as arachnids and possibly extending as far as other phyla including nematodes with implications for control of gene expression in science, agriculture and medicine.

CRediT authorship contribution statement

David A. Winkler: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Ronald J. Hill:** Writing – review & editing, Writing – original draft, Validation, Investigation, Conceptualization.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2025.106585.

Data availability

No data was used for the research described in the article.

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