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Discovery and Synthesis of Crop Protection Products

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ACS SYMPOSIUM SERIES **1204**

Discovery and Synthesis of Crop Protection Products

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Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from the ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previous published papers are not accepted.

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Preface

Modern agribusiness is one of the main generators of employment and income worldwide and plays a vital role in improving the production, quality, and quantity of food, feed, fiber, and fuel ensuring our world has the safest and most nutritious, abundant, and sustainable food supply possible. The global agribusiness industry with its offerings such as insecticides, herbicides, and fungicides as well as biotechnology products contributes to growing public expectations for food security and agricultural sustainability while addressing the industry's global challenges, such as population growth and rising caloric consumption, increasing environmental stresses across the globe, a changing regulatory landscape, development of resistance to existing active ingredients and traits by investing in effective R&D programs and inventing new solutions.

This book contains a collection of papers presented by representatives of academia and industry at the IUPAC and ACS-AGRO co-sponsored 13th IUPAC INTERNATIONAL CONGRESS OF PESTICIDE CHEMISTRY held in San Francisco, August 10-14, 2014 from the sessions "Discovery and Synthesis" (Organizers: J. Coats, P. Maienfisch, X. Yang, A. M. Rimando and T. Stevenson) and the Crop Protection Research Director's Forum (Organizers: C. Corsi, T. Stevenson and P. Maienfisch).

The book provides an update on state of the art crop protection research and highlights the pivotal role of novel chemistries for modern crop protection. Recent research and new directions in the synthesis and chemistry of agrochemicals, as well as new research approaches, tools and directions in the crop protection field including nematicides, biologicals and natural products are described and details on the design, synthesis, biology and/or structure-activity relationships of a series of new chemical entities targeting fungicides, insecticides, herbicides and nematicides provided. Furthermore future directions for advancing research and regulation of agricultural chemistry and pest management science, promoting public health, and preserving environmental quality are covered as well.

In the early days of crop protection research, new products were often a result of trial and error. Today, the task of inventing and developing novel compounds addressing unmet market needs demands a combination of many scientific disciplines and novel technologies. This includes, for example, high throughput screening, combinatorial chemistry, molecular modeling, biochemistry, genomics and bioinformatics. It is expected that the huge R&D efforts of the companies active in crop protection research will result in the discovery of a series of novel chemical classes and innovative products with excellent biological activity, novel mode of action and favourable safety profile. Some recent examples of such innovative chemical classes are highlighted is this book.

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This ACS book is designated to inform the reader about current trends in the search for modern agrochemicals. It presents a unique look into selected research activities of academia and leading crop protection companies and highlights the discovery, optimization and mode of action of new chemical classes in the field of modern fungicide, insecticide, herbicide and nematicide science. Overall, the chapters of this book serve to show that many novel and exciting innovations in chemical crop protection research have been made in recent years and that new compounds with advantages over existing products continue to be discovered and introduced.

This book could only realized due to the high engagement and commitment of the authors and the technical editors from ACS books. We would like to express our great thanks to all of them.

The cover picture shows adults of *Euschistus heros* (Neotropical Brown Stink Bug) on soybean. The stink bug has become one of the most important pests in this crop over the last decade. The picture has kindly been provided by Roland Reist, Research Biology, Syngenta Crop Protection Münchwilen AG.

We wish the readers an enjoyable and informative reading of this ACS book on the "DISCOVERY AND SYNTHESIS OF CROP PROTECTION PRODUCTS".

August 2015

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Editors' Biographies

Peter Maienfisch

Dr. Peter Maienfisch, Research Portfolio Manager Insecticides & Seedcare, studied chemistry at the ETH in Zürich, Switzerland, where he received his PhD in 1983. After a post-doctoral stay at Caltech (Pasadena, CA, USA) Peter joined Animal Health Research of Ciba-Geigy in Basel in 1984 and became group leader in Insecticide Research in 1988. Following the creation of Novartis in 1996, he was appointed Head of Chemistry 2 in Crop Protection Research and with the creation of Syngenta in 2000 he became Group Leader Insecticide Chemistry, Analytics & Automation. In 2007 he took over the responsibility for portfolio management of the insecticide research projects. Since 2011 he leads the Insecticides & Seedcare Research Portfolio teams, responsible for research strategies and projects. In 2015 he became Honorary Professor of the East China University of Science and Technology (ECUST), Shanghai, China. His track record of innovation and successful introduction of new technologies includes authorship of more than 160 scientific papers and patents. Furthermore he is the inventor of thiamethoxam (Actara®, Cruiser®, one of the leading insecticides world-wide) and a co-organizer of many international congresses, including IUPAC Crop Protection congresses and the International Symposium on Fluorine in the Life Sciences on Bürgenstock. He is a member of the Swiss & American Chemical Societies and the CHIMIA Editorial Board.

Thomas M. Stevenson

Dr. Thomas M. Stevenson was born in Murphysboro, Illinois. Stevenson graduated with a B.S. in chemistry from Saint Louis University in 1979 where he carried out undergraduate research with Harold A. Dieck funded by a Monsanto Summer Fellowship. He received a Ph.D. in organic chemistry from the University of Illinois in 1983, under the supervision of Nelson J. Leonard. After postdoctoral research at the University of Geneva from 1983 to 1985 with Wolfgang Oppolzer, Stevenson joined DuPont Crop Protection as a research chemist, rising in ranks to his current position as senior research fellow.

As an undergraduate he won the Merck Index Award as outstanding senior chemistry major at St. Louis University. During his doctoral studies he held a University of Illinois Graduate Fellowship. A member of ACS, Stevenson has been active in organization of technical sessions in both the AGRO and Organic divisions. His honors include DuPont Pedersen Medal and the ACS Heroes of Chemistry Award. He has also received the DuPont Bolton-Carothers Innovative Science Award (twice), the DuPont Sustainable Growth Excellence Award, and the

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R&D 100 Award, as well as the ACS Award for Team Innovation, the Philadelphia Organic Chemists Club Industrial Award, and the IPO National Inventor of the Year. The DuPont Crop Protection Scientific Leadership Award which he received in 1994 allowed him to spend a sabbatical in the labs of Paul Knochel at Phillips-Universität Marburg in Germany during 1996.

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Chapter 1

Modern Agribusiness - Markets, Companies, Benefits and Challenges

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The global agribusiness market which includes insecticides, herbicides, and fungicides as well as biotechnology products is valued at almost $ 78 billion. 90% of the worldwide sales in this market are achieved by only 10 companies. However despite the benefits of today's modern crop protection products and traits, the future of the agribusiness market faces important challenges which need to be overcome by effective R&D programs in the industry. Examples of these challenges include an increasing demand for food driven by population growth and rising caloric consumption, increasing environmental stresses across the globe, a changing regulatory landscape, development of resistance to existing active ingredients and traits, increasing R&D costs, and societal pressures to provide sustainable products. These challenges ensure that new and innovative solutions to allow growers and farmers to "grow more with less" must and will be invented.

Agribusiness Market Overview

Since the earliest days of agriculture, weeds, diseases, and infestations by insects have always been, and still are, major reasons for yield losses. Today modern chemical crop protection and GM traits together with fertilizers, mechanization, and precision farming are contributing in a decisive manner to secure the sustainable production of food, feed, and fiber. In 2014, the value of

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the agribusiness market, which includes herbicides, insecticides, fungicides, as well as biotechnology products, reached $ 77.7 billion, to which crop protection products contributed $ 56.7 billion and GM seeds $ 21.0 billion, respectively (Figure 1) (*1*). In addition crop protection products are widely used in the non-crop agrochemical market which comprises the following sectors: home & garden, pest control operators, turf, nursery and ornamentals, rodenticides, wood preservation, material preservation, stored grain, public health, post-harvest protectants and industry outlets. In 2014 these non-crop agrochemical market contributed another $ 6.6 billion sales to which insecticides contributed $ 2.45 billion (37.4%), herbicides $ 2.3 billion (35.2%) and fungicides $ 1.7 billion (25.6%) (*1*).

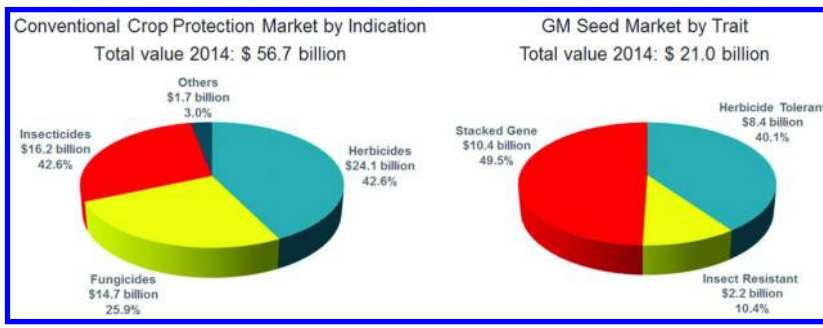

Figure 1. Agribusiness market 2014. Total value $ 77.7 billion (1).

Today, crop and non-crop agrochemicals still represents over 75% of sales for inputs into the agribusiness and there is no doubt that agrochemicals will continue to be a vital component of crop protection, even in the face of novel biotechnological solutions and emerging technologies like biologicals, RNAi and others.

In the time period from 2000 to 2009 the conventional crop protection market declined by 0.8% per year in real terms, however, it has shown strong growth since 2010 with annual average growth rates of 7.6% per year in real terms. Major factors influencing this growth are attributed to commodity price increases and rising crop demands from developing nations, however, the market is regarded to be quite vulnerable, which is resulting in a high level of uncertainty regarding the future development. Major crops of the conventional crop protection market are: fruit & vegetables (24.0% of the total market), soybean (17.0%), cereals (16.2%), corn (11.4%) and rice (8.9%).

GM technology was introduced to the market in 1996 and since then the GM seeds market has grown constantly. In the last few years the strong initial growth has started to flatten, but is expected to remain at the current growth rate of 2-3% (*2*). So far GM technology is mainly applied in only a few crops: corn (45.5% of total GM seed market 2014), soybean (32.8%), cotton (8.5%) and canola (3.7%) and mainly in the USA (40.3%), Brazil (23.6%), Argentina (13.9%), Asia

(11.4% and Canada (5.9%). In the EU the regulatory framework based on Council Directive 91/414/EEC (entry into force: 26 July 1993) and on Regulation (EC) No 1107/2009 (entry into force: 14 June 2011) has triggered the suspension of EU approved GM products in individual countries and an endless delay of EU approval of GM products with the result that GM seeds are currently rarely used in Europe. As a consequence European growers can currently not benefit from the modern GM technologies.

Leading Agribusiness Companies

Although the agribusiness market has grown remarkably over the past three decades, a strong consolidation of the industry has taken place (*3*, *4*). Well known research-based agrochemical companies like Ciba Geigy, Novartis, Maag, Sandoz, Zeneca, Hoechst, Schering, Rhone Poulenc, Aventis, Shell, and others have disappeared mainly due to mergers. Today, more than 90% of the worldwide sales in the agribusiness market are achieved by only 10 companies (Table 1) (*1*).

Among these companies Monsanto, Syngenta, DuPont and Bayer invest more than $ 1 billon every year in Research and Development. With annual R&D budgets between $ 630 and 835 million Syngenta, Bayer and BASF are leading the investments in Crop Protection R&D, whereas Monsanto makes by far the highest investments into Seeds & Traits R&D, followed by DuPont, Syngenta and Bayer. Considering the overall agribusiness, certainly Syngenta and Bayer show the highest commitment to R&D in both business areas – Crop Protection and Seeds & Traits. Companies like MAI (Adama), Nufarm and FMC are more focused to deliver new solutions to growers based on established products rather than engaging in new a.i. research-driven approaches. Besides the US and Europe based companies, Sumitomo has established itself as the leading company in Asia Pacific. Beyond these leading enterprises a series of smaller companies with mainly a regional business focus are active in the crop protection area.

Benefits of Crop Protection Products and GM Traits

Now in the 21st century, agrochemicals and GM traits are part of intergrated solutions and are vital components in agricultural practices. The benefits of these tools have been well documented over many years (*5*, *6*).

The most important benefits are:

- To contribute to a sustainable production of food, feed and fuel
- To secure the most efficient use of land, water and energy
- To protect crop yields
- To increase food quality
- To enable an efficient and effective production of food at the lowest possible costs
- To contribute to sustainable incomes of farmers

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Table 1. Leading agribusiness companies: 2013 Sales & R&D expenditures

Rank	*Company*	*Sales 2013 ($m)*			*R&D Expenditure 2013 ($m)*		
		Crop Protection	*Seeds & traits*	*Total*	*Crop Protection*	*Seeds & traits*	*Total*
1	Monsanto	4‘804	10‘261	15‘065	50 (0.3%)[a]	1‘546 (10.3%)	1‘596 (10.6)
2	Syngenta	11‘413	3‘204	14‘617	835 (5.7%)	510 (3.5%)	1‘345 (9.2%)
3	DuPont	3‘558	8‘181	11‘739	295 (2.5%)	837 (7.1%)	1‘132 (9.6%)
4	Bayer	10‘418	1‘293	11‘711	680 (5.8%)	458 (3.9%)	1‘138 (9.7%)
5	Dow	5‘523	1‘614	7‘137	340 (4.7%)	290 (4.1%)	630 (8.8%)
6	BASF	6‘942	0	6942	623 (9.0%)	200 (2.9%)	823 (11.9%)
7	MAI (Adama)	2‘876	0	2‘876	33 (1.15%)	0	33 (1.15%)
8	Nufarm	2‘297	98	2‘395	43 (1.8%)	5 (0.2%)	48 (2.0)
9	FMC	2‘146	0	2‘146	101 (4.7%)	0	101 (4.7%)
10	Sumitomo Chemicals	2‘020	0	2‘020	150 (7.4%)	0	150 (7.4%)

[a] % of total sales.

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Modern crop protection products and traits help to safeguard crops and to generate extra yields. Crop plants must compete with 30,000 species of weeds, 3,000 species of nematodes and 10,000 species of plant-eating insects (*7*). The increased use of agrochemicals since the 1950's is a major factor for the huge productivity increases achieved. As demonstrated by Oerke (*8*, *9*) the proper use of agrochemicals results in yield gains of 22-53% in 6 of the most important crops (Figure 2). This is a striking example of the value generation achieveable with agrochemicals, which certainly has a positive impact not only on food production but also on the farmer's income.

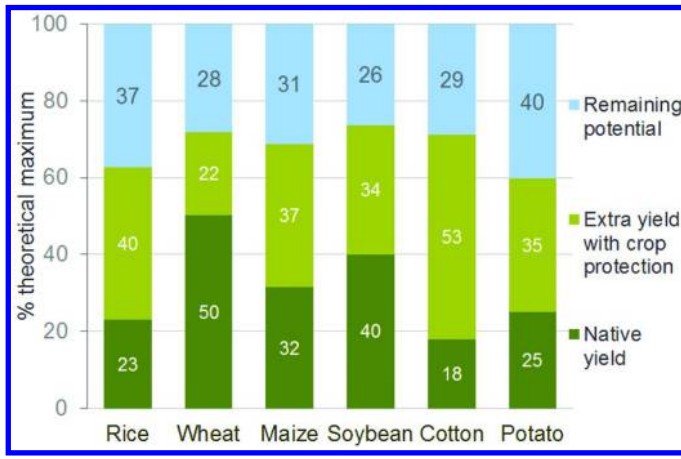

Figure 2. Contribution of modern crop protection to crop yields (8, 9)

Current Challenges for Growers and the Agribusiness Industry

Today, growers and the agribusiness industry are facing a series of challenges. Some key challenges are discussed below:

- Demand for food is increasing driven by population growth and rising calorie consumption
- Environmental stresses are increasing
- To grow more from less
- Changing regulatory landscape
- Resistance development to existing active ingredients & traits
- Increasing R&D costs
- Societal pressures
- Global financial instability regarding commodity prices and credits

Food Demand

Demand for food is increasing driven by population growth and rising calorie consumption (Figure 3) (*10*, *11*). In 1950, the world's population was just 2.5 billion, in October 2011, it hit 7 billion. Today, the world's population

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is increasing by 200,000 every day and by 2050 there will be two billion more people on the planet. We are facing the challenge of feeding 9 billion people by 2050. Additionally we observe a trend of diet changes in emerging markets, where populations are fast growing. As a consequence grain demand is expected to increase by +50% by 2050. This cannot be achieved only by productivity gains - a holistic approach building on new technologies (new agrochemicals with novel mode of action, traits, biologicals and other technologies such as RNAi), improved farming practices and monitoring while considering environmental and societal demands is required. For example, research on new agrochemicals needs to focus on delivering products which are efficacious, safe, and affordable, broadly applicable and can be used in integrated crop solutions. Furthermore, they need to meet the requirements of future regulatory systems and are easily producible (*12*).

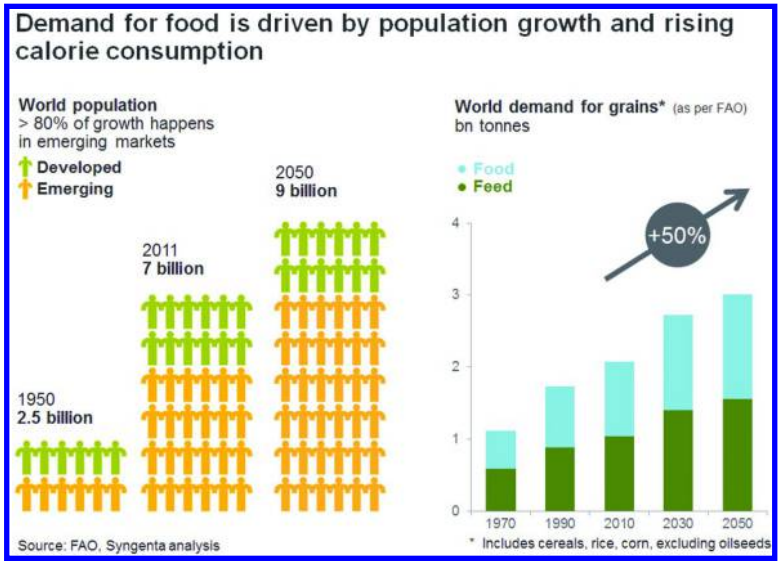

Figure 3. Population growth and rising calorie consumption (10)

Environmental Stresses

Environmental stresses are increasing due to climate change, weather volatility, water scarcity and land degradation. Today, growers must contend with all these dramatic environmental changes. As indicated on the world stress map (Figure 4) (*10*, *13*) the change in climate is already reducing water and arable land. Two thirds of the world's surface are facing high or medium climate change impact.

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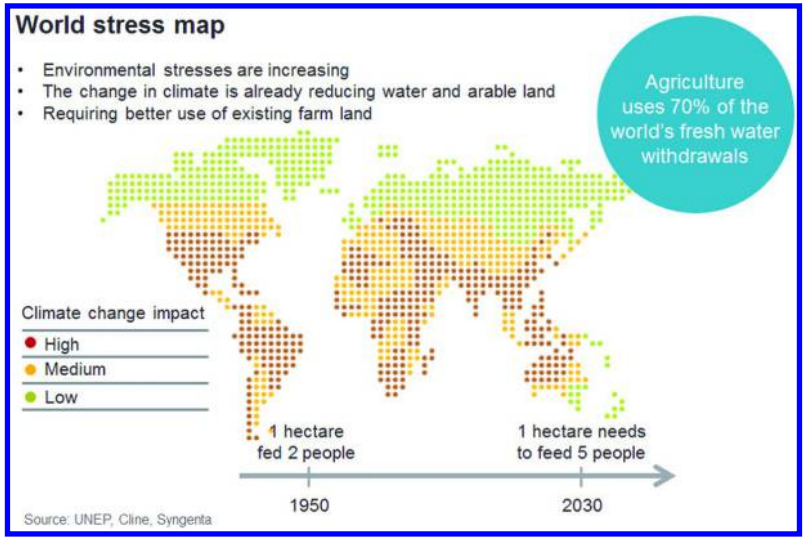

Figure 4. Population growth and rising calorie consumption (10, 13)

Population growth and land scarcity will lead to the need for a further increase in food production per hectare of land. In 1950, one hectare could feed two people, by 2030 it will have to feed five. Food demand is already outstripping supply. Farmers must grow more from less.

Grow More from Less

As a consequence of increasing environmental stresses combined with the expected population growth and rising calorie consumption, growing more from less has become an imperative (visit: www.growmorefromless.com for more information).

The agribusiness industry needs to help growers to deliver greater food security in an increasingly crowded world in an environmentally sustainable way. This means with fewer resources, while protecting nature, and at the same time helping people in rural communities live better lives. Additional sustainable production systems need to be made available that link technology, people and land to deliver better solutions to further improve farm productivity while reducing agriculture's environmental footprint through resource efficiency and allowing rural economies to build sustainable prosperity.

Changing Regulatory Landscape

The regulatory environment varies enormously around the world, reflecting economic development, political priorities and the importance of agriculture. Over the last years the regulatory process for the registration of new active

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ingredients and the re-registration of established products became increasingly complex, costly (*14*) and of lower predictability, especially in the EU where a continuous shift from a risk-based to a hazard-based approach took place, mainly as a result of Regulation (EC) No 1107/2009 (entry into force: 14 June 2011).

Today, the regulatory process leading to the registration of a new agrochemical molecule takes an average of 9.8 years between the first research tests and the registration of the product (*14*, *15*).

Despite the EU's Strategy 2020 aspiration to 'grow innovation', the reality for our industry is quite the opposite and with the consequence that European growers will potentially not be able to benefit from all new and modern technologies, with GM technology as a first example, leaving them at a disadvantage, compared to growers in other regions (*16*, *17*).

Regarding today's EU regulatory framework the following points deserve to be considered:

- A regulatory framework that seeks to safeguard human health and the environment is welcomed. However the complexity in the EU regulatory framework allows for misuse of the precautionary principle.
- Regulatory requirements for a new product should be proportionate to the potential risks. This philosophy must be applied to newer areas of research to avoid the EU losing further ground in new technology area.
- A regulatory framework that demands such excessive cost, time and effort yet provides so little certainty in relation to product approvals and timescales is a disincentive to investment in technology development.

Overall, a more globally harmonized risk assessment framework for agrochemicals and traits and a universal approach to the "Principles of Regulation" is certainly desirable (*18*).

Resistance

Resistance development to agrochemicals and traits is a well-known risk. Some chemistries are more robust towards resistance development than others, however, in the long run most, if not all, chemistries and traits are affected. Figure 5 highlights the development of resistance towards major selective and non-selective herbicides demonstrating that herbicide resistance has now become a major problem of global importance (*19*). In the fungicide and insecticide area the situation is quite similar. For examples, new resistant insect species are appearing all the time. Since 1984, four to five new resistant species have been identified each year (Figure 6) (*20*).

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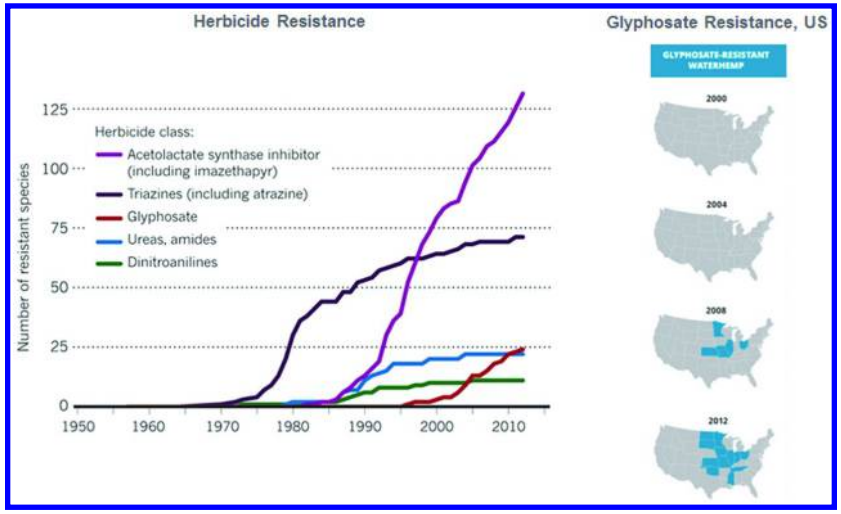

Figure 5. Development of herbicide resistance (19)

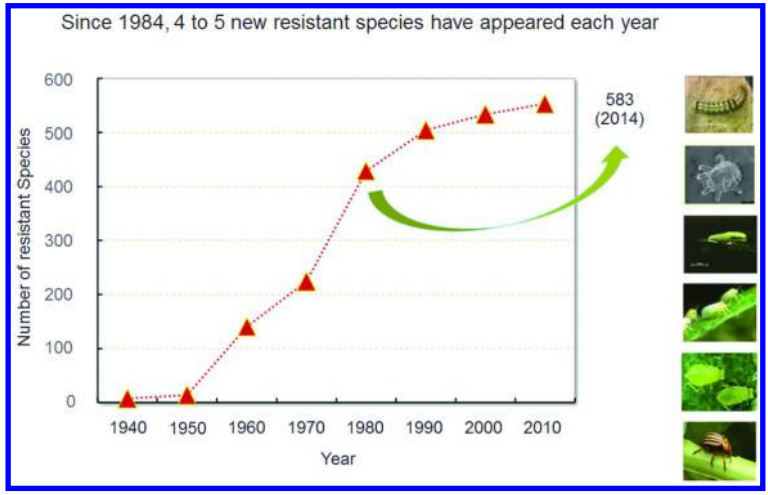

Figure 6. Development of insecticide resistance (20)

Loss of performance due to resistance can be costly to the grower, crop protection companies and the environment. New innovative solutions to fight resistance are a key driver of current crop protection and traits research and are urgently required.

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In addition suitable resistance management strategies to maintain long-term efficacy and use of active ingredients and traits have been developed and are constantly reviewed and improved by all major suppliers of agrochemicals and traits. Resistance management strategies aim to minimize the risk of resistance development and include:

- Avoidance of exclusive use - restriction of the number of treatments applied per season if required
- Recommendation of spray regimes including products with different resistance mechanisms / modes of actions
- Use of mixtures
- Field monitoring programs on efficacy of products and early indication of resistance development

R&D Costs

A study reported by Phillips-McDougall (*14*, *15*) has shown that the cost of bringing a new active ingredient to the market has strongly increased over the last years: Between 1995 and 2005, the total cost for R&D and registration has raised on average by 68.4% from $ 152 million to $ 256 million, research costs by 18.0% (from $ 72 million to $ 85 million) and development costs by 117.9% (from $ 67 million to $ 146 million). The cost split reported for 2005-8 is highlighted in Figure 7.

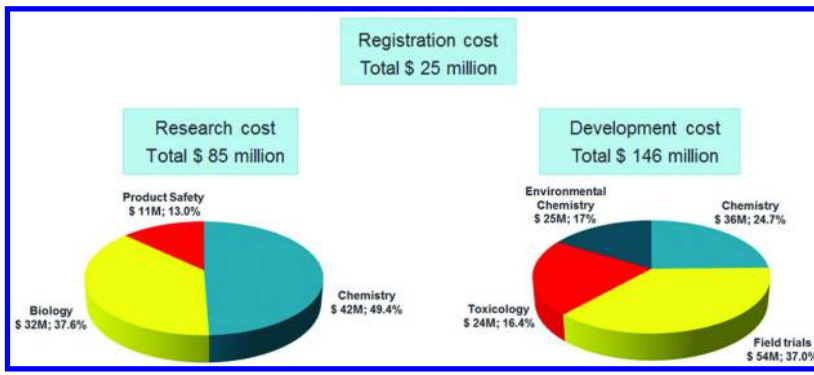

Figure 7. R&D cost of bringing a new active ingredient to the market (14, 15)

Multiple factors have driven this tremendous growth of development cost among them cost for global field testing and additional toxicological and environmental studies required by the more stringent regulatory systems had certainly the highest impact.

In contrast, the cost for bringing a plant biotechnology trait to the market in the 2008-2012 was with $ 136 million significantly lower (*21*).

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Societal Pressures

Developing economies are creating a growing rural-urban disconnect. Food preferences and lifestyle choices in cities are disconnected from the challenges farmers face to grow quality food. Urban consumer and pressure groups are challenging the need for crop protection technology leading to political rather than science based government intervention based on a precautionary principle that is biased towards risk avoidance rather than risk management. The neonicotionoid bee toxicity discussion is just one recent example. Agribusiness companies have developed a broad understanding of societal demands and are putting in place management practices to deal with such challenges. However the uncertainty of public acceptance of modern technology is increasing.

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Global Financial Instability - Commodity Prices and Credit

While crop prices have remained generally high, their continuing volatility makes growers' decision-making processes more difficult. Furthermore, currency fluctuations have an impact on the relative competitiveness of agriculture in different regions of the world. Overall, the global financial uncertainties makes predictions about future developments and consequently farmers' lives difficult.

Summary and Conclusions

Modern chemical crop protection and GM traits together with fertilizers, mechanization, and precision farming have been contributing for decades in a decisive manner to secure the sustainable production of food, feed, and fiber. The current value of the agribusiness market, which includes herbicides, insecticides, fungicides, as well as biotechnology products, is $ 77.7 billion, to which crop protection products contributed $ 56.7 billion and GM seeds $ 21.0 billion, respectively.

More than 90% of the worldwide sales in the agribusiness market are achieved by only 10 companies, of which only 7 are research-driven and invest more than 7.4% of their annual sales into R&D. With annual R&D budgets of more than $ 1 billon Monsanto, Syngenta, DuPont and Bayer are globally leading R&D investments.

The benefits of crop protection products and traits are well documented. Most importantly they contribute to a sustainable production of food, feed and fuel, help secure the most efficient use of land, water and energy, increase crop yields and food quality, and enable an efficient and effective production of food at the lowest possible costs.

Today, growers and the agribusiness industry are facing a series of challenges which include increasing food demand, environmental stresses, R&D costs and global financial instability as well as fast resistance development towards the established solutions and the tighter regulatory landscape.

The agribusiness industry has taken the responsibility to contribute to societal needs by using their capabilities and resources to address these current challenges. It is obvious that despite the modern arsenal of agrochemicals and traits available

to growers and farmers today, new innovative solutions are still required and if both, the agribusiness industry and regulators, work together towards the common objective to "grow more from less", the necessary inventions will be made and introduced to the market.

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Chapter 2

Building a Successful Crop Protection Pipeline: Molecular Starting Points for Discovery

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The discovery and development of new products for crop protection begins with a healthy pipeline of molecular starting points for discovery. Strategies and processes associated with the identification of new starting points will be discussed.

Protection of the global food supply is essential as world population grows from approximately seven billion today to an estimated nine billion people by the year 2050. Global food protection will take the combined efforts of industry, government, farmers and a host of others that influence world food production. The stakes are high and incorporate enormously large issues such as efficient management of land and water. Crops will further require continued protection from the pressures of disease, insects and weeds that seek to compete. The changing dynamics of pest populations and the development of resistance are among some of the factors that demand new innovation and new technology for farmers the world over.

The health of the crop protection industry as well as a crop protection business is directly then related to its new product pipeline. A healthy pipeline insures a renewable source of new products for plant protection and provides the farmer new tools to meet changing needs. It further aids in the combat of pest resistance through the support of resistance management strategies that frequently employ rotation of products acting by differing modes-of-action. At DuPont we recognize that for the health of our own Crop Protection business it is essential that we provide a consistent stream of new products into our pipeline.

We have clearly defined “product concepts” focused on critical success factors, including customer needs, societal needs and regulatory requirements.

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Our goal for a new crop protection product is to create a new molecule that has attributes beyond what is currently on the market. Depending on the product and the market, our search for new products will be molecules that provide some combination of the following: an improvement in pest control, enhanced toxicological or environmental profile, differentiating attributes (e.g. fast acting, improves yield, quality, etc.), or provides a new tool in integrated pest and resistance management.

The identification of new crop protection products begins with the identification of new molecular starting points (Hits). Typical new starting points are generally weakly active on the pest and little is known of their profile with respect to a host of parameters including spectrum as well as their environmental and toxicological profiles. Once identified, however, the optimization of chemistry through the judicious choice of synthetic analogs, coupled with a feedback loop for new analogs based on evolving structure-activity profiles, can lead to exquisitely active compounds with well understood ecological and toxicological profiles. The course of optimization frequently requires solutions to a variety of issues and our experience shows that only a small fraction of molecular starting points are successfully optimized to new products. It is essential, therefore, for any successful crop protection pipeline to identify and maintain a healthy and robust supply of new starting points. The pipeline in principle then begins first with identification of new Hits.

We operate on the principle that a balanced approach to the discovery of new molecular starting points is essential and focus on three main streams of input (Figure 1).

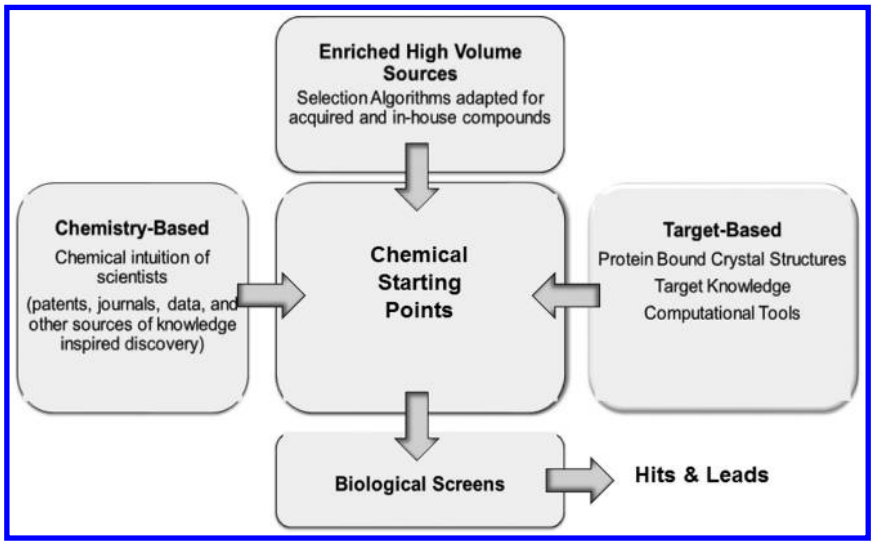

Figure 1. Primary streams of input for chemical starting points

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1. **High Volume Sources (HVS)** provide a rich supply of new molecules for screening. Over the years we have refined our selection algorithms to maximize the diversity of the compounds acquired and fill gaps in our existing compound libraries. To further increase the chances of success, a variety of algorithms have been developed to seek compounds with "Ag-Like" properties in keeping with rules such as those from Lipinski and Tice as well as proprietary models developed internally. Independent analysis of properties associated with the diversity of active crop protection chemistry gives additional insight into compounds and compound libraries we might acquire.

   Related to our high volume sources are the million plus compounds we have in our in-house chemical libraries. These serve as a rich source of chemistry as the insecticide, fungicide and herbicide screens evolve in testing protocols or change as new species are added. The addition of high volume screens further allows re-testing (retro-screening), or in some instances first time testing, of compounds designed for a specific set of screens (e.g. herbicides) that have yet to be screened broadly. Over time we have seen numerous examples of compounds designed to control one particular area will show surprising activity on another and the literature supports that this is not an uncommon occurrence.
2. **Chemistry-Based Discovery (CBD)** is a term we use to describe new Hits identified through the knowledge and intuition of our scientists based on inspiration from known molecular starting points. The enormous volume of information that provides clues to biologically active molecular entities makes this a particularly challenging task as one must choose a starting point for ideas. The ideas of course must ultimately be reduced to practice, i.e. a molecule that can be screened and tested. Information provided through patents, journals, meetings and other sources of knowledge such as natural products or the pharma literature along with a rich source of in-house data on millions of biological tests afford a multitude of inputs for information to be distilled. The aid of computational tools and the input of colleagues provide a valuable source of added insight and grounding.
3. **Target-Based Discovery (TBD)** has proven to be a useful tool in the identification of new molecular starting points and a variety of interesting new molecular types have been discovered. Issues associated with translation (i.e. the translation from *in vitro* to whole organism activity) have however proven to be a vexing problem. The issue is in fact two-fold. First, at this point in time the percentage of successful Hits we have identified through Target-Based Discovery is significantly lower than those identified either through Chemistry-Based or High-Volume strategies. Second, for those Hits that have been successfully identified we have yet to realize an advanced pipeline candidate, let alone a new product. Nevertheless, we continue to seek to solve this problem as we believe the strategy has great value if translation issues can be resolved.

   Chemistry to biochemical mechanism is a related approach that first relies on the identification of a new molecular starting point followed by

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identification of the biochemical mechanism of action. It is not unusual to find that many of the new Hits which show activity in the crop pest screens act by known biochemical mechanisms. These are determined through a panel of tests designed to evaluate known modes-of-action for existing Hits and Leads. However, in the rare instances where new biochemical modes-of-action are identified, the establishment of *in vitro* screens can be a valuable tool in the search for new molecular starting points. These starting points typically face the same issue of translation but have the advantage of being coupled with validation that the particular mechanism can express *in vivo* pest activity based on knowledge of the original Hit.

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Within any of the three discovery platforms described above we find the common thread that drives innovation is the people associated with discovery. At DuPont we are fortunate to have a highly experienced staff with a broad knowledge base of the crop protection business. We allow for a free flowing exchange of ideas and seek to provide training and experience for all of our discovery scientists across all indication areas. As we hire new employees we further set up long term mentoring relationships to insure the knowledge base is passed on. Some of the attributes associated with the scientific staff that we believe hold high value include the following:

- Knowledge: Possess deep knowledge in the field of technology, the underlying science, and the associated business
- Creativity: Ability to bring seemingly disparate pieces of information together to create something new
- Perseverance: Ability to work through problems and to know when to stop pursuing an idea and shift to a more promising course
- Team oriented: Ability to work within groups and across functions
- Externally focused: Ability to garner support for ideas and engage the best resources
- Market focused: Possess a clear understanding of product concepts, customer needs and market opportunities
- Know the competition: Have a thorough understanding of the competitive landscape and the patent landscape.

The examples that follow serve to illustrate some of the strategies used in the discovery of new molecular starting points. In the first example we highlight the High Volume Source strategy (Figure 2). The initial Hit for the new fungicide oxathiapiprolin (DuPont™ Zorvec™) was discovered through the screening of a 300 plus combinatorial library acquired from Tripos. The library was composed of diamides derived from 4-thiazolylpiperidinecarboxylic acids. While there was very little activity detected for the library as a whole, one compound (DP-010) was found to demonstrate some activity on grape downy mildew and in particular showed modest levels of curative control. Further, DP-010 was the only compound that contained both phenyl acetamide and benzyl amino substituent groups making it a unique chemotype within the library. Synthesis of a subset

of analogs confirmed oomycete activity for a wide spectrum of compounds with derivatives derived from pyrazole acetamides showing the most promise. Further constraining of the 2-methylbenzylamino group into a 2-aminotetralin produced the analog DP-020 which showed a 1000-fold improvement in biological activity with good curative control. Extensive optimization through over 1200 analogs led to the discovery of oxathiapiprolin, the most potent oomycete fungicide to date with remarkable preventive and curative activity (*1–3*).

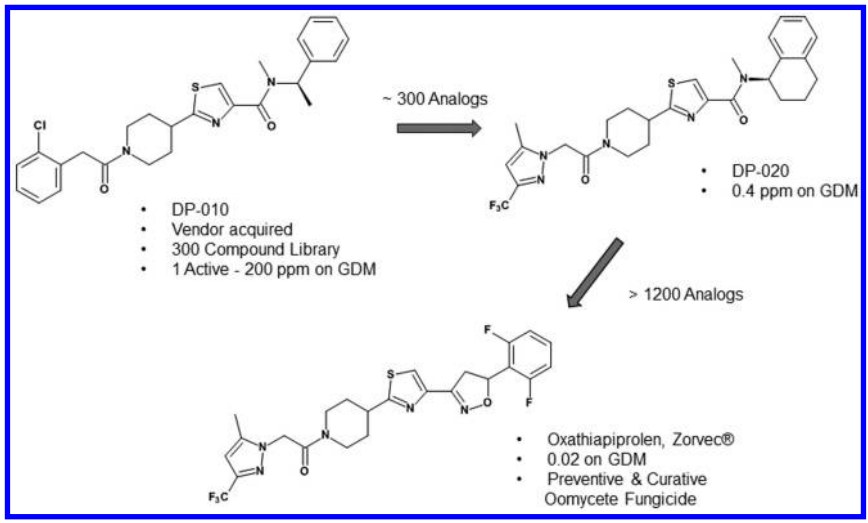

Figure 2. High Volume Sourcing Strategy, Zorvec® Fungicide

Important lessons here come not only from the confirmation of High Volume Sources as a valuable strategy but also to the importance of critical evaluation of the biological data and the keen insight required to extract a weakly active Hit from an otherwise inactive library. It would have been easy to dismiss the initial Hit given that activity was found for only a single analog.

The next example stems from a discovery made in the retro-screening of our in-house libraries leading ultimately to the new insecticide triflumezopyrim (Figure 3). This strategy is a form of directed High Volume Sources where random screening is intended to fill in data gaps, test on new species or seek internal compounds with specific physical properties. The compound DP-030 was found to possess moderate levels of activity on both lepidopteran and hemipteran pests. It was determined that these compounds acted by a new mechanism at the nicotinic receptor with good activity on pests resistant to neonicotinoids. While the traditional chloropyridyl and chlorothiazolyl heterocycles demonstrated good activity it was observed that the unsubstituted thiazoles provide better hopper control. Extending this observation into other heterocycles we found that the unsubstituted pyrimidine of triflumezopyrim stood out. Triflumezopyrim is in fact the most potent compound discovered for the control of planthopper pests in rice with outstanding activity in both contact and systemic applications (*4–6*).

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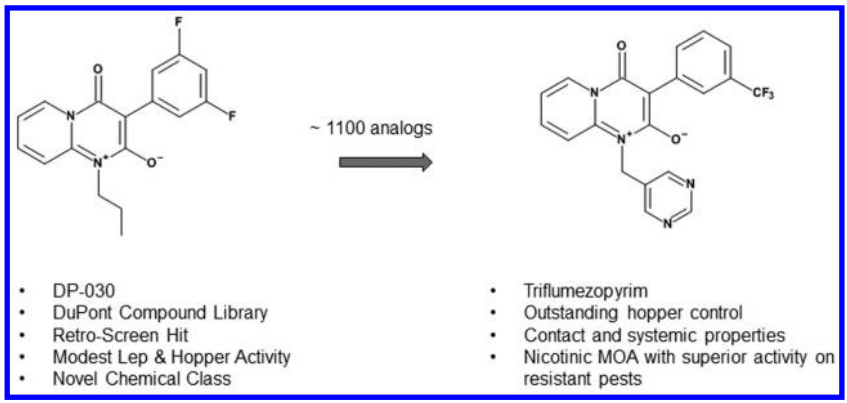

Figure 3. Retro-Screening of Internal Compound Files, Triflumezopyrim Insecticide

Another rich source of Hits and Leads comes from a strategy directed toward the design of novel chemistry based on knowledge extracted from the expansive pool of chemical and biological information found in the open literature and internal sources, i.e. Chem-Based Design (Figure 4). The publication of insecticidal activity associated with a new class of phthalic diamides by Nihon Nohyaku prompted exploration in adjacent chemical spaces including anthranilic diamides. We were able to identify modest levels of activity on important lepidopteran species for anthranilamides such as DP-040 which provided our initial internal molecular starting point for optimization. At the outset, the other interesting observation was that the biochemical mechanism was not identified in our mode-of-action panels suggesting that the mode-of-action was new. Through a two year program aimed at optimization of the chemistry we were able to identify a potent class of pyridylpyrazole anthranilic diamides on a wide spectrum of Lepidoptera. We further determined these compounds worked through inhibition of the ryanodine receptor, a novel mode-of-action for synthetic insecticides first identified for the natural product ryanodine. From this group of pyridylpyrazole anthranilic diamides, chlorantraniliprole (Rynaxypyr®) was found to possess the optimum properties for exceptional pest control. Rynaxypyr® has proven to be a remarkable product for farmers around the world (*7–9*).

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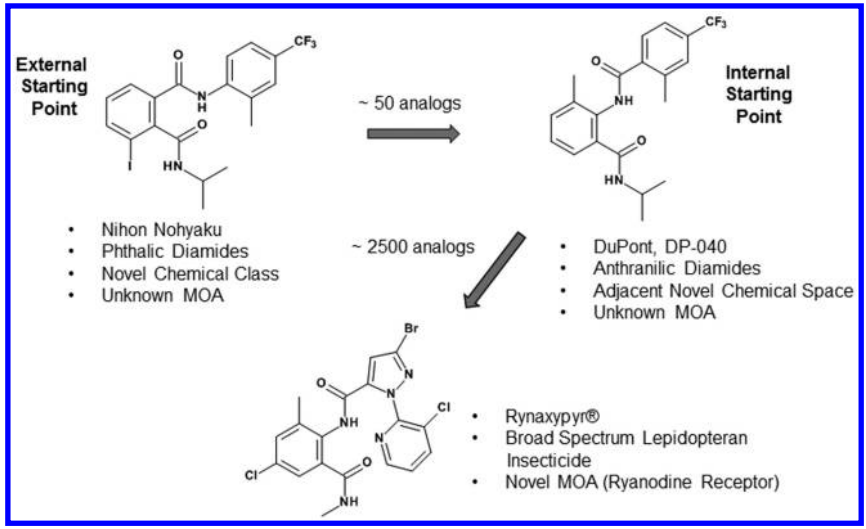

Figure 4. Chem-Based Discovery, Rynaxypyr® Insecticide

The final example is an extension of Chem-Based Discovery in the search for molecular starting points through physical property modification of existing leads or products (Figure 5). As noted above, chlorantraniliprole was found to be exceptionally active on lepidopteran pests. While there were indications of activity on a broader pest spectrum, including insects from the order Hemiptera, the lack of significant systemic activity precluded significant utility in this market. In an effort to improve systemicity, compounds with improved aqueous solubility, as measured by reduction of their calculated log P, were examined. After the evaluation of approximately 1500 analogs it was determined that introduction of a cyano group as a replacement for chloro on the anthranilic ring provided a range of compounds with improved systemic properties and with broad activity over a wide spectrum of lepidopteran and hemipteran pests with cyantraniliprole (Cyazypyr®) as most preferred. In the particular case of Cyazypyr® the measured logP was found to drop a full log unit as compared to Rynaxypyr® and this reduction in logP is largely responsible for the improved systemic properties. Obviously, it was particularly important that activity be retained for compounds with reduced logP as we worked through many modifications that improved water solubility but simultaneously lost activity. Cyazypyr®, in its second full year of sales, promises to be an exceptional product for cross-spectrum pest control (*10*, *11*).

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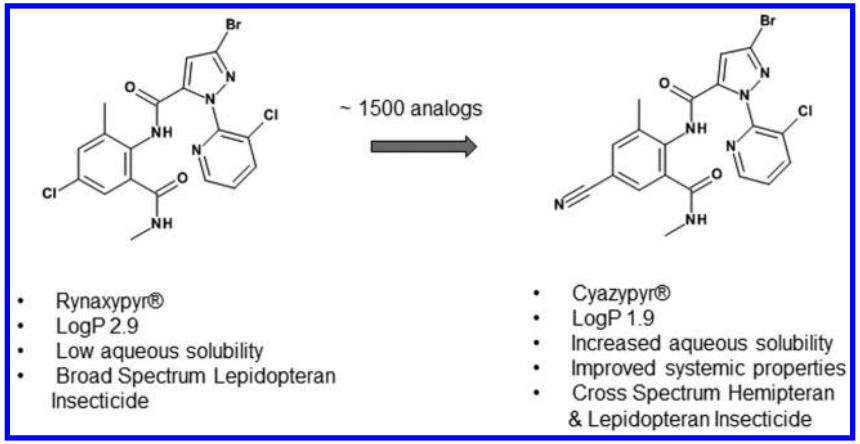

Figure 5. Physical Property Modification, Cyazypyr® Insecticide

Critical Success Factors for New Product Commercialization

In addition to the technical challenges of discovering new crop protection chemicals, at DuPont we believe that the governance of the process is critical to the overall success of the technical effort.

Within the business we charter decision boards with cross functional business representation, including marketing, supply chain and business development, in addition to research and development. The purpose of the decision boards is to have a holistic view of the end to end process from discovery to commercialization. The decision boards are accountable for oversight of the Staged-Gated process that works collaboratively with R&D to manage the discovery & development pipeline, including assessing the market opportunity and value of the new crop protection products.

The decision boards also enable strong collaboration between R&D and marketing to occur throughout the development process, ensuring that the new product is meeting a market need that is valuable to the grower and is aligned with our product concepts.

With all the tools we have developed and teams we have chartered, the success and value of our pipeline is truly the output of the people who work in our research and development organization. Discovering and developing a new crop protection products takes years, sometimes almost a decade of effort and dedication. Ultimately, our success lies with the people who dedicate their time to this goal, who bring their creativity, their technical skills, and are empowered with a strong sense of urgency to achieve our goals. No one person or team can achieve this goal, and our team members work in and sustain collaborative team environments equipped with the necessary tools and processes to get the job done.

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Chapter 3

New Paradigms in Crop Protection Research: Registrability and Cost of Goods

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The development and launch of new crop protection agents with novel modes of actions remains an important task for the research-based agrochemical industry to support the global agriculture in its goal to provide food security. However, it seems that major selection criteria for such active ingredients have changed over time, whereas in earlier decades biological activity against target species was the main driver, in our days registrability and cost of goods seem to be in the foreground.

Introduction

Since the earliest days of agriculture, humans have had to protect their crops against yield loss from weeds, plant diseases and insect pests. Seedlings are in competition with undesired vegetation (weeds) for light, space, nutrients and water. Harvest devastation by insects and fungal diseases has been known since biblical times. Many of these problems could be solved over time, but feeding a steadily growing human population remains one of the biggest global challenges. Currently 7 billion people are living on earth, a population which is forecasted to reach 8 billion people in 2025, 9 billion in 2050 and the 10 billion mark at the end of this century, every single day the world's population increases by 200.000 human beings (*1*). All kind of improved technologies, such as state-of-the-art agrochemicals, seeds, fertilizers, mechanization and precision farming have to be integrated into modern agriculture to provide food security also in the future. But at the same time the agricultural yields are more and more under pressure because of increasing environmental stresses of crop plants (e.g. drought, heat, cold, salinity) caused by the climate change and shrinking arable land caused by

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continuing urbanization. The best possible use of existing farm land is a vital necessity, because the number of people fed per hectare will double from 3 in 1974 to almost 6 in 2020 (Table 1) (*2*). Not surprisingly, the global demand for grains, such as maize, rice and wheat, also doubled within the same time frame Table 1) (*3*), further forecast project a 70 % increase from 2000 to 2050 (*4*). Major drivers for this development are

- fuel: because of the rising production and usage of biofuels
- food: based on the already mentioned human population increase
- feed: triggered by changing eating habbits, in particular higher meat consumption because of economic prosperity in emerging markets. It is forecasted that the global meat demand will triple from 1974 to 2020 Table 1) (*3*).

Table 1. Increasing challenges for the global agriculture 1974 – 2020 (*2*, *3*).

	1974	*1997*	*2020*
Number of people fed per ha of planted land	3.0	4.3	5.6
Global grain demand (million metric tons)	1208	1843	2497
Global meat demand (million metric tons)	109	208	327

Regarding the latter topic, the environmental footprint of different meat varities differs significantly and therefore also impacts the global grain demand (Table 2). Poultry requires with 2 kg of grains and 3500 liters of water per kg meat the least amount of agricultural and environmental resources, beef with 7 kg of grain and 43000 liters of water per kg of meat the highest amount (*5*, *6*).

Table 2. Grain and water requirements for the production of different meat varities (*5*, *6*).

	kg's of grain required for	*liters of water required for*
1 kg of poultry	2	3500
1 kg of pork	4	6000
1 kg of beef	7	43000

For six of the most important agricultural crops a scientific study evaluated the influence of chemical crop protection on the yield (Table 3) (*7*, *8*). As it turned out, the application of agrochemicals triples the yield of rice and cotton and doubles the yield of maize, soybean and potato, always compared to the yield without any crop protection. However, it was also estimated that each of these crops currently

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reach only ca. 60 – 75 % of their theoretical maximum yield, mainly based on weeds, pests and diseases which

- cannot be controlled with the available arsenal of agrochemicals
- develop resistance against the marketed mode of action classes

Table 3. Estimated crop losses on six major crops (% of theortical maximum) (*7*, *8*).

	Rice	*Wheat*	*Maize*	*Soybean*	*Cotton*	*Potato*
Native yield without crop protection (%)	23	50	32	40	18	25
Extra yield due to crop protection (%)	40	22	37	34	53	35
Remaining potential, lost to weeds, pests and pathogens (%)	37	28	31	26	29	40

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The development and launch of new crop protection agents with novel modes of actions therefore seems a vital task for the research-based agrochemical industry to support the global agriculture in its goal to provide food security also in the future (*9*, *10*). However, it looks as if major selection critieria for such active ingredients have changed over time, whereas in earlier decades biological activity against target species was the main driver, in our days registrability and cost of goods seem to be in the foreground. This review will explain, how this trend impacts crop protection research.

Registrability

Crop protection chemistry has come a long way from its "alchemic" beginnings in the late 19th century to a high-tech science that supports agriculture and public health. We have witnessed huge advances in terms of shrinking application rates, increased potency and spectrum as well as greater margins of safety to consumers and the environment. Since the introduction of the first modern agrochemicals, the conventional crop protection market has grown considerably and was valued at over 54 billion USD in 2013. However, the number of research-based agrochemical companies has halved within almost twenty years, from 34 companies in 1995 to 17 in 2012 (*11*). And, even more significant for the grower, in 2000 there were 70 new active ingredients in the development pipeline of these companies, whereas in 2012 there were only 28 (*11*). Possible reasons for this decline of development compounds and product launches are

- agrochemicals have generally become increasingly effective (use rates of 10 – 20 g/ha possible in all three indications, compared to kg amounts per hectare several decades earlier), raising the bar for each new generation of products
- hazard-based EU regulation and drive for higher safety margins complicates the search for single compounds fulfilling many different requirements (see further paragraphs in this subchapter)
- increasing research and development costs which have to be invested during the itself increasing time between discovery and first sales of a compound require higher sales volumes and profitability (blockbuster concept). Within this investment enhancement the greatest rise was seen in the costs of field trials (Table 4) (*12*)

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Table 4. Increasing research and development costs and time to market of agrochemicals (*12*).

	1995	*2000*	*2005*
Research chemistry (million USD)	32	41	42
Research biology (million USD)	30	44	32
Research safety studies (million USD)	10	9	11
Process chemistry (million USD)	18	20	36
Field trials (million USD)	18	25	54
Human safety (million USD)	18	18	32
Environmental safety (million USD)	13	16	24
Registration (million USD)	13	11	25
Total (million USD)	**152**	**184**	**256**
Time between 1st synthesis and 1st sale (years)	8.3	9.1	9.8

Not only that these factors are responsible for a smaller number of developmental products in the pipelines of the agrochemical industry, the requirement for manufacturers of established crop protection agents to submit additional data to regulatory authorities as part of re-registration procedures is resulting in the removal of a significant number of active ingredients from the market. This is because some of these older chemicals no longer meet the regulatory standards of today or do not warrant the costly generation of new data. This process is causing growers, particularly of some minor crops, considerable concern as they increasingly find that there are no longer agrochemicals registered to address their needs. For research-based companies this situation is on one hand threatening when revenue streams are phased out, but on the other hand also a great opportunity to invent and commercialize the next generation of highly

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efficacious and even safer products. But overall a lower number of developmental products and therefore also a lower number of new market entries combined with the removal of older market products results in a smaller arsenal of compounds for the control of weeds, insects and fungal diseases (*13*).

To overcome this dilemma the future research and development strategy of agrochemical companies needs both a left-hand and a right-hand shift.

- The left-hand shift focuses on the registrability and the cost of goods of new active ingredients. It addresses success criteria for the product profile.
- The right-hand shift focuses on the re-registration of existing active ingredients. It integrates stewardship and sustainability offers into existing product solutions to maintain them on the market.

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The agrochemical industry experiences the most dramatic changes regarding the registrability of agrochemicals in the European Union. Already the council directive 91/414/EEC, which went into force in 1993, reduced the number of agrochemicals registered in the EU from 831 to 218, which means a loss of more or less ¾ of all agrochemicals!

Of even greater concern to the agrochemical industry is currently the shift in regulatory philosophy from a risk-based assessment to a hazard-based approach, as embodied in the new EU agrochemical registration regulation 1107/2009. Here, a product will not achieve registration or re-registration if it is deemed to be mutagenic, carcinogenic, toxic to reproduction or, as planned for the future, an endocrine disruptor, regardless of the level of the compound that may be encountered. Under the previous legislation, if the expected exposure level that may be encountered following correct application was minimal and well within safety limits, then the risk was considered acceptable and the active ingredient could be registered. Under 1107/2009, any exposure, regardless of level, is deemed unacceptable when a compound triggers the hazard criteria. This situation is expected to lead to a further reduction of the number of EU approved active ingredients. For several established and useful products the classification has changed to the worse which eventually may result in an EU ban of the compound – not because of new data but only due to more conservative interpretation of the existing data. Some of these hazard-based EU cut-off criteria, e.g. linked to endocrine disruption or to concentrations of the active ingredient itself or its metabolites in groundwater, are neither applied in the US, where the EPA still works with a scientific risk-based approach, nor in Japan or Brazil. But regulation 1107/2009 not only impacts the number of available crop protection agents by banning established products, also new active ingredients suffer from huge delays in the EU approval process due to unexpected additional requirements. Some new products are not even submitted in Europe due to a risk of failure and the unpredictability of the approval process. The full implication of this regulation within the EU are still not clear yet, nor whether other regions such as NAFTA, Asia Pacific or Latin America will follow suit. A recently published study forecasts already potential problems for the UK in the control of *Peronospora destructor* (downy mildew) in onions and of *Alopecurus*

myosuroides (black-grass) in cereals because of banned unavailable agochemicals (*14*).

The increased regulatory testing needs can be best demonstrated at the hand of environmental toxicity and environmental fate data required by European and US agencies from 1950 till today (Table 5). Not only the number of test species has grown significantly, also several higher tier refinements, such as detailed studies with all metabolites formed in a content higher than 5 % have increased over the years cost and efforts for the submission of a dossier for a new active ingredient (*15*).

Table 5. Environmental toxicity and fate data requirements of developmental products between 1950 and 2015 (*15*).

	1950	*1990*	*2015*
Environmental toxicity:			
Birds	acute toxicity	acute oral toxicity (2 spp) 8-day dietary toxicity (2 spp) reproductive effects	acute oral toxicity (2 spp) 8-day dietary toxicity (2 spp) reproductive effects
Fish	acute toxicity	acute toxicity (3 spp) early life stage tests bioaccumulation	acute toxicity (3 spp) early life stage tests bioaccumulation
Daphnia		acute toxicity reproduction	acute toxicity reproduction
Shrimp		acute toxicity	acute toxicity
Algae		acute toxicity	acute toxicity
Bees	acute toxicity	acute toxicity	acute toxicity chronic toxicity
Beneficial insects		toxicity	toxicity
Earthworms		toxicity	toxicity
Soil fauna		effects	effects
Non-target terrestrial plants			effects
Aquatic macrophytes			effects
Soil microorganisms		effects	effects
Soil macroorganisms			effects

Continued on next page.

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Table 5. (Continued). Environmental toxicity and fate data requirements of developmental products between 1950 and 2015 (*15*).

	1950	*1990*	*2015*
Sediment dwelling organisms			effects
Environmental fate:			
Soil	residues	adsorption/desorption	adsorption/desorption (increased country requirements for local soils)
		leaching	leaching
		photodegradation	photodegradation
		metabolism (identification of metabolites >10%)	metabolism (identification of metabolites >5%)
			adsorption/desorption and degradation rate studies with metabolites >5%
			anaerobic degradation rate studies with metabolites >5%
Water		mineralization	metabolism/ mineralization
		photolysis	photolysis
		hydrolysis	hydrolysis
			photolysis and hydrolysis rate studies on significant degradates
Soil/water		sediment studies	sediment studies
		biodegradation rate	biodegradation rate
			sediment studies on significant degradates

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The challenge of satisfying increasingly precautionary registrability criteria has stimulated a fundamental shift of emphasis in agrochemical invention. Historically, this focused mainly on biological performance; today, however, equal importance is placed on registrability as an aspect of chemical design, placing particular attention on both environmental fate and behavior and (eco)toxicological hazards. For example, the physicochemical properties required to achieve good herbicidal performance thorugh movement *in planta* may also lead to soil mobility and potential leaching issues. Early exploration of the mutually acceptable chemical space in this respect relies on use of a tiered approach, beginning with simple predictions of relevant properties at the earliest stage of the projects, followed by a suite of increasingly sophisticated medium- to high-throughput assays which enable a more precise evaluation and manipulation of soil behavior alongside efficacy as part of the design-synthesis-test-analysis (DSTA) cycle. In a similar vein, avoidance of undesirable toxicological hazards is being achieved through the application of physiologically-based pharmacokinetic (PBPK) models. Already widely used in pharma in support of efficacy optimization, the same approaches can be applied in agrochemical design to reduce systemic exposure to levels below toxicity thresholds. Such methods are well-suited to the design phase of projects, since they rely on readily accessible pharmacokinetic inputs that can be generated *in vitro*, thus reducing the burden for animal testing, and the outputs can be correlated either directly with *in vitro* measurements of potency against known receptors or indirectly with *in vivo* measurements of biomarkers of toxicity. The emergence of additional areas of increasing challenge for registrability, for example non-target species hazard, will create yet further demand for early-stage inputs that are able not only to identify problem areas but also to provide solutions through contributions to chemical design. This left-hand shift in the application of safety- and registrability-related science represents a new challenge to the industry.

Cost of Goods (CoGs)

The manufacturing cost of an agrochemical is basically linked to its molecular structure. This means that each new design phase in the DSTA cycle (*9*) of a chemical class has its impact on the cost of goods (CoGs) of a potential future product. Some general trends regarding the chemical composition of modern agrochemicals are important to know. More than 70 % of active ingredients introduced to the market within the past 30 years possess at least one heterocyclic scaffold (*16*), which is in general positive because of many existing methods for a quick and efficient assembly of even multisubstituted heterocycles. A similar number (70 %) of todays crop protection agents contain at least one halogen substituent (*17*). Although these halogen atoms play crucial roles either as pharmacophoric function or, in many cases, as ideal substituents for the finetuning of physico-chemical properties of the active ingredient towards a required lipophilicity, the manufacturer has to pay a price, as the very popular fluorine atoms are often not easy to introduce. Figure 1 shows the two highly halogenated insecticides broflanilide (**1**) and noviflumuron (**2**), in which the

numerous halogen atoms play a significant role to achieve the desired biological activity, but certainly also increase the total product cost.

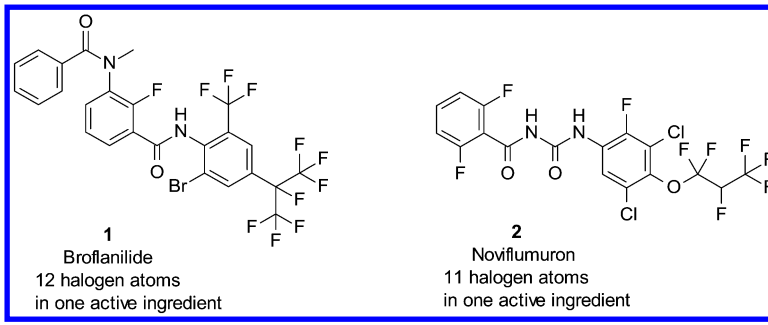

*Figure 1. The multi-halogenated insecticides broflanilide (**1**) and noviflumuron (**2**) (17).*

Around 35 % of all agrochemicals are chiral compounds, a trend that appears to be increasing, however ¾ of all chiral market products are applied as racemates, only ¼ of them is marketed in enantiopure or enantio-enriched form (*18*, *19*). Amongst them are some of the most important and most abundantly manufactured active ingredients, such as the herbicide (*S*)-metolachlor, the fungicide (*R*)-metalaxyl and the insecticide (*S*)-indoxacarb. However, the occurrence of chiral centers in agrochemicals is both opportunity and burden. Often, not always, only one of the different enantiomers or diastereomers is responsible for the desired biological activity and therefore the active principle of the racemic mixture which means that the application of only this specific stereoisomer reduces the use rate and in general the amount of chemicals brought out into the environment. But the large-scale production of such enantiopure or enantio-enriched active ingredients is in many cases much more complicated and expensive than the manufacturing route to the corresponding racemic mixture.

The above mentioned problems of cost-enhancement by number of halogens or chiral centers are only two of several factors contributing to increasing CoGs for agrochemicals. Possibilities to steer against this trend are

- Integration of CoGs-thinking from the beginning into the design of novel compounds
- Dedicated process research resources for each research project starting with the first field trials
- Adequate cost analysis at different project stages
- Continuous investment in CoGs-reducing technologies

A paramount example for the power of CoGs-reducing technologies and a true masterpiece of large-scale crop protection chemistry is the manufacturing of the grass herbicide (*S*)-metolachlor (**5**), which has its basis in the Ir-catalyzed enantioselective hydrogenation of the imine **3** to the (*S*)-metolachlor precursor **4** (Scheme 1). This process is used on a production scale of >10,000 tons/year, for

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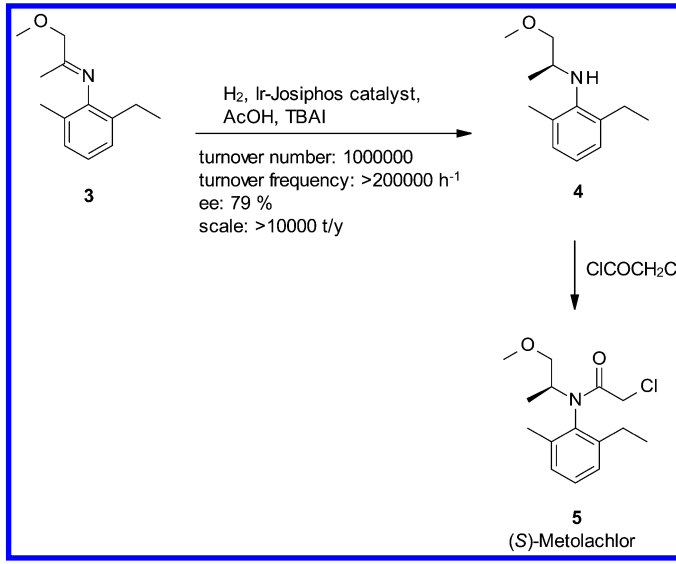
the synthesis of each 10 tons of the chiral amine **4** only 34 g of Iridium and 70 g of Josiphos ligand are required (*20*, *21*).



*Scheme 1. Enantioselective hydrogenation of the imine **3** to the amine **4**, a direct precursor of the grass herbicide (S)-metolachlor (**5**) (20, 21).*

The power of process research and development for the often dramatic reduction of CoGs of an active ingredient and therefore enabling its development and launch to the market is nicely demonstrated by pinoxaden (**16**), a new herbicide for the selective postemergence control of grass weeds in cereals, which inhibits acetyl-coenzyme A carboxylase (ACCase), an important enzyme in the fatty acid biosynthesis. The first synthesis of this graminicide, which was also used for obtaining larger amounts for the first field trials and toxicology studies, was rather lengthy, as most of the altogether ten steps were dealing with the introduction of a methyl group into the *meta*-position of the two ethyl substituents at the phenyl ring and especially with the installation of the required malonate at the phenyl carbon atom between the two ethyl groups (*22*). The phenyl malonate **14** had to be tediously built via 4 different intermediate functionalities, the benzyl chloride **8**, the benzyl cyanide **9**, the phenylacetic acid **12**, the ethyl phenylacetate **13** , because the introduction of a malonyl equivalent into such a highly sterically hindered position of a benzene ring was hitherto unknown. The allocation of massive process research resources enabled the discovery of the transition-metal catalyzed C-C coupling reaction between the aryl bromide **18** and malononitrile, delivering the arylmalonitrile **19**, which can be converted in only three further steps into pinoxaden (**16**) (*23*). The number of steps required for the synthesis of pinoxaden could be exactly halved from ten in the first-generation synthesis to five in the current technical synthesis (Scheme 2).

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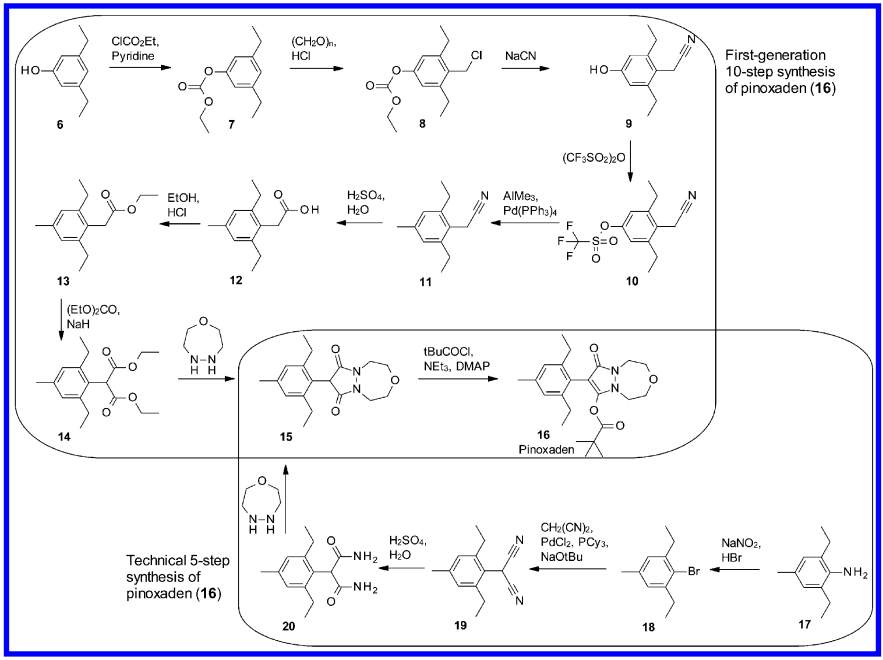

*Scheme 2. First generation synthesis (10 steps) and technical synthesis (5 steps) of pinoxaden (**16**) (22, 23).*

Conclusion

Population growth, rising calorie consumption, resistance development, pest shifts, abiotic stresses and de-registration of older active ingredients will ensure that the agrochemist has plenty to do in the coming decades. The challenge for crop protection research, but also its promise, is to deliver new agrochemicals which

- are efficacious, safe, affordable and can be built into sustainable production systems
- meet requirements of a demanding and uncertain future regulatory environment
- can be used broadly and integrated into crop solutions

To address these mentioned challenges, the new paradigms in crop protection research are

- conducting global regulatory assessments earlier (left-hand shift)
    - toxicology
    - soil mobility and persistance as well as groundwater leaching
    - aquatic ecotoxicity

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- integrating CoGs considerations early in the project lifetime
- developing enantiomerically enriched compounds rather than racemates
- complementing the offer with biologicals

The ideal molecule will give an optimal balance between biological efficacy, registrability and CoGs.

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Chapter 4

Pesticide Invention Model in a Japanese Company

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For R&D-type pesticide companies, it is important and required to supply new products continuously. Under such circumstances, many new pesticides of high originality have been discovered by Japanese companies. For the invention of new pesticides, a deep and wide knowledge, serious attitude towards working on research, and the penetrating insight of researchers are not only desirable but necessary conditions. In this chapter, the author introduces invention models in a Japanese company using two examples of Sumitomo Chemical's approaches. One is the insecticide pyridalyl and the other is the fungicide fenpyrazamine.

Introduction

For R&D-type pesticide companies which discover, develop and commercialize new active compounds, the human resources who carry out R&D, facilities for that purpose, and financial support (R&D funding) are, needless to say indispensable. For carrying out R&D and getting fruitful results, generally speaking, it is more advantageous to secure large budgets. However, the R&D budget is naturally limited. The quality of researchers is therefore important to use the limited budget efficiently and appropriately, and get excellent results. For the invention and discovery of new pesticides, the deep and wide knowledge, serious attitude about working on studies, and penetrating insight of researchers are very necessary conditions. It may be said that in the case of Japanese companies such as Sumitomo Chemical, R&D expenditures are low compared with Western multinational companies. However, many new pesticides of high originality have

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been discovered by Japanese companies. In a recent article titled "An Agchem Innovator" (subtitle: Small by global standards, Japanese agrochemical firms are prolific inventors) in Chemical & Engineering News published November 10, 2014, the author (Jean-François Tremblay) states "dwarfed by their competitors abroad, Japanese pesticide companies punch above their weight when it comes to innovation" (*1*).

In this chapter, the author (N. Sakamoto) introduces invention models in a Japanese company using two examples of Sumitomo Chemical's approaches; one is the insecticide pyridalyl and the other is the fungicide fenpyrazamine. Each of the two pesticides is the first one which belongs to a new chemical class for commercialized pesticides.

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What Kind of a Compound Is Desirable as a New Pesticide?

What kind of new pesticides should researchers pursue in R&D studies? It's necessary for a newly developed pesticide to be complementary to existing pesticides, and further, to possess excellent features substituting for existing ones. It's also an important point that it's a completely new type which is unprecedented in terms of the mode of action and/or the chemical structure.

In the R&D process, it is important to discover new pesticides which correspond to needs that have emerged. Furthermore, it is also important to discover or create potential needs promising as R&D targets.

Comparison between Multinational and Japanese Companies Concerning R&D of New Pesticides

What are the differences between multinational and Japanese companies concerning R&D of new pesticides? The author tries to compare these companies from several points of view. First, multinational companies have big budgets and, without question, surpass Japanese companies in sales force and development capability. Secondly, the number of launched products is also large in multinational companies. Thirdly, the passion is the same between multinational and Japanese companies. It may be comparable between multinational and Japanese companies. Finally, Japanese companies have recently developed many unique chemistries, especially in insecticide fields. Next, some products discovered by Japanese companies are mentioned briefly.

Figures 1, 2, and 3 respectively show new classes of insecticides (including miticides), fungicides and herbicides discovered in Japan, with the names (at that time) of the companies where they were discovered, the launch years and modes of action (*2–5*). The pesticides shown in the Figures are not exhaustive and some old products are also included among them. Many companies developed and launched analogues of imidacloprid, flubendiamide and so on.

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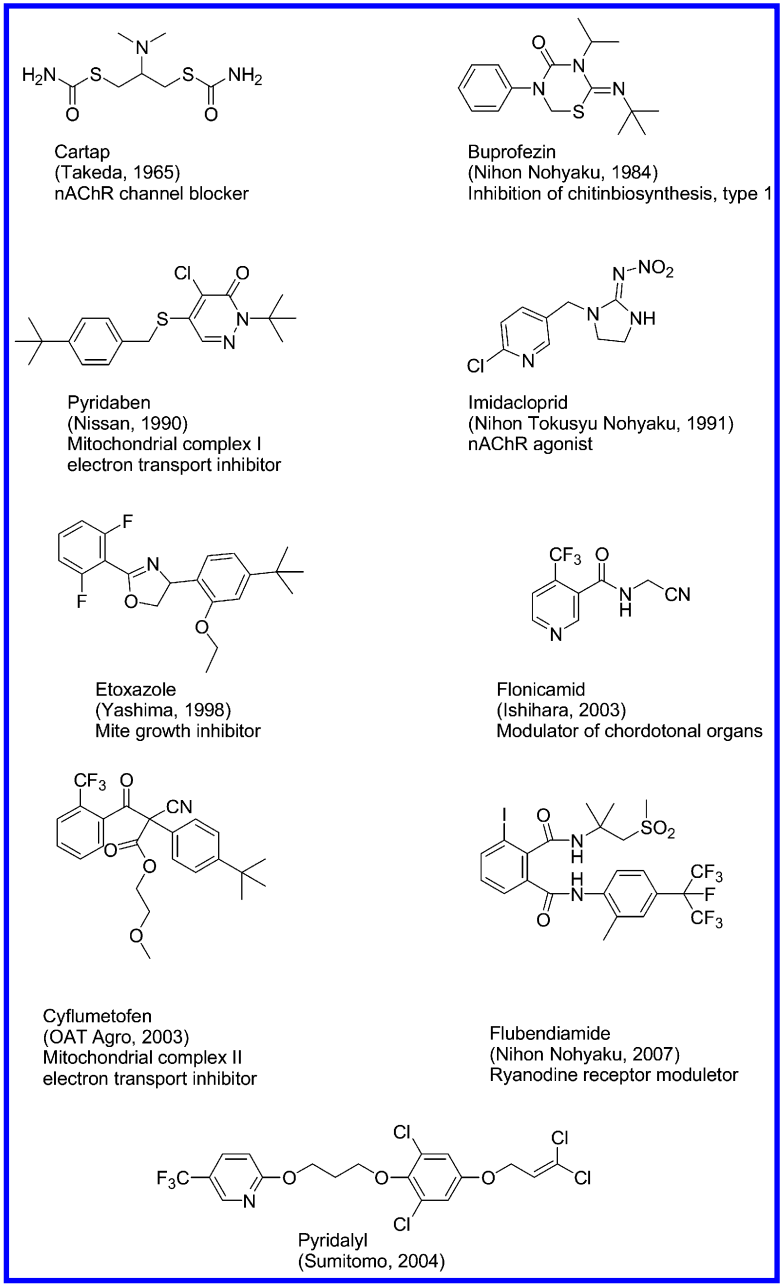

Figure 1. New classes of insecticides discovered in Japan

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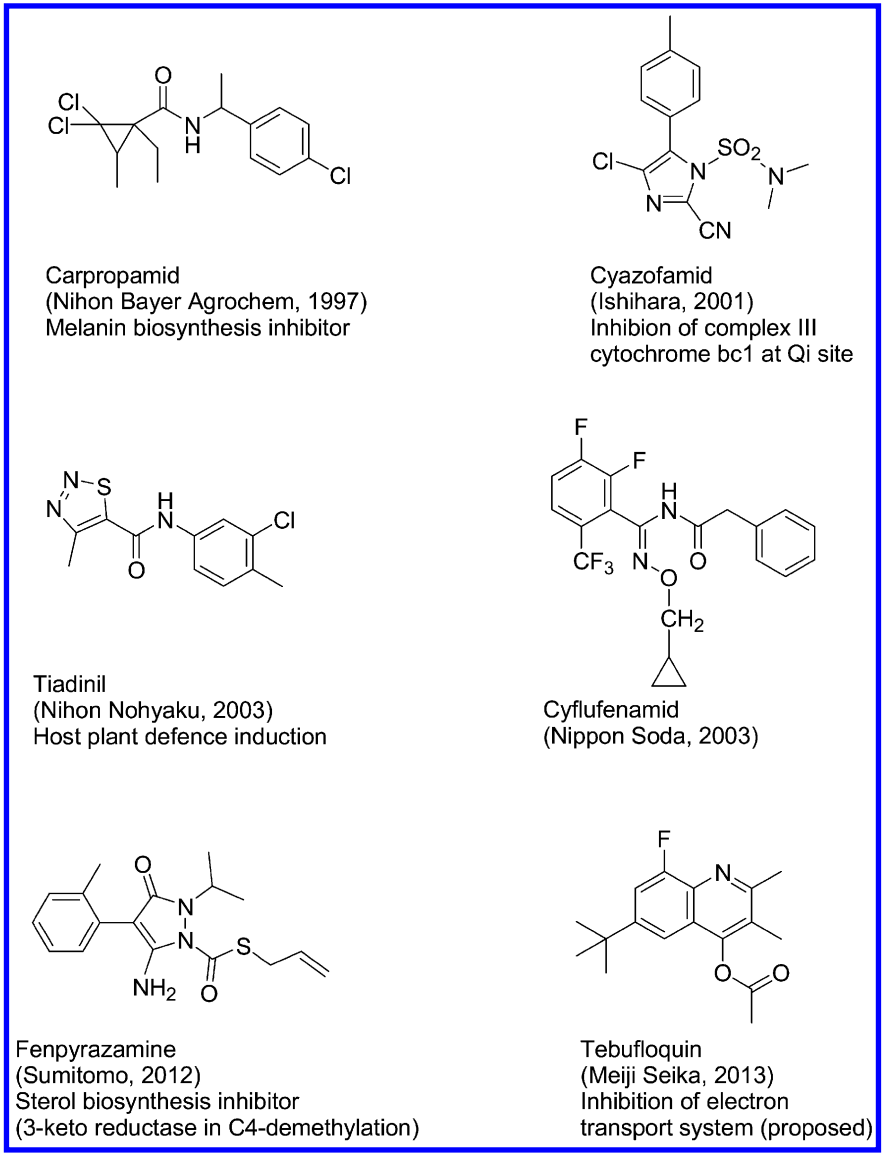

Figure 2. New classes of fungicides discovered in Japan

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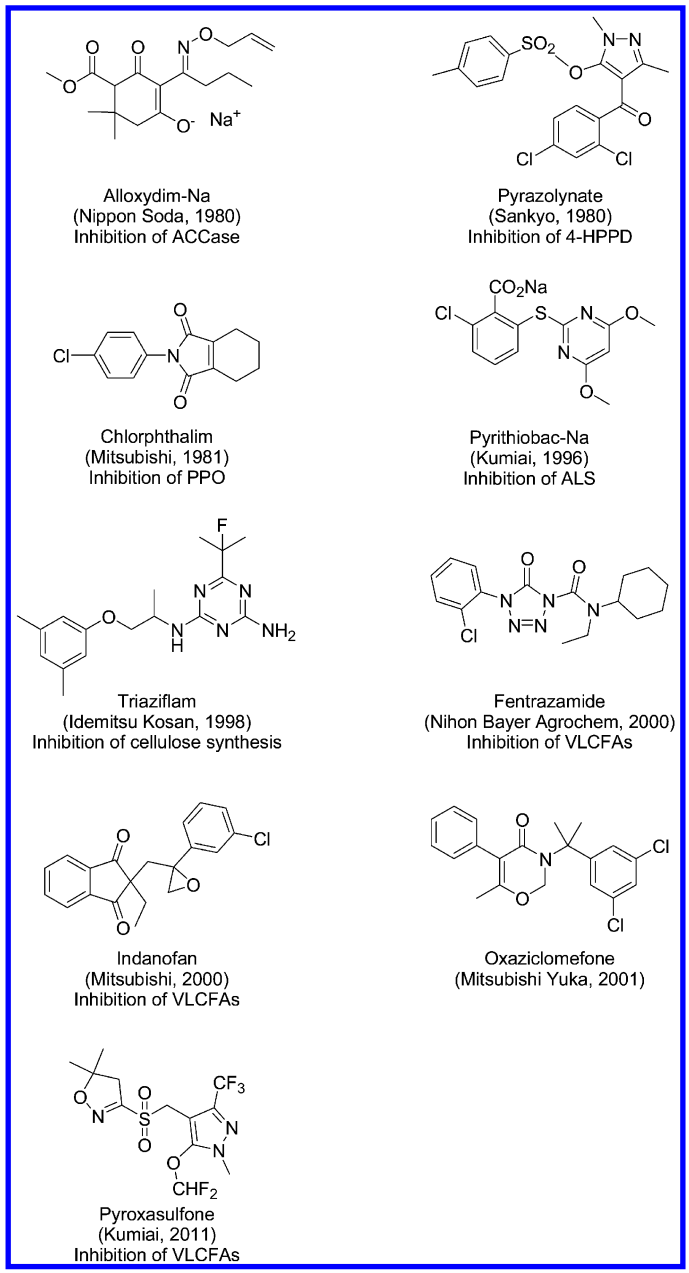

Figure 3. New classes of herbicides discovered in Japan

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What Is a 'Japanese Way'?

As mentioned above, the scale of Japanese companies is by no means large compared to multinational companies. Nevertheless, they have created many new promising chemistries. Why? In the author's opinion, they have a 'Japanese way' as shown in Table 1. First, regarding R&D targets, "Selection and Concentration" is essential under limited budgets. Secondly, regarding organization, (moderate) research human resources are flexibly allocated. When talking about the means for thinking, Japanese companies put importance on conventional technologies as well as advanced technologies. When advancing a study, close observation with an "eagle eye" is very important. Persistence toward the target is also needed.

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Table 1. Japanese way in R&D of new pesticides

Targets	"Selection and Concentration" under limited budget
Organization	Flexible allocation of (moderate) research human resources
Thinking	Digital (advanced) + Analog (conventional)
Research attitude	Close observation with an 'eagle eye'
Research spirit	Persistence

Discovery Pathways

From here, the author explains Japanese invention models with examples in Sumitomo Chemical. Figure 4 represents the upstream stage of pesticide discovery pathways leading to the acquisition of hit compounds. The discovery process can be broadly classified into a target-based one by advanced technologies and a ligand-based one by conventional technologies. The first process needs a precise bridging between in vitro experiments and in vivo ones, but the throughput is higher. On the other hand, the main evaluation from the conventional process is conducted by in vivo experiments and the throughput is lower. R&D in Sumitomo Chemical is carried out using both approaches.

Hit Sources and Their Characteristics

So, where do new hit compounds come from? Hit sources include Scientific journals, Patents from other companies and institutes, General and Proprietary compound libraries, etc., as shown in Table 2.

These hit sources have the characteristics respectively as shown in Table 3. In the case that the hit source is "Scientific journals", "IP FTO" is generally "Good". In "Patents", generally, "Activity level as hits/leads" and "Time to leads" are respectively "Good" and "Short". In "Proprietary library", "IP FTO" is "Good".

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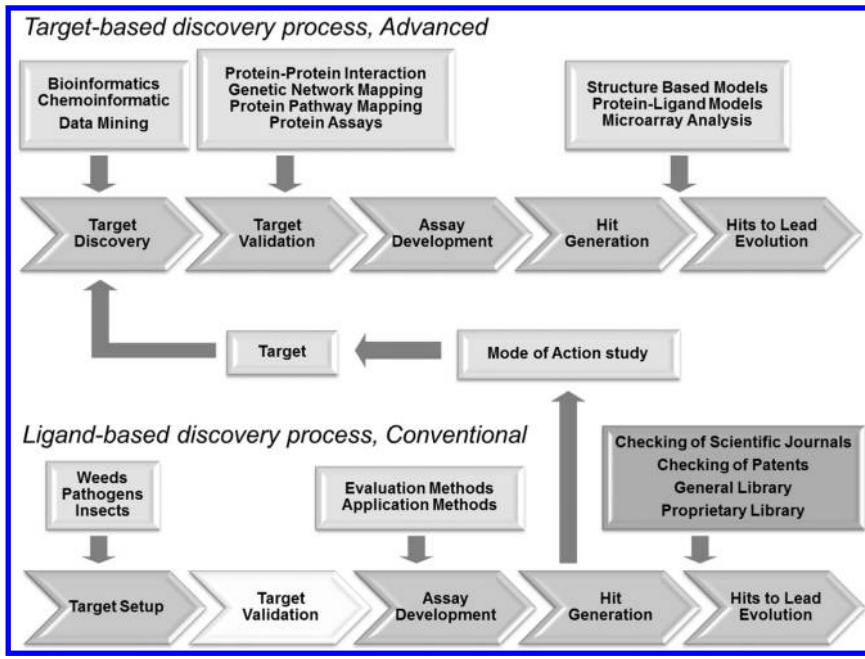

Figure 4. Pesticide discovery pathways in the upstream stage

Table 2. Hit sources in pesticide discovery pathways

Hit sources	*Comments*
Scientific journals	Synthesized compounds which usually have weak activity Natural products
Patents	Newly synthesized compounds which occasionally have good activity
General library	Low level of assurance of activity Available to all
Proprietary library	Originally synthesized intermediates and compounds Some level of assurance of activity Not available to all Unique library by collaboration with academia

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Table 3. Characteristics of different hit sources

Hit sources	*Activity level as hits/leads*	*Chances for new MOA*[a]	*IP Freedom To Operate*	*Time to leads*
Scientific journals	Poor	Case-by-case	Good	Rather long
Patents	Good	Low	Poor	Short
General library	Poor	Case-by-case	Moderate	Rather long
Proprietary library	Moderate	Case-by-case	Good	Long

[a] MOA: Mode of action

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Discovery of Pyridalyl

In the R&D of pyridalyl (see Figure 1 for the chemical structure) (*6*), which is the first and only launched insecticide having a dihalopropenyloxy structure, the hit compounds originated from scientific journals (*7*, *8*). The process leading to the discovery of pyridalyl from the finding of the hit compounds is described below.

Lead Generation

In the R&D of new insecticides leading to the discovery of pyridalyl, we tried to utilize particular known compounds for generating an appropriate lead compound. The researchers first chose lepidopterous pests on cotton, vegetables, and fruits, which had already developed resistance to many existing insecticides at that time, as the target insect pests. Then we carefully examined and screened a number of scientific articles on bioactive compounds. As a result, we were able to identify dichloropropenyl alcohol derivatives, A1 (*7*) and A2 (*8*) shown in Figure 5, which had been reported to regulate insect growth, as hit compounds. Compound A1 had been described to possess anti juvenile hormone activity against lepidopterous species (*7*). On the other hand, Compound A2 had been reported to possess juvenile hormone activity against insects tested (*8*). They assumed that "3,3-dichloro-2-propenyloxy group" as a common structure in both compounds would contribute in some way to the biological activity. Under such a working hypothesis, they initiated the design and syntheses of some 3,3-dichloro-2-propenyloxy compounds to generate a new lead compound.

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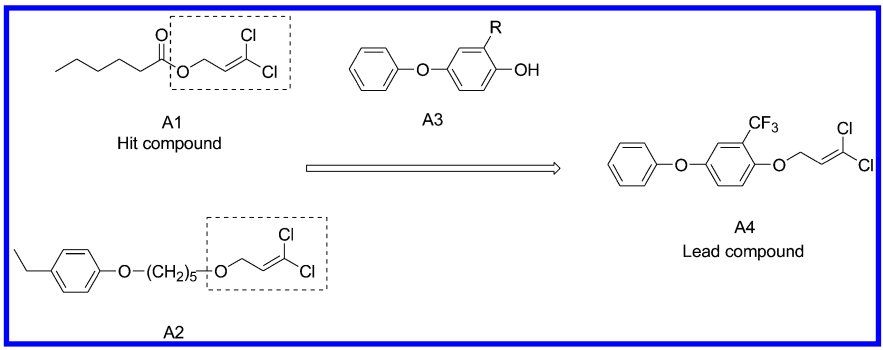

Figure 5. Structure development to the lead compound from the hit compounds

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Each of the compounds A1 and A2 contains a 3,3-dichloro-2-propenyloxy moiety respectively in the form of a substituted alkenyl alkanoate and a substituted alkyl alkenyl ether. In the syntheses of these compounds, we had noticed that the compounds were somewhat chemically unstable. Thus, chemical stabilization was attempted by changing their molecular structures to substituted phenyl alkenyl ethers. We synthesized a variety of derivatives using 4-phenoxy-2-substituted phenols (A3 in Figure 5), synthetic intermediates related to a previous research on insect growth regulator (IGR). Among the derivatives synthesized, 4-phenoxy-2-(trifluoromethyl)phenyl-(3,3-dichloro-2-propenyl) ether (A4 in Figure 5) showed mortality 60% at 500 ppm against tobacco cutworm (Spodoptera litura) larvae. Furthermore, the compound A4 demonstrated certain other interesting characteristics such as unique lethal symptoms, which appeared especially in a topical assay. We turned an eagle eye to the somewhat different lethal symptoms as compared to various existing insecticide standards. We did not overlook the symptoms. As a result, compound A4 was picked out as a lead compound and structure optimization was sought out (*9*).

From the Lead Compound to Pyridalyl

We initiated an optimization program on the lead compound **A4** having two benzene rings, which can be subdivided into 4 parts as shown in Figure 6. It can be said that the right-side benzene ring, to which 3,3-dichloro-2-propenyloxy group is connected, is linked to the other left-side benzene ring via an oxygen atom as a linker.

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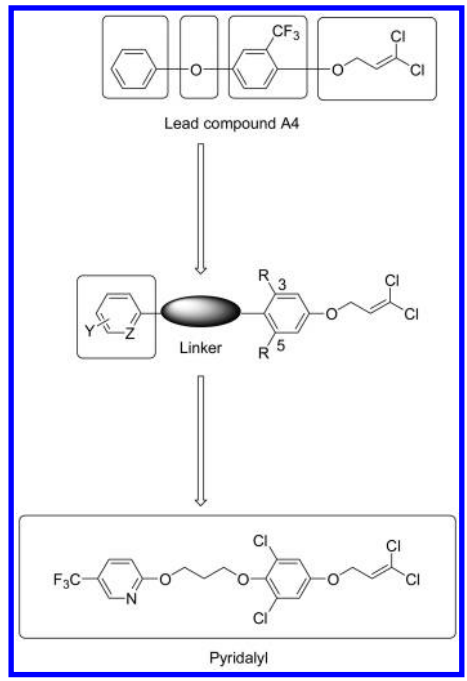

Figure 6. Structure development to pyridalyl from the lead compound

First, structural modifications were performed on the propenyloxy moiety, as well as on the right-side benzene ring. As a result, the flexibility for the modification on the propenyloxy moiety was very small and 3,3-dichloro-2-propenyloxy group was considered to be essential for the activity. For the benzene ring, it was concluded that "having substituents at the 3- and 5-positions" in addition to the 3,3-dichloro-2-propenyloxy group was most favorable for the activity. This effect of the introduction of the substituents at the 3- and 5-positions suggested the possibility that biological activity may be correlated with molecular conformation.

Next, further changes on the left-side benzene ring and the optimization of the linker moiety, which is most likely to affect the molecular conformation, were tried. We found that converting the benzene ring moiety into a pyridine ring or various aromatic heterocycles and the introduction of various substituents on the aromatic ring were acceptable from the point of view of insecticidal activity (*9*). Furthermore, it was found that 1,3- or 1,4-alkylene dihydroxy linker would bring greater insecticidal activity (*9*).

We advanced structure optimization based on the findings described above. Finally, from among some highly active compounds, pyridalyl was selected as the compound for development, based on overall considerations of efficacy, safety, environmental impact, and manufacturing cost (see Figure 6).

Characteristics of Pyridalyl

Pyridalyl having a novel 3,3-dichloro-2-propenyloxy benzene structure exerts excellent control effect against lepidopterous and thysanopterous pests on cotton, vegetable, and fruits (*6*). It shows no cross-resistance to many types of existing insecticides such as organophosphates, pyrethroids, benzoylureas, etc. Furthermore, this insecticide causes quite unique insecticidal symptoms. Therefore, it is suggested that pyridalyl has a different mode of action from any other existing insecticide, although it has not yet been identified (*2*). Pyridalyl is also highly safe to mammals and various beneficial arthropods such as bees, fitting well to IPM (Integrated Pest Management) programs.

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Discovery of Fenpyrazamine

In the R&D of fenpyrazamine (see Figure 2 for the chemical structure) (*10*), which is the first and only launched amino-pyrazolinone fungicide, the hit compound was discovered from a chemical library through collaboration with an external academic (*10*). The process leading to the discovery of fenpyrazamine from the finding of the hit compound is described below.

Beginning of the Study

A compound having a unique chemical structure from collaboration with an external academic showed a disease controlling efficacy on powdery mildew in wheat. Then amino-pyrazolinone compounds **B1** shown in Figure 7, which were conceived from this hit compound, were chosen as the chemical target of discovery research of a new fungicide, and screening processes were initiated.

Discovery of a Lead Compound and Structure Development

The first, on compounds **B1** having *t*-butyl group as each of the substituents R^1 and R^2, we investigated carefully the effect of the substituents R^3n on the benzene ring. As a result we found that compound **B2** (Figure 7), where a chlorine atom was introduced at the *ortho* position (R^3 = Cl), showed a disease control effect against gray mold (*Botrytis cinerea*). This finding was an extremely important one, because the next generation of gray mold controlling agent was being sought in the company at the time. Then compound **B2** was chosen as a lead compound for discovering a new fungicide effective against gray mold.

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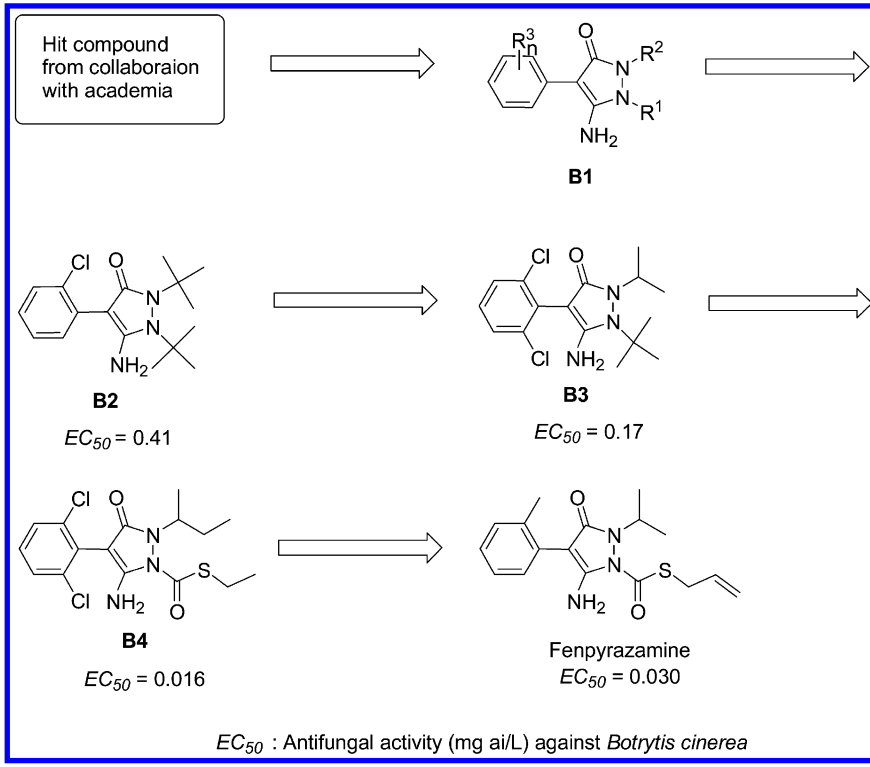

Figure 7. The process leading to discovery of fenpyrazamine

As a result of careful investigation, the outline of structure-activity relationship for gray mold controlling efficacy became clear (*10*). First Structure-Activity Relationship (SAR) was that the introduction of Substituent (R3) at the ortho position of the benzene rings was important to high activity. The second one was that the carbonyl structure in the pyrazolinone ring was essential to high activity. The third one was that it was favorable to high activity that substituents R1 and R2 on the pyrazolinone ring were branched hydrocarbon substituents. And the last one was that the bonding of an amino group (NH2) at the fifth position in the pyrazolinone ring was essential to high activity.

Based on this structure-activity relationship, the researchers synthesized hundreds of compounds with the aim to improve the biological activity and evaluated the activity of these compounds. As a result, they discovered compound **B3** (Figure 7), antifungal activity (mg ai/L) of which against *Botrytis cinerea* was stronger 2-3 folds than that of compound **B2** (*10*). Here, each of substituents R^1 and R^2 of compound **B3** is a branched hydrocarbon group.

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Further Structure Development

Through further improvements toward structure optimization, we found that conversion of the branched hydrocarbon group R^1 to methylthiocarbonyl group drastically improved the efficacy against gray mold. After the re-evaluation of a variety of combinations of the substituents (R^1 and R^2) on the pyrazolinone ring and the substituent R^3 on the benzene ring, they paid attention to compound **B4** (Figure 7), which had excellent activity in gray mold disease control. The activity of compound **B4** appeared to be comparable with or greater than those of benchmarked products. Therefore, it was determined to proceed with development of compound **B4** from the standpoint of biological activity.

However, degradation of compound **B4** in the soil was slower than we expected. Because of this, it was determined that further improvements were required.

Further Study toward Discovery of Fenpyrazamine

In order to solve the problem of the unexpected persistence of **B4** in the soil, we carried out simple soil residue evaluations in parallel with further structure modifications. As a result, we discovered fenpyrazamine, which has a methyl group as the substituent R^3 at the *ortho* position on the benzene ring, and propenylthiocarbonyl and isopropyl groups respectively as R^1 and R^2 on the pyrazolinone ring.

While fenpyrazamine has high activity substantially comparable with compound **B4** in gray mold disease control, it is rapidly degraded in the soil.

As a result of comprehensive consideration to various development criteria, fenpyrazamine was finally selected as the compound for development.

Characteristics of Fenpyrazamine

Fenpyrazamine having a novel amino-pyrazolinone structure exerts high controlling effect especially against gray mold, stem rot, and brown rot in fruits and vegetables (*10*). It has good fungicidal properties, such as high antifungal activity, preventive efficacy, translaminar ability, inhibition activity in lesion development, long lasting activity, and short pre-harvest intervals. Fenpyrazamine also shows safer profiles for human health and the environment.

Fenpyrazamine inhibits 3-keto reductase, as the biochemical target site, involved in the C-4 demethylation during fungal ergosterol biosynthesis as shown in Figure 8 (*10*). The other launched fungicide, whose mode of action is the same as fenpyrazamine, is only fenhexamid (*11*) having hydroxyanilide structure.

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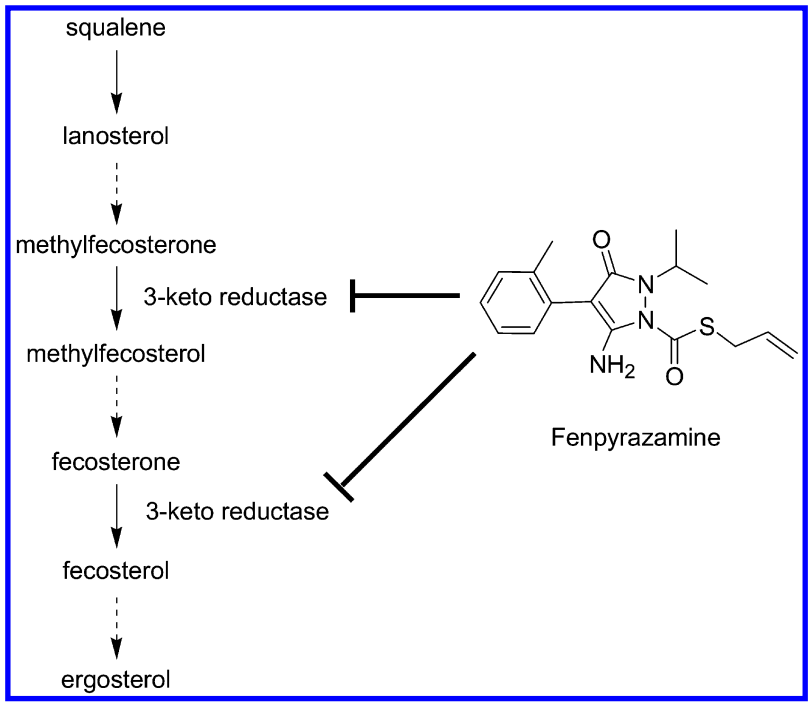

Figure 8. Biochemical target site of fenpyrazamine

We have shown two examples of successful R&D's of our company. Based on these results including other unique studies, we understand that it is important to accept experimental results faithfully and not to expect strong activity at early evaluating stage. And the shortest way to success should be to ordinarily generate hypotheses and implement PDCA (Plan-Do-Check-Act) cycles.

Future Challenges in Discovery Research

As long as research resources and the number of researchers are limited, it is impossible to cover all potential R&D targets. In advancing discovery research, if there are multiple research seeds which could be a subject of study, what should we do? To ascertain whether those compounds have potential, we consider them all tentatively and advance studies on them. But such approaches usually have their limits and only a few can be given priority. Based on what should the order of priority be decided? It is a major problem. Developing insights as to what type of pesticides will be needed worldwide now and in the future is very important. Furthermore, in order to promote discovery research successfully, the close cross-functional cooperation among all members involved is very important.

Finally, for R&D-type pesticide companies, regardless of whether they are Japanese or Western multinationals, it is vital to supply new chemicals continuously. Especially in the case of Japanese companies, they need to accelerate their overseas operations to take greater advantage of their own

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products. The scale of overseas operations (including developments and sales) of Japanese companies is still smaller than one of multinationals. Recently, Japanese companies have increased overseas operations. But most of these operations depend on business alliance with the multinationals. It will be important for Japanese companies which are smaller scale than multinationals to expand overseas regardless of their company scale. Further, it is desirable to shift from being mere technical grade pesticides suppliers to total-solution providers in agriculture as a whole.

Acknowledgments

The author is grateful to the many colleagues within Sumitomo Chemical Co., Ltd. and Valent USA Corporation who have contributed to the discovery and development of pyridalyl and fenpyrazamine, and he appreciates the help received from Dr. Chiyozo Takayama and Mr. Fujio Mukumoto with valuable discussion and assistance in preparation of the manuscript.

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Chapter 5

Natural Products for Crop Protection: Evolution or Intelligent Design

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Natural products have long been a component of agricultural pest control and are considered among the most effective biologic pest control solutions available. Discoveries of natural products, albeit impactful, are rare and taken in the aggregate make it difficult to justify a full scale natural product effort. However, when considering semi-synthetic and nature-inspired discoveries that result from natural product research, the investment is leveraged and has resulted in 50% of the existing modes of action used today. Combined with new techniques in Synthetic Biology, the productivity of discoveries has potential to increase exponentially. Evolution AND Intelligent Design have combined to provide many important pest control products - many more are anticipated.

Natural resources have been employed as pest control tools since the earliest days of agriculture as a means to minimize yield loss from insects and plant diseases. Of course, the discovery of these tools was largely through serendipity and was limited to the use of inorganic chemicals (e.g., sulfur) and botanicals such as nicotine and essential oils. The effectiveness was low by modern day standards, but any level of yield protection (pre and post harvest) was linked to survival of the population (indeed the species), was welcomed.

As the world now anticipates a human population of over 9 Billion by 2050 (*1*, *2*), the importance of pest control for survival continues to be a relevant topic, but the role of natural products per se in an era of chemistry, genetic engineering, and integrated pest management (IPM) is a point of debate. In this chapter, the question

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of whether or not natural products will continue to be of value to agriculture and, if so, in what form, will be addressed. In addition, the future focus for natural products will be explored, with attention on the design of products that build upon nature's own discoveries.

Categories of Biologics

Natural products can be considered as one category of a larger group of pest control products often referred to as Biologics.

As suggested in Figure 1, the wide range of technologies under the biologics umbrella necessitates clarification of what is included in the category of "natural products". In their purest view, natural products must be naturally produced secondary metabolites of organisms created in nature. These are usually insecticidal or fungicidal compounds produced by bacteria or fungi, but examples exist of compounds with nematicidal and herbicidal activity as well (*3*). In the context of IPM, all categories of biologics may be of use, but natural products – by a large margin – have been the most widely used and adopted due to their typically high efficacy, speed of action, and compatibility with conventional pesticide delivery systems. By contrast, the average efficacy and speed of action for most microbial pesticides lags behind conventional and natural product pesticides. Similarly, botanicals, pheromones, and predator/parasites all have some promise in IPM systems, but have significant shortcomings (e.g., supply chain complexity, speed of action) for large-scale agriculture as well as large-scale agricultural product companies. So, natural products seem to hold the most promise, at least in the near future, and warrant further discussion as a target for product discovery.

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Natural, Semi-Synthetic, and Nature-Inspired Products

Gerwick and Sparks (*4*) published an analysis of the role, value, and future of natural products in pest control and outlined clearly their impact to agriculture and the agricultural products marketplace of natural products, semi-synthetic products (referred to by Gerwick and Sparks as Natural Product Inspired) and nature-inspired products (referred to by Gerwick and Sparks as Natural Product Model). In addition, they provided an exhaustive list of examples, some of which are highlighted in this chapter.

The discovery for natural products that meet product performance criteria is a rare event indeed. Notable discoveries over time include abamectin and spinosad (Figure 2), both highly efficacious insecticides in wide use for over 20 years in large scale agriculture, horticulture, and in some cases organic agriculture (*5*). These discoveries, and others like them, have changed agriculture and clearly validated the hypothesis that nature can produce potent pest management tools that meet modern expectations for efficacy while fitting within multi-tactic IPM systems.

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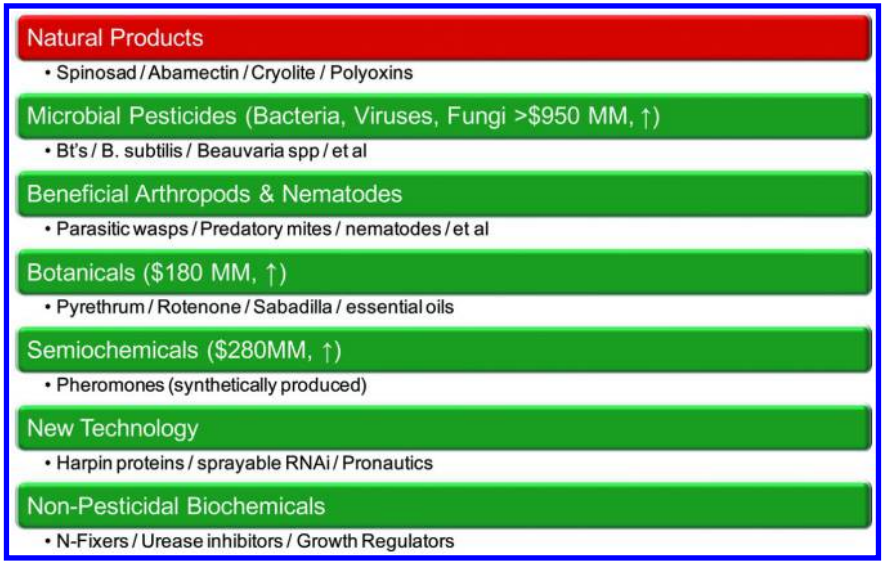

Figure 1. Categories of Biologics used in agriculture for pest control and other areas focused on productivity.

The frequency of discovery makes this proposition very expensive and risky, so adaptation and expansion of the natural product chemistry with limited conventional synthesis has resulted in much more productivity for the chemistry. In fact, natural product chemistry, known as "semi-synthetic" has been far more prevalent in agriculture, leading to outstanding products built upon their natural precursors such as emamectin benzoate and spinetoram. (Figure 3).

In addition to the semi-synthetic modifications to natural products as a means to enhance efficacy (spectrum, duration, and/or potency), toxicology, ecotoxicology, or other product attribute, the natural product scientists have studied the mechanism of action for natural products and formulated hypotheses to construct fully synthetic products that leverage the core mechanism for the natural molecule. Perhaps the most compelling of these natural products leading to naturally-inspired chemistry has been strobiluran-A. This natural compound has inspired the entire class of strobilurin fungicides, none of which actually utilize strobilurin-A as a starting structure. Other compelling examples include the herbicide glufosinate, which is based off of the natural compound bialaphos or the insecticide chlorfenapyr which is derived from the natural compound dioxapyrrolomycin (Figure 4).

The prevalence of these three categories of products (natural, semi-synthetic, nature-inspired) is often under recognized. Gerwick and Sparks (*4*) conducted a survey and identified that 15% of the known modes of action (MoA) are from natural products, 33% of the MoA from natural and semi-synthetic, and 61% of the MoA from the combined natural, semi-synthetic, and nature inspired (Figure 5).

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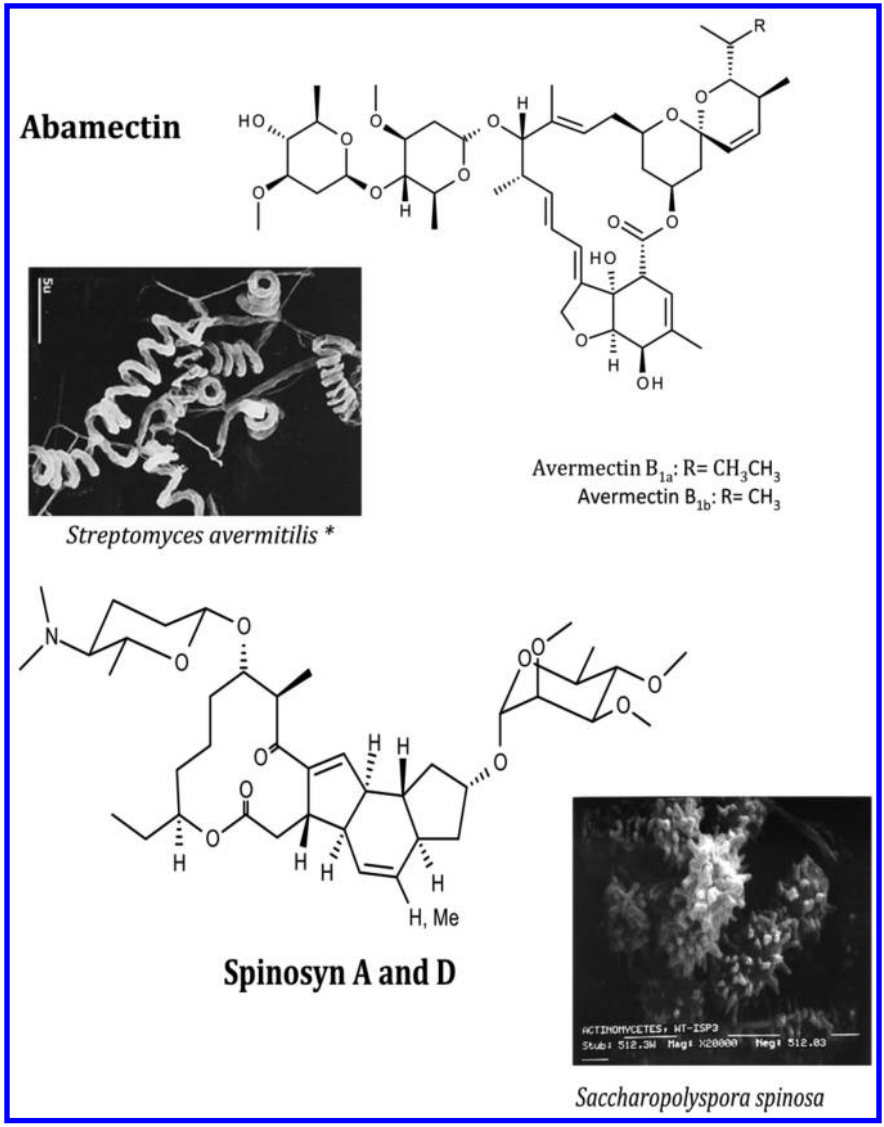

Figure 2. Chemical structure of two natural product insecticides, abamectin and spinosad, along with the respective photographs of the bacterial species.

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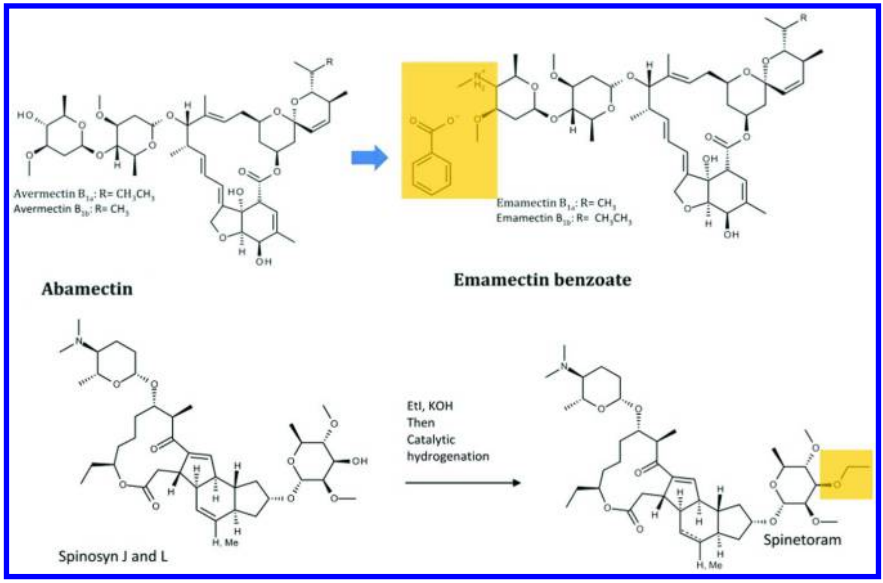

Figure 3. Chemical structure modifications of abamectin and spinosyn to create the semi-synthetic emametin benzoate and spinetoram molecules, respectively.

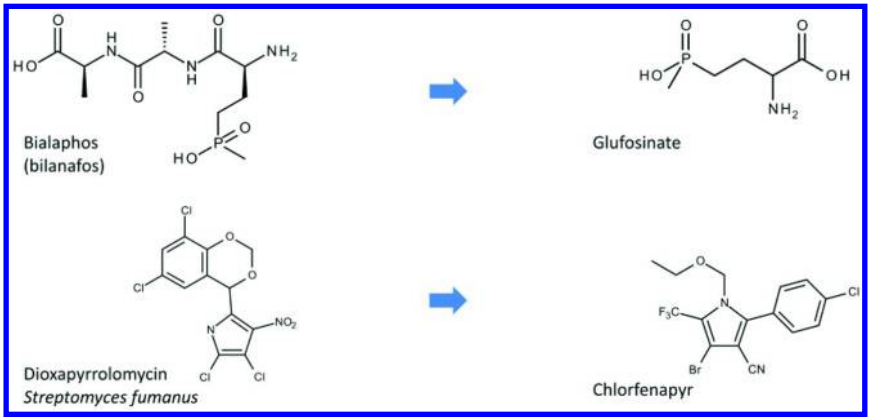

Figure 4. Chemical structures for the fully synthetic herbicide glufosinate and the fully synthetic insecticide chlorfenapyr, inspired by the natural products bialaphos and dioxapyrrolomycin, respectively.

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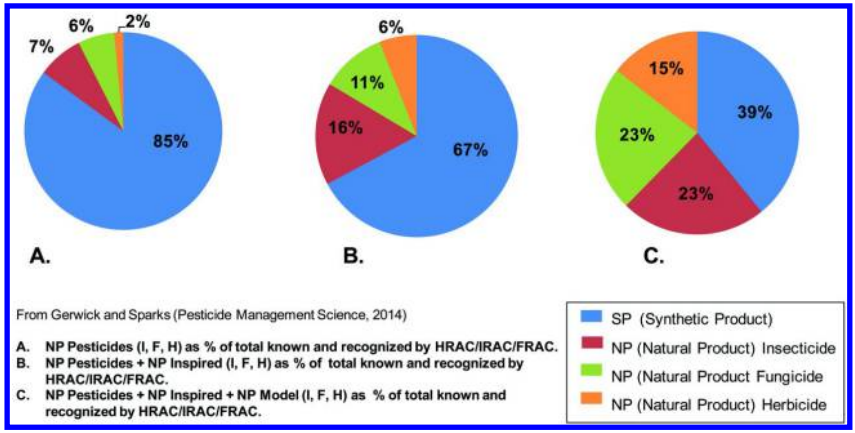

Figure 5. Breakdown of the existing Modes of Action in the crop protection marketplace from natural, semi-synthetic, and nature-inspired approaches to discovery research of insecticides, fungicides, and herbicides.

In terms of impact to the crop protection industry, the products in these categories have been immense – representing up to 50% of the total sales (Figure 6). In contrast, the classical natural product category only represents 7% of the total market, which demonstrates the importance and productivity to the industry of leveraging nature's inventions to meet agricultural challenges through semi-synthetic modification or nature-inspired hypotheses.

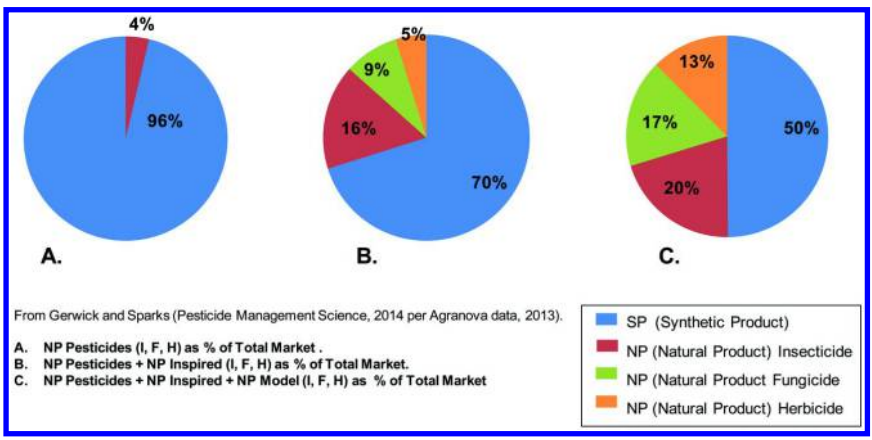

Figure 6. Breakdown of crop protection sales from natural, semi-synthetic, and nature-inspired approaches to discovery research of insecticides, fungicides, and herbicides.

These data clearly demonstrate that natural products have established a solid position as sources for pest control in agriculture. The evolution of these mechanisms and naturally-produced products has taken millions of years of

co-evolution, which is difficult to imitate synthetically. However, understanding the biochemical and biological basis for these products and their interaction with other species has allowed chemists to make rational modifications synthetically to create semi-synthetic natural products that have resulted in improved attributes. Perhaps most compelling is that we have learned from nature and been inspired to formulate hypotheses on fully synthetic compounds that we would have likely not conceived without deep appreciation for their natural state. These methods are excellent examples of "leveraging" nature's evolutionary learning to address modern challenges in agriculture and IPM.

Imagine the learning and progress demonstrated in moving from natural products to nature-inspired products advancing at logarithmic rates. This is the promise and demonstrated potential of synthetic biology – a science that utilizes the principles of engineering and synthetic chemistry to construct biological systems with new or improved functions. Progress with product design is the product of the number of attempts x the quality of attempts. Whereas traditional genetic engineering can produce dozens of constructs in a month/scientist, the advanced materials and algorithms for synthetic biology can produce 10x that amount. In product discovery, speed and frequency of success is a key success factor for all research-based companies – the ability to conduct macromolecular design on this scale is truly game-changing.

The potential for Synthetic Biology is still clearly in the "imagination" phase, but research explorations go far beyond agricultural pest control products and expand into environmental restoration, creation of microbes that clean air, soil, or water, and of course the engineering of crops themselves. In addition, parallel progress is envisioned with advanced materials, cellular network design, and of course health and medicine applications which may intersect with agriculture.

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Summary and Conclusions

Agricultural science must continue to expand the productivity of food production per unit of land to meet the growing demand of the population, which is anticipated to reach 9 Billion by 2050. Pest control, used in a manner that respects the environment, will be critical to our ability to sustain this growth in productivity and will, necessarily, include a wide range of synthetic and biologic pest control methods (*6*). In the wide range of biologic products, natural products produced by microorganisms have been the most impactful and holds the greatest promise. Moreover, expanding the focus to natural products which are modified to be semi-synthetic results in a wide expansion of product mix available to growers. And, creating fully synthetic products based on nature-inspired modes-of-action or structures has expanded the value of a natural product investment broadly. New technologies in the broad category of synthetic biology have dramatically expanded the productivity of engineering organisms to product more or different metabolites, which has potential to truly change the current productivity of discovery for the broad application and utility of natural products – and has potential well beyond pest control products.

Coevolution among organisms over millions of years has created the promise of using nature to address pest control in agriculture (as well as medicine and other applications). But the process of identifying and advancing these natural products to commercial reality is slow, costly, and highly risky – driving most companies toward fully synthetic approaches. However, the value of natural product chemistry goes beyond the occasional isolation of a single winning metabolite, it includes the learning and leverage of applying chemistry and biochemistry to build on nature's template for the betterment of agriculture and mankind – in other words – Intelligent Design. Evolution, combined with Intelligent Design, has proven fruitful and productive and promises to continue to lead to discoveries that will address the agricultural productivity challenge.

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Chapter 6

Leveraging Mammalian Therapeutic Research To Identify Novel Lead Chemistries for Crop Protection

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Driven by pest resistance and the ever changing demands of the market place, the discovery of new modes of action and lead chemistries with the potential to evolve into novel agricultural products is of prime importance to any crop protection discovery program. One proven inspirational resource for hypotheses which can unearth new agriculturally relevant lead chemistries is the medicinal literature. With the tremendous amount of basic and applied research being conducted to understand as well as exploit mammalian biology, biochemistry and the pathogens that act on them, new therapeutic modes of action, target site ligands and/or biologically privileged chemical motifs continually find their way to the open literature. In this paper some strategies to exploit this wealth of information to identify novel insecticidal and fungicidal lead chemistries will be discussed. Several case studies will be given to illustrate some of the advantages and drawbacks in this lead generation approach.

Introduction

The constant development of resistance to commercial pesticides, the emergence of new pest pressures (*1*, *2*) and the changing regulatory environment governing pesticides are among the factors that drive and shape the research towards the discovery of new commercial pesticidal chemical families with novel

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modes of action (MoAs). Among the most challenging steps on the road to discovering new products are the early stages in which an *in vivo* bioactive entity or "hit" is found and exploited resulting in the identification of a structure-activity vein which then proceeds to the discovery of more potent "lead" analogs. Many different strategies, listed in Figure 1, can be employed in finding and in the early exploitation of a hit. Each has its strengths and weaknesses. Some, such as a Target Site approach, are more directive, in that at the outset of the investigation the research team can focus on a commercially underexploited mode of action (MoA). All, however, have proven useful in generating hits and leads at Dow AgroSciences.

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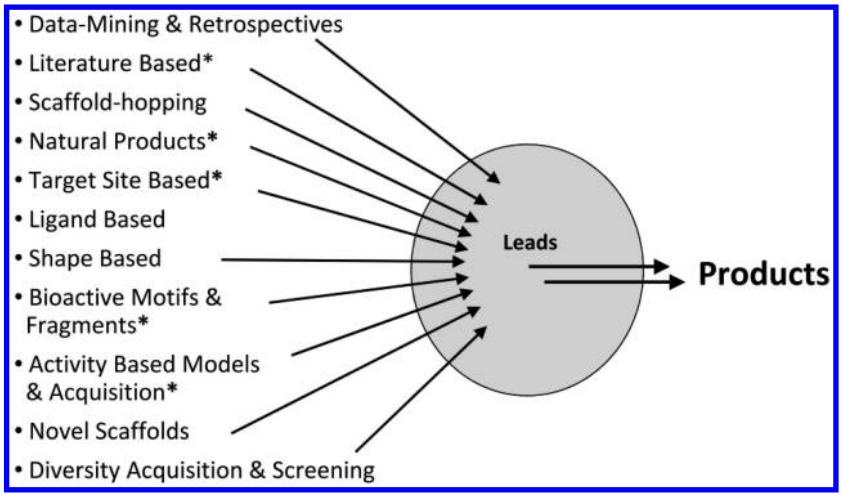

*Figure 1. Agrochemical hit/lead generation strategies. *Strategies encompassed when leveraging the medicinal literature.*

Leveraging the literature pertaining to mammalian therapeutics in the lead generation process is an overarching approach which brings together many of the aforementioned strategies. By definition it is literature based, and since much of human medicinal therapeutic research is target based (*3*), that strategy is often brought in as well. Publications pertaining to screening campaigns based on a cellular or biochemical assay not only report discreet chemical hits, but also families containing privileged bioactive motifs (*4*) which can modulate the target of interest. The hit identified via a screening campaign need not be of synthetic origin. In many cases the first indication that a natural product may have some medicinal utility comes from a target site assay on the pure entity or a complex matrix containing the natural product (*5*). The question arises, does the information from hit/lead generation strategies to support medicinal research have any relevance in identifying chemical starting points for crop protection discovery research?

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Relevance to Agrochemical Research

A number of examples can be found in which common molecules, families of chemistries and/or MoAs have been exploited for agricultural and medicinal purposes. Tobacco, with its active component nicotine (Figure 2), when first brought over from the New World to Europe was touted as a cure for many an aliment (*6*). Now it is the active component in numerous smoking cessation products (*7*). In the interim, nicotine found use as an insecticide, with multi-tons used post World War II (*8*). A more pertinent insecticide example involved different families of molecules which inhibit a common pathway. Fenazaquin (*9*), a member of the quinoline and quinazoline mitochondrial electron transport complex I inhibitors, was developed and launched as a miticide. The complex I respiratory inhibitor Gaunacoentin (*10*), and the acetogenins in general (*11*), have been investigated for their use as oncology therapeutics. In addition, biologics containing acetogenins as a key component have been used medicinally and insecticidally (*12*). Likewise mitochondrial electron transport complex II inhibitors have attracted some interest by cancer researchers (*13*), and have been utilized by the agricultural industry as fungicides (*14*). Finally, the most commercially successful crossover chemical family and mode of action are the fungicidal azole sterol biosynthesis inhibitors. The agricultural triazole Propiconazole and the human antifungal Voriconazole being representative of the numerous commercial offerings from this class of pathogen control agents (*15*).

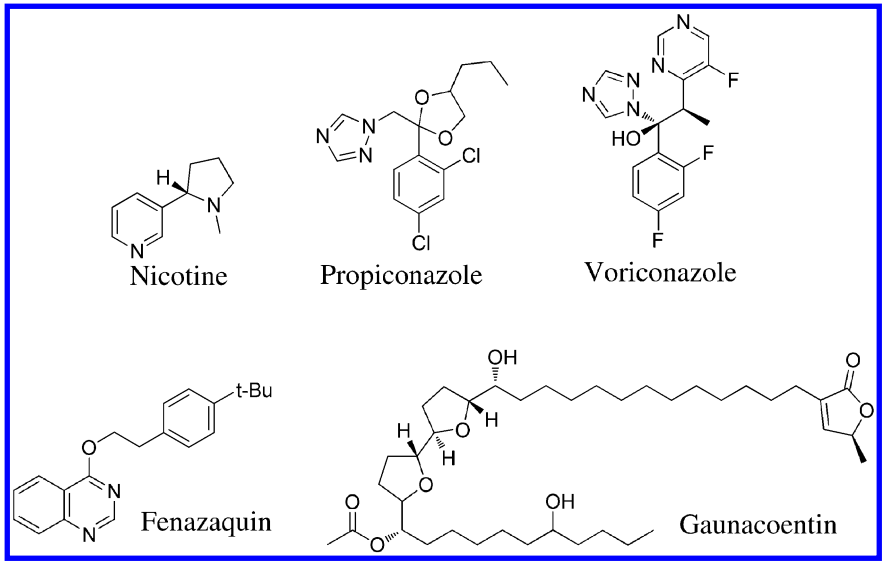

Figure 2. Molecules, chemical families and/or compounds exhibiting a common MoA that have been exploited or investigate for their utility as a pest control agent and medicinal therapeutic.

The examples just given are for chemistries and MoAs that pertain to products. They do suggest that chemistries and/or MoAs that are fully validated with a

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therapeutic product may be a good starting point for agricultural research. But more generally, chemistries and MoA at all stages of the discovery/commercial validation process that are reported in the pharmaceutical literature may be worthwhile to pursue from an agricultural perspective. One advantage of following up on the early stage research is that the number and diversity of opportunities increases as compared to focusing on just commercialized medicinals. Consider in 2011, greater than 80 billion dollars were budgeted by corporations (*16*) and institutes (*17*) with the goal of gaining a better understanding of the human biological and biochemical condition, as well as identifying remedies to address unmet needs, so as to obtain a piece of the 900 billion dollar pharmaceutical market (*18*). The pharmaceutical industry alone used their research dollars to support over one hundred and fifteen thousand research and development (R&D) employees (*19*). The R&D output for 2011 resulting from previous years endeavors allowed for the worldwide development of 5400 compounds (*16*), the filing of greater than 69,000 pharmaceutical and 40,000 biotechnology patent applications (*20*), as well as enough scientific information to fill the requirements of roughly 400 journals focused on medicinal chemistry, biology, biochemistry and/or molecular biology (*21*). So clearly, just considering research pertaining to humans, there is an incredible amount of data that one interested in agricultural applications could sift through and potentially repurpose. The real challenge may be in devising a set of defining principles that increases the probability of finding the gem of a starting point in the sea of information.

Advantages and Hurdles of the Approach

Lead generation strategies can be challenging and risky in that each individual attempt has a low probability of generating an exploitable novel lead. Leveraging mammalian therapeutic research to identify novel lead chemistries for crop protection is no different in that respect. It then becomes important that each hypothesis is readily testable, so that many ideas can be cycled through quickly to find that elusive mammalian bioactive chemistry or MoA that is relevant to pest control. Reports of new mammalian bioactives are almost always coupled to how they were derived either synthetically, or in terms of a natural product, the source from which they were derived. Should similar biologically interesting analogs be reported in multiple studies, it is not unheard of for a commercial compound vendor to offer focused chemical libraries comprised of members which occupy the chemical space surrounding those bioactives. By judiciously choosing to pursue chemistries constructed via concise synthetic routes, or better yet, biologically privileged motifs (*4*) for which vendor libraries are available, one can efficiently obtain compounds needed to test hypotheses related to agronomic utility.

In the interest of cycling through hypotheses quickly, one should choose to leverage assays already in place. Unlike pharmaceutical research, agrochemical research utilizes *in vivo* bioassays directly on the commercial pests of interest. Elucidating the structure activity relationships (SARs) about a medicinally derived

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starting point based on *in vivo* activity offers two distinct advantages. First, time and resources in developing and running an *in vitro* biochemical assay are saved. More importantly, if activity against the organism of interest is observed, it is evident that although the pharmacokinetic properties of the molecule may not be optimal, they are within an acceptable range that enables the compound to translocate to the biochemical target site. The downside with only following *in vivo* activity is that the reason for a ligands failure to elicit the desired agronomic phenotype is unclear. Inability of the ligand to get to the target site, poor binding to the biochemical target, or modulating a pathway incapable of delivering a lethal event, could all result in inactivity. If one can afford the time and resources to run a biochemical assay concurrent with *in vivo* studies, one should consider prioritizing chemical series reported to act via a MoA for which an assay is already known. Often such an assay can be reworked so that enzymes, receptors or channels with higher structural homology to the commercial agricultural pest of interest can be used.

Once a therapeutic MoA or chemotype has been chosen to examine, one is most often faced with a number of options as to which putative ligands to prepare. The most obvious, but not always most appropriate would be to test the material which was reported to be the most potent in the study being leveraged. Unfortunately, these ligands may be more drug-like than lead-like (*22*). The high degree of compound functionalization that comes with a drug-like compound might mean that it is overdesigned to accommodate homology differences between the medicinal biochemical target site and the analogous one found in the agronomic organism. Therefore, one might consider preparing analogs with a range of potencies within space define by Lipinski's rule of 5 (*23*) or an agrow-derived variant (*24*, *25*). Also important, especially when only following a chemical series SAR via *in vivo* assays, is to pick a range of physical properties. This can potentially increase the odds of identifying a ligand which not only modulates a target site, but just as importantly, exhibits the required pharmacokinetic properties to reach the biochemical target *in vivo*. Some additional confidence that delivery issues are not the determinate cause for the lack of activity in the greenhouse can be achieved if compounds are chosen which have demonstrated the ability to pass through a biological membrane. So, prioritizing ligands from a medicinal study that elicit the desired phenotype in a cell-based assay or whole animal model system may be appropriate.

At its best, this approach works by judiciously selecting a medicinal therapeutic pathway and corresponding ligand, identifying an *in vivo* pesticidal hit, and developing that hit into a fruitful SAR vein that yields a lead series which manifests its activity via a MoA analogous to the medicinal derived starting point. With forethought as to what enzyme, receptor or channel family is chosen to follow at the outset; the outcome can be biased towards outputting a pesticidal family of chemistries which act on a commercially underexploited MoA. This need not be the case, however, especially when *in vivo* activity alone, drives the SAR. Just like new drugs can be developed by optimizing a side effect of an existing drug (*26*), a new pesticidal series could evolve from optimizing a secondary biological effect. Likewise, jumping to a totally new MoA cannot be ruled out. Dow AgroSciences' experience with Microcolin A, may be illustrative

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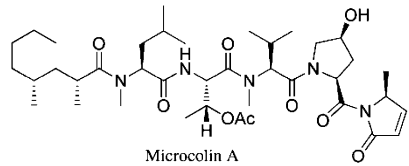
of the latter scenarios. The tetrapeptide isolated from cyanobacterium (*27*), was reported to be immunosuppressant agent over 100 fold more potent than cyclosporin A in *in vitro* assays (*28*). When subsequently assayed at Dow AgroSciences, chewing and sucking pest activity comparable to the commercial standard spinosad was observed (Table 1) (*29*). Although there certainly was an element of coincidence in a material exhibiting insect and immunosuppressive activity, one may argue that focusing research on chemotypes in biologically relevant chemical space predisposes serendipitous outcomes.

Table 1. Insecticidal activity of microcolin A relative to the commerical standard spinosad



Microcolin A

Compound	Tobacco Budworm LC_{50} (ppm)	Twospotted Spider Mite LC_{50} (ppm)
Microcolin A	8	1
Spinosad	1	2

The desired outcome from this lead generation process is not just a chemical series with bioactivity against relevant agricultural targets. Other attributes such as structural novelty from an intellectual property perspective, and safety towards non-target organisms is also desirable in a lead series. In both cases, this could require that at some point the structure activity relationship of the new lead series relative to mammalian and agricultural organisms diverge. Considering that the starting point could have a common MoA on an enzyme, receptor or ion channel that is highly conserved across taxonomic kingdoms and phyla, obtaining SAR divergence can be challenging. In determining if SAR divergence has been achieved from an intellectual property perspective, one only needs to do computer searches to ensure that composition of matter uniqueness has been achieved. Determining if an evolving new lead series is biologically selective may require setting up parallel screens to assay target as well as off target efficacy. The hurdle of achieving selectivity will be further illustrated in the following case studies.

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Case Studies

Using the principles outlined above, a number of chemistries in the literature which are reported to modulate a validated or potentially useful therapeutic pathway have been screened at Dow AgroSciences for insecticidal, fungicidal and/or herbicidal activity. Some of the purported biochemical targets of these chemistries are highlighted in Figure 3. The putative ligands explored include receptor agonists and antagonists, as well as enzyme inhibitors, receptor ligands, and ion channel activators and blockers. Both allosteric inhibitors and chemistries that are believed to act at the same active site as a protein's endogenous ligand have been considered. In the following sections, two examples from Dow AgroSciences Discovery Research will be briefly described and some of the learnings from those endeavors will be discussed. The first case study pertains to an insecticidal lead program that originated from a medicinally derived muscarinic agonist, while in the latter example a reported kinase inhibitor evolved into a fungicidal lead.

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G-protein Coupled Receptors (GPCRs)
- Muscarinic acetylcholine receptors
- Adrenergic receptors (α_2, etc.)

Enzymes
- Acetyl-coenzyme A carboxylase
- Kinases (p38, etc.)
- Synthases (tRNA synthase, thymidylate synthase)
- 2,3-Oxidosqualene-lanosterol cyclase
- Transferases (*N*-myristoyltransferase, farnesyltransferase, geranylgeranyltransferase, etc.)

Ion Channels
- Ligand-gated (nicotinic acetylcholine receptors)
- Voltage-gated (Na^+, Ca^{++})

Figure 3. Ligands that are reported to modulate the mammalian target sites above have been screened for pesticidal activity.

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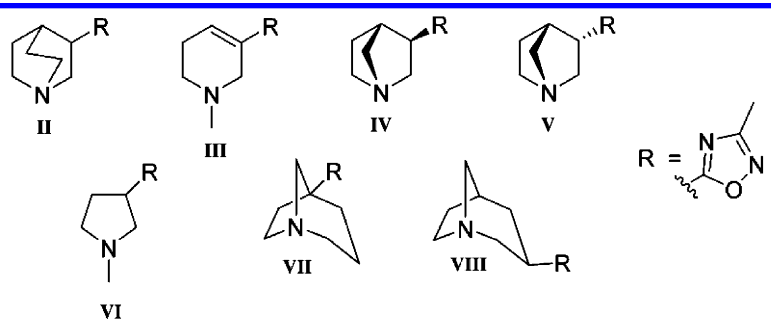
Table 2. Insecticidal activity of cyclic alkyl amines substituted with a 5-methyl-1,2,4-oxadiazole ring



Compound	Brown Planthopper LC_{50} (ppm)	Green Leafhopper LC_{50} (ppm)	Twospotted Spider Mite LC_{50} (ppm)	Cotton Aphid LC_{50} (ppm)
II	4.6	29	3.1	11
III	4.1	14	185	50
IV	0.03	1.6	0.2	0.4
V	NT*	NT*	0.7	2.5
VI	> 400	>400	50	~100
VII	4.9	45	10	6.4
VIII	19	140	20	200

* NT – not tested

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Azabicyclic Insecticides

Researchers at Merck originally reported the 1,2,4-oxadiazole quinuclidine **II**, as a high affinity muscarinic agonist in their study to identify a potential therapy for the treatment of Alzheimer disease (*30*). The fact that insects have muscarinic receptors, that this particular MoA was underexploited from an agricultural perspective and the leadlikeness of these putative muscarinic ligands, prompted an investigation into the quinuclidine chemistry. Very encouraging results were immediately realized with the observation that quinuclidine **II** was potent *in vivo* against a wide variety of pests which feed by sucking the fluids out of plants (Table 2) (*31*). A SAR investigation was initiated in which the

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preferred relative spatial orientation between the 1,2,4-oxadiazole and the basic cyclic amine was probed. Of the six cyclic amines tested, analogs containing the azabicyclo[2.2.1]heptane moiety were most active across the spectrum of sucking pests tested. These results parallel Merck's medicinally driven SAR (*32*). In addition the endo isomer **IV** was slightly more active that the exo isomer **V**. This trend held true for the initial set of analogs prepared. Accordingly, the endo isomer was the diastereoisomer most of interest throughout the rest of this study. Upon expanded biological characterization against non-target organisms, the promiscuous nature of oxadiazole **IV** was identified as an undesirable trait. Analogs with a narrower biological spectrum targeted towards sap feeding pests were sought. Replacement of the 3-methyl group of the 1,2,4-oxadiazole with other substituents did not uncover an analog with compelling sucking pest activity concurrent with decreased off target activity.

In the initial discovery of quinuclidine **II**, the introduction of the 1,2,4-oxadiazole grew out of the Merck research teams' desire to find a hydrolytically stable ester bioisostere for the muscarinic inhibitor arecoline (Table 2, amine **III** when R = CO_2Me) (*30*). As part of the effort to uncover insect and off target SAR divergence, the exploration of other heterocycles as an ester bioisostere was undertaken in the azabicyclo[2.2.1]heptane series. Some of the heterocycles probed are shown in Figure 4. The most promising, from an insecticidal perspective, were the tetrazoles **IXe**, especially those functionalized at the 5-position with an amine. When the 5-amino residue (NR_1R_2) contained one or two small aliphatic groups (Table 3), twospotted spider mite and cotton aphid activity similar to that of the 5-methy-1,2,4-oxadiazole **IV** was observed. Unfortunately, the desired therapeutic index between the insect activity and the off target bioactivity was not obtained. The research in the area was ultimately discontinued when attempts to realize the desired selectivity using a proinsecticide approach analogous to what was successfully employed with the carbamate insecticides (*33*) failed to achieve the project goals.

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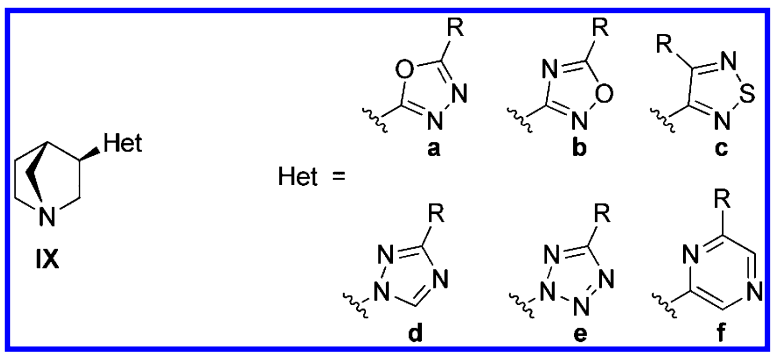

Figure 4. Sampling of 3-endo substituted 1-azabicyclo[2.2.1]heptanes that were investigated for insecticidal activity.

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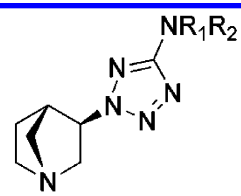
Table 3. Insecticidal activity 5-amino substituted endo 3-tetrazolazabicyclo[2.2.1]heptanes



Compound	R_1, R_2	Twospotted Spider Mite LC_{50} (ppm)	Cotton Aphid LC_{50} (ppm)
X	H, H	1.0	1.7
XI	H, Et	0.1	0.3
XII	Et, Et	0.3	0.2
XIII	H, Pr	0.5	0.7
XIV	H, CH_2cyclPr	0.3	0.6
XV	H, CH_2Ph	2.5	6.2

Interestingly, biochemical assays using housefly tissue demonstrated that the azabicyclic insecticides interact with housefly muscarinic receptors. Furthermore, highly correlated regression relationships between the housefly muscarinic binding affinity and the insect activity of the azabicyclo[2.2.1]heptanes were found. This suggested the toxicity of theses insecticides is directly related to their interaction with the muscarinic receptor (*31*). This then demonstrates one of the potential advantages of this lead generation approach suggested earlier. MoAs can be maintained as the chemical series, designed initially to meet a medicinal need, are morphed to maximize agricultural utility. Again, this can be a powerful tool to bias research towards developing pesticides which act on commercially underexploited MoAs. This case study on the azabicycles also demonstrates two significant drawbacks with the approach. First, when one deals with ubiquitous MoAs, obtaining selectivity in bioactivity can be problematic. Secondly, finding the initial bioactive hit, from which a lead series is grown, can be a low probability event. Indeed, subsequent screening of a number of other chemotypes reported to be mammalian muscarinic agonists or antagonists did not uncover any other insecticide hit that exhibited any significant *in vivo* insecticidal

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activity in the most sensitive standard screens at Dow AgroSciences. Some of the motifs explored are shown in Figure 5.

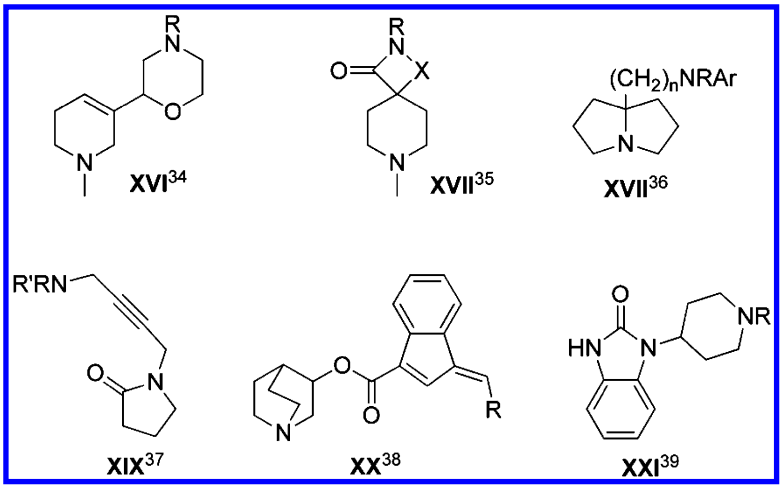

Figure 5. Chemotypes of putative muscarinic antagonists and agonists which exhibited no significant insecticidal activity in Dow AgroSciences primary screens.

Cyclopropylaminopyrimidine Fungicides

Although many different kinases that enable critical cell functions are present in fungi, ligands which selectively inhibit a kinase or non-selectively inhibit a family of kinases have not been exploited to any significant extent commercially for control of agronomically important pathogens (*40*). Analogs of pyrimidine **XXII**, in which the distal phenyl and pyridyl rings are functionalized, were identified as serine/threonine p38 kinase inhibitors by GlaxoSmithKline (GSK) researchers while searching for anti-inflammatory agents (*41*). Allowing for the possibility that the ligands reported by GSK were too specifically designed for this mammalian mitogen-activated protein (MAP) kinase orthologue, the bare pentacyclic core was prepared and assayed. Pyrimidine **XXII** exhibited reasonable *Septoria tritici* (leaf blotch) protectant activity, and a hint of fungicidal spectrum (Table 4). Curative activity, an attribute deemed important for this particular research program, was not noted. It was hypothesized that physical property characteristics of pyrimidine **XXII**, such as low water solubility and high melting point, were limiting factors adversely affecting bioactivity. Thus working toward analogs that conform more closely to the Briggs rule of three (*25*) for systemic compounds, the phenylpyrazolo[1,5-a]pyridine tricycle was replaced with various phenyl or single ring heterocycles groups targeted to improve compound delivery with a more water soluble, lower melting inhibitor. Marginal progress toward project goals was achieved with the 4-thiazolylpyrimidine **XXIII** and 4-phenylpyrimidine **XXIV**. The former exhibited increased potency as a *Puccinia recondita* (brown rust) protectant and improved *Septoria* curative

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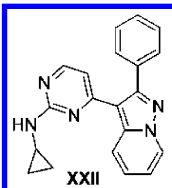
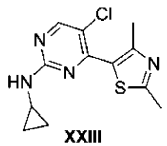
control was observed in the latter. Inverting the pyrimidine core yielded analogs with melting points 50 to 100 °C lower than pentacycle **XXII**. A significant gain in *Puccinia* protectant efficacy was observed with the 2-arylprimidines (**XXV - XXVIII**), but only pyrimidine **XXVIII** exhibited any notable increase in *Septoria* protectant control. More importantly, though progress toward designing fungicidal compounds with targeted physical property attributes was made, no significant curative activity was achieved. Since these analogs prepared were not able to deliver on that key project goal for this series, efforts around this lead were discontinued.

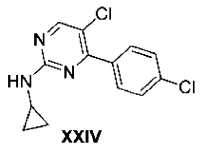
Table 4. Fungicidal activity of cyclopropylamine pryimidines



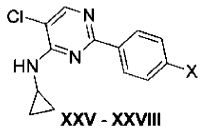
XXII



XXIII



XXIV



XXV - XXVIII

Compound	*Septoria tritici* Percent Control 1 Day Protectant (50 & 200 ppm)	*Puccinia recondita* Percent Control 1 Day Protectant (50 & 200 ppm)	*Septoria tritici* Percent Control 3 Day Curative (50 & 200 ppm)	Substitution X
XXII	67/90	0/50	0/0	-
XXIII	29/39	38/85	0/0	-
XXIV	61/91	0/53	50/50	-
XXV	38/90	99/99	0/0	H
XXVI	65/95	97/99	0/0	Me
XXVII	74/85	97/99	0/0	OMe
XXVIII	90/97	93/97	0/0	Cl

Through the entirety of the cyclopropylaminopyrimidine fungicide investigation, the research direction of the project was driven by greenhouse biological observations. No kinase assay was employed, so it is unclear if the fungicidal 4-cyclopropylaminopyridines **XXV-XVIII** are MAP kinase inhibitors, let alone selective for p38. What can be stated is that the research ultimately led to fungicidal compounds of interest with novel composition of matter (*42*).

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Conclusion

Leveraging mammalian therapeutic research to identify novel lead chemistries for crop protection is a validated approach which brings together several strategies for the identification of leads. Like many lead generation strategies, this approach has a low success rate in uncovering an initial bioactive hit and evolving it into a research vein that could potentially deliver a commercial agrochemical product. To help mitigate the low probability of finding the key chemical starting point from the vast sea of pharmaceutical related information, several tactics should be considered. From a chemistry as well as ligand uptake and delivery perspective, compounds derived from medicinal research that discloses bioactive lead-like structures, have physical properties in the range of commercial agricultural products and that have shown the ability to cross biological membranes should be prioritized. From a biochemical perspective, one can focus on mammalian therapeutics shown to modulate enzymes, receptors, and ion channels that have isoforms or analogous pathways in agriculturally relevant organisms. While there are no guarantees that the agriculturally directed lead will share the same MoA as the medicinally derived starting point, results disclosed here demonstrate that it is possible. Therefore, as older commercial agrochemical MoAs fall out of favor or become ineffective due to pest resistance, the quest to replace them with active ingredients that act on underexploited biologically pathways will continue. Thus, we anticipate this approach to remain an important modus operandi of the agrochemical discovery scientist.

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Chapter 7

Discovery of New Herbicide Modes of Action with Natural Phytotoxins

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About 20 modes of action (MOAs) are utilized by commercial herbicides, and almost 30 years have passed since the last new MOA was introduced. Rapidly increasing evolution of resistance to herbicides with these MOAs has greatly increased the need for herbicides with new MOAs. Combinatorial chemistry and gene knockout approaches have not led to commercial herbicides with new MOAs. The question arises as to how many good herbicide MOAs exist. The relatively little that we know of the MOAs of natural phytotoxins that can effectively kill plants suggests that there are herbicide MOAs yet to be exploited. Some of these targets are questionable because of potential toxicological problems, but many others are not. The MOAs of natural phytotoxins are discussed and strategies to maximize discovery of new MOAs with natural products that might be used as herbicides are presented.

Introduction

New herbicide modes of action (MOAs) are needed to counter the rapidly increasing evolution of herbicide resistance (*1*). The last new MOA was introduced almost thirty years ago, when herbicidal inhibitors of hydroxyphenylpyruvate dioxygenase (HPPD) reached the market (*2*). The number of herbicide MOAs of commercial herbicides has remained around 20 for

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decades, while those for commercial insecticides and fungicides have risen to 28 and 41, respectively (*3*–*5*).

The reasons for the paucity of herbicide MOAs have been debated among those involved in discovery of new herbicides. Clearly, investment in new herbicide discovery waned considerably after the highly successful combination of glyphosate with glyphosate-resistant crops removed a large part of the profit from the global herbicide market. The question of how many viable herbicide molecular target sites exist has been debated without resolution. Have we passed the point of diminishing returns? Molecular biology studies and natural products research suggest that there are still many unexploited target sites. Numerous new potential herbicide target sites were identified by Bayer and other scientists by knocking out expression of genes for production of these potential target sites (reviewed in ref. (*6*)). But, no new herbicides have resulted from these studies, apparently because of difficulties in finding good chemical inhibitors (cost effective, highly active, and safe) for any of these sites.

There are highly effective natural phytotoxins that kill plants by MOAs that are entirely different than the 20 currently used MOAs (*7*–*9*). Thus, we have knowledge of other good molecular targets (i.e., MOAs), but this has not translated into new products that interact with these sites. This chapter will briefly chronicle the few successes with natural products translating into new commercial herbicides with new MOAs, followed by some examples of target sites of nautral products that could result in new products. We will then discuss some of the hindrances and opportunities in using natural products for MOA discovery and new product development.

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Examples of Past Successes

Of the 20 MOAs used, only two have come from natural products. They are glutamine synthetase (GS) and HPPD. The very successful non-selective herbicide glufosinate is the synthetic version of the *Streptomyces* spp. product L-phosphinothricin (*10*). The synthesized version of the compound is a racemic mixture of L- and D-phosphinothricin, of which, only the L-form is active. A tripeptide (two alanines and phosphinothricin), bialaphos (aka bilanafos), produced by fermentation has been a minor herbicide in Japan. This compound is a proherbicide with no activity as a GS inhibitor, but is converted into L-phosphinothricin by the target plant. Transgenic, glufosinate-resistant crops are increasing the use of glufosinate, especially in places where glyphosate-resistant weeds have become a major problem. Although there are several other good natural GS inhibitors (*e.g.*, tabtoxin, oxetin, and methionine sulfoximine – aka glabrin), glufosinate and bialaphos are the only commercialized herbicides with this MOA.

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Two major categories of HPPD inhibitors appeared at about the same time; the triketones and the isoxazoles (*11*). Of these, the triketones were discovered as the result of the study of natural triketones, starting with the putative allelochemical leptospermone (*12*). No natural triketones are sold as herbicides, even though they possess many of the physicochemical properties of commercial herbicides (*13*), and one of them, grandiflorone, a constituent of the essential oil of *Leptospermum scoparium*, is almost as active as the commercial triketone herbicide sulcotrione against HPPD in an *in vitro* assay (*14*). Natural oils rich in HPPD-inhibiting triketones have herbicide activity in soil, causing bleaching of emerging plants (*15*).

There are other herbicides that may have natural products origins. For example, the structure of the minor herbicide cinmethylin is closely related to the natural phytotoxin 1,4-cineole (discussed in 8), but no clear connections between the two compounds have been published. A recent report suggests that the target site of cinmethylin is tyrosine aminotransferase (*16*). There are structural similarities between several of the other herbicide classes and natural compounds, but discovery and MOA links do not exist or have not been divulged. Furthermore, there are a number of natural phytotoxins that have MOAs of commercial herbicides. But the discovery of the synthetic herbicides was independent of any knowledge of natural products. For example, one of the first herbicide MOAs introduced was inhibition of photosytem II (PSII) of photosynthesis, with examples such as diuron and the triazines. Subsequent to the discovery of PSII as a MOA, several natural PSII inhibitors from both plants (*e.g.*, sorgoleone and sarmentine (*17*, *18*)) and microbes (e.g., cyanobacterin (*19*)) have been found. Gerwick and Sparks (*20*) detail a number of similar examples of natural compounds with MOAs in common with commercial herbicides. In summary, only two of the approximately 20 commercial MOAs can clearly be shown to have come from natural phytotoxins.

Gerwick and Sparks (*20*) recently analyzed the role of natural products in the discovery of all pesticides. Of the commercial MOAs they found approximately three-fold more fungicide and insecticide MOAs to come directly from natural products than herbicide MOAs. Considering all the pesticide MOAs, about 6, 11, and 16% of the commercial MOAs were from natural products or natural product-inspired compounds for herbicides, insecticides and fungicides, respectively. However, when adding MOAs that could have been derived from natural compounds with those MOAs, many other MOAs could have come from the study of natural compounds (Figure 1). They have calculated that although less than 0.1% of the $US 24.9 billion 2012 herbicide market are natural compounds, 10.2% of the market are synthetic compounds that had natural compound starting points. The percentage values in Figure 1 are of total pesticides. When calculated as a percentage of 2012 herbicide sales, about 33% of that market is natural products, natural product-inspired compounds, plus MOAs that could have been derived from a natural compound (*20*).

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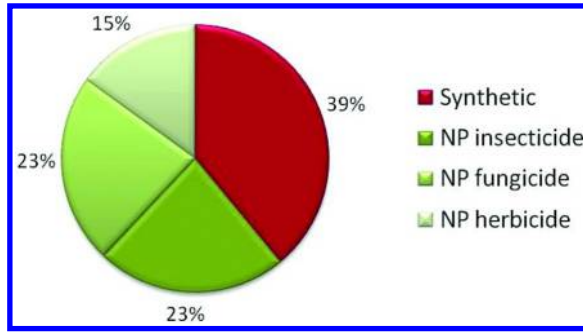

Figure 1. The percentages of known commercial MOAs of pesticides that were derived from natural products + natural product-inspired products + products that could have been discovered by study of natural products as a percentage of the total compounds known and recognized by HRAC, IRAC and FRAC. Redrawn from (20). (see color insert)

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The Potential for New Modes of Action

The MOAs of most natural phytotoxins have not been determined, but of those that are known, many are not those of commercial herbicides. These many novel MOAs have been detailed in previous publications (*e.g.,* (*7*, *9*)). Table 1 provides a sampling of novel MOAs of natural phytotoxins. There are more examples of active compounds than those provided for some of the target sites listed. Some of the MOAs would be of little interest because of the clear toxicity concerns. For example, AAL-toxin is highly toxic to plants, but its MOA, inhibition of ceramide synthase (CS) (*51*), is the same as the mode of action of a series of mycotoxins that are highly toxic to mammals such as the fumonisins, which are also quite phytotoxic (*52*, *53*). Efforts to find strong inhibitors of plant CS with very low effects on animal CS have not been successful (*53*). Industry is wary of any compounds (*e.g.*, aphidicolin, hydantocidin) that might affect such universal processes as nucleic acid or protein synthesis. Despite this, the pesticide industry generated numerous patents on analogues of hydantocidin as herbicides.

Table 1. Some phytotoxins and their non-commercial herbicide molecular target sites (expanded from ref. (*8*)).

Compound	*Target site ir MOA*	*Ref.*
AAL-toxin	Ceramide synthase	(*21*)
Acivicin	Glutamate synthase	(*22*)
Actinonin	Plastid peptide deformylase	(*23*)
Anhydro-D-glucitol	Fructose-1,6,-bisphosphate aldolase	(*24*)
Aphidicolin	DNA polymerase α and δ	(*25*)

Continued on next page.

Table 1. (Continued). Some phytotoxins and their non-commercial herbicide molecular target sites (expanded from ref. (*8*)).

Compound	*Target site ir MOA*	*Ref.*
Bestatin	Aminopeptidase	(*26*)
Carbonic coformycin	AMP deaminase	(*27*)
Cerulenin	β-Ketoacyl-ACP synthase	(*28*)
Cornexistin	Aminotransferase	(*29*)
Coronatine	Jasmonate receptors	(*30*)
Cyperin	Enoyl reductase	(*31*)
7-Dehydrobrefeldin A	Golgi assembly	(*32*)
Fosmidomycin	1-Deoxy-D-xylulose-5-phosphate reductoisomerase	(*33*)
Gabaculin	Glutamate-1-semialdehyde aminotransferase	(*34*)
Gostatin	Amino transferase	(*35*)
HC-toxin	Histone deacetylases	(*36*)
Helminthosporium carbonum-toxin	Lysine deactylases	(*37*)
Hydantocidin	Adenylosuccinate synthase	(*38*)
Hymeglusin	2-Hydroxy-3-methylglutaryl CoA synthase	(*39*)
Lactacystin	Proteasome interference	(*40*)
5-Methyl-tryptophan	Tryptophan synthase	(*41*)
Mimosine	Ribonucleotide reductase	(*42*)
Ophiobolin A	Calmodulin antagonist	(*43*)
Phaseolotoxin	Ornithine transcarboxylase	(*44*)
Rhizobitoxin	β-Cystathionase	(*45*)
Rhizoxin	β-Tubulin destabilization	
Streptomycin	Plastid 30S ribosomal subunits	(*46*)
T-toxins	Membrane destabilizer	
Tagetitoxin	Plastid RNA polymerase	(*47*)
Taxol	Microtuble hyperstabilization	
Tentoxin	CF_1 ATPase	(*48*)
Thaxtomin	Cellulose synthase	(*49*)
Toyocamycin	Auxin signaling	(*50*)

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In addition to natural phytotoxins with known MOAs, there are many potent phytotoxins for which the MOA is still unknown. In several cases, there are indications that the compounds have unique MOAs. One example is ascaulitoxin aglycone (AscA) (Figure 2), a phytotoxin from the plant pathogen *Ascochyta caulina* (*54*). It is highly toxic to both host and non-host plant species. It is more effective as a growth inhibitor to duckweed (*Lemna paucicostata*) than many commercial herbicides (*55*, *56*). Supplementation of growth media with most amino acids reverses the effects of this potent phytoxin (*e.g.*, Figure 2) (*55*). Certain tricarboxylic cycle intermediates also reversed or reduced the effects. Metabolomic analysis of the effects of AscA revealed drastic effects (both up and down regulation) on pools of some amino acids. But, other metabolic disruptions were also found. The metabolic fingerprint caused by AscA was unlike that caused by herbicides with known MOAs that BASF had on file, indicating that it has a unique MOA.

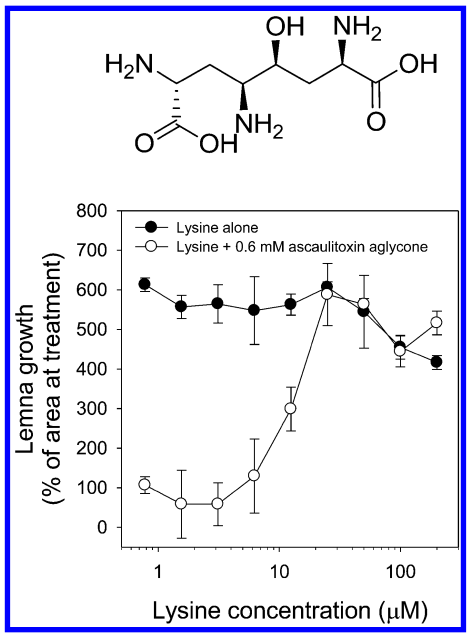

Figure 2. Structure of ascaulitoxin aglycone (AscA) and the effect of L-lysine on AscA-inhibited growth of Lemna paucicostata after 7 days exposure to both chemicals. Reproduced in part with permission from reference (55) Copyright 2011. Publisher Elsevier.

Mevalocidin (Figure 3), a microbial product of *Fusarium* DA056446 and *Rossellinia* DA092917 (*57*), is a close analogue of mevalonic acid (Figure 3), an intermediate of the mevalonic pathway for terpenoid synthesis. The phytotoxin is readily taken up by plants and is phloem mobile, producing symptoms of chlorosis (Figure 4). In the mevalonate pathway, mevalonate is phosphorylated to mevalonate-P and mevalonate-PP by two consecutive kinases. Mevalonate-PP

is then decarboxylated to form isopentenyl-PP (the basic terpene building block) by mevalonate-PP-decarboxylase. Considering the structure of the herbicide, the mode of action may be inhibition of one or more of the three enzymes of the mevalonic pathway that use mevalonate or one of its phosphorylated forms as a substrate, an entirely new MOA. If so, like hydantocidin (*58*), mevalocidin might be a proherbicide that must be phosphorylated in order to be an active enzyme inhibitor.

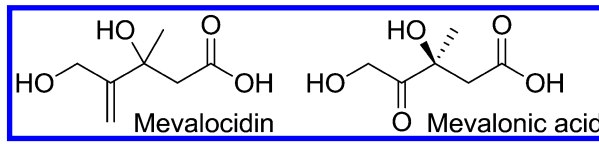

Figure 3. Structures of mevalocidin and mevalonic acid.

Figure 4. Symptoms of mevalocidin treatment on Xanthium strumarium (cocklebur) treated 16 days prior to the photograph. Reproduced with permission from reference (57). Copyright 2013. Publisher Springer. (see color insert)

All isoprenoids originate from isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) building blocks (*59*) (Figure 5). IPP was originally thought to be derived exclusively from mevalonic acid (MVA), a ubiquitous metabolite synthesized by 3-hydroxy-3-methylglutaryl (HMG) CoA reductase in eukaryotic organisms. However, this paradigm was challenged early on in studies that showed that mevilonin (a natural compound that is a cholesterol-lowering statin also known as lovastatin), a specific HMG-CoA reductase inhibitor, reduced plant growth and sterol synthesis but had no effect on carotenoid synthesis (*60*). They postulated that chloroplasts held a different form of this enzyme. It took the same research group more than a decade to discover that chloroplasts contained an entirely different pathway to IPP synthesis (*61*). In the chloroplast, IPP and DMAPP are derived from 1-deoxy-d-xylulose-5 phosphate, which is subsequently converted to methyl-d-erythritol-4-phosphate.

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This pathway, called either DOXP or MEP, is the target of the synthetic herbicide clomazone (actually a proherbicide that is active as keto-clomazone) and the natural product fosmidomycin (Figure 5). Consequently, effort is being pursued to discover inhibitors of other steps of this pathway (*62*).

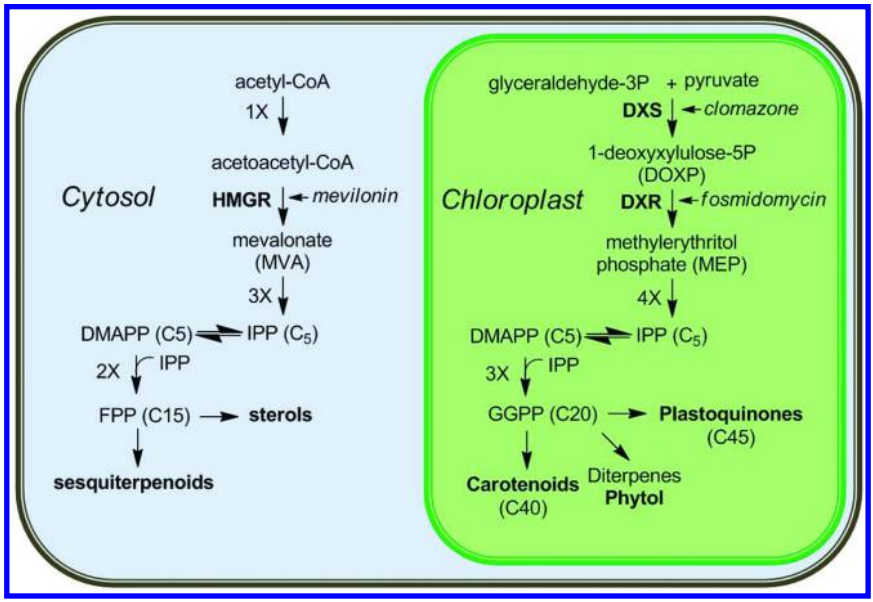

Figure 5. The two isoprenoid biosynthesis pathways in plants. The mevalonate (MVA) pathway is localized in the cytosol involves 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), the target of mevilonin. The DOXP or MEP pathway is compartmentalized within the chloroplast and involves 1-deoxy-D-xylulose 5-phosphate synthase (DXS), and 1-deoxy-D-xylulose 5-phosphate reductase (DXRP), the targets of clomazone and fosmidomycin, respectively. GGPP, geranylgeranyl diphosphate; FPP, farnesyl diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate. (see color insert)

These are only a couple of examples of potent phytotoxins that have unexploited MOAs. The pesticide industry has patents on a number of natural phytotoxins for which the MOA is either unknown or not disclosed. Examples of potent natural phytotoxins explored by industry, but with unknown MOAs, are macrocidin (*63*, *64*), cinnacidin (*65*), and albucidin (*66*). Natural phytotoxins with unknown MOAs such as agropyrenol (*67*), phomentrioloxin (*68*), and pyrenophoric acids (*69*) are being discovered at a rapid pace. Considering the already established wide range of MOAs not associated with commercial herbicides that are possessed by natural compounds (Table 1), one might expect that even more new MOAs are still to be discovered. We emphasize that all of these compounds are highly phytotoxic, indicating that their MOA could be viable for a commercial herbicide MOA.

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Why has the information above not resulted in new herbicides with new MOAs? Gerwick (*70*) summarized several reasons for this: inadequate efficacy and reliability, as well as safety issues with non-target organisms. We mentioned the potential safety problems of some of these natural phytotoxin MOAs above. One could add to Gerwicks's list that many natural compounds are structurally complicated (*e.g.*, multiple stereiogenic centers), making the cost of synthesis out of reach for an agrochemical. Just as for pharmaceuticals based on natural compounds, simplifying the structure while maintaining biological activity has not worked for some natural phytotoxins. For example, no simplified CF_1ATPase inhibitors with good activity have been generated from the very potent natural cyclic tetrapeptide, tentoxin (*e.g.,* (*71*)). There are two parts of the structure-activity puzzle that must be solved: activity at the molecular target site and the physicochemical prerequisites for movement to that target site. However, advances in fermentation have made complex pesticide molecules such as the spinosyns commercially successful, and similar solutions may be possible for structurally complex natural phytotoxins. Most natural phytotoxins are not as active as synthetic herbicides, however, the triketones provide an example of the natural compound providing the structural template for more active synthetic compounds.

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Summary

The information above provides an abbreviated rationale for a stronger effort to exploit natural phytotoxins in the quest for badly needed new herbicide MOAs. Numerous highly effective natural phytotoxins have been found that kill plants by MOAs that are not used by commercial herbicides. If the natural compound cannot be a successful herbicide (*e.g.*, high cost or inadequate physicochemical properties), a new herbicide with the new MOA can be produced by either structural modification of the natural compound or by searching for new compounds that are effective on the new target site with *in vitro* screening. The growing herbicide resistance problem should hasten the increased use of this strategy.

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Chapter 8

What Makes a Good Compound against Sucking Pests?

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Good field control of sucking pests requires in particular effective control of individuals feeding on the underside of leaves; i.e. translaminar pest control. Insufficient control of individuals which were less exposed to the spray would otherwise result in too rapid rebuilding of pest population. Translaminar control of several sucking arthropods was shown to be determined by correlation of the distribution of the insecticide within foliar tissues with the feeding behaviour of target pests. We apply a model to calculate intracellular localization of compounds, allowing the prediction of translaminar distribution, and thus potential for good field control of sucking pests. Once the modeling information predicted appropriate distribution of the compound within leaf tissues, the insecticide quantity exposed to feeding pests might be increased by formulation. Here, we present a new methodology ('artificial leaf') to rapidly optimize formulations for good leaf uptake of aphicides. This approach could identify formulation components which enhance the cuticle penetration, and could therefore be described as adjuvants, i.e. enhancing the biological effect.

Keywords: pesticide design; intracellular localization; vacuole trapping; translaminar; sucking pests

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Introduction

Insecticides are often classified according to their target spectrum, i.e. against sucking and chewing pests (e.g. (*1*)). This simplification reflects the feeding behavior of insects on plant tissues. So called sucking pests feed on specific tissues (vascular system, mesophyll, epidermis) (*2*) whereas so called chewing pests do not discriminate between foliar tissues and bite off entire pieces of foliage. The physiology of the target insect can determine which species are controlled by particular insecticides, and a huge variety of broad and narrow spectrum insecticidal activity is found among small molecules in agrochemical research.

In addition to intrinsic activity, the bioavailability *in planta* of an insecticide is essential for good efficacy. Since sucking pests show diverse feeding behaviors, often very specific for particular tissues and cellular compartments, inappropriate compartmentalisation of insecticides on treated crops can lead to insufficient field performance.

An insecticide is said to be "systemic" if it moves within the plant vascular system (phloem and xylem) where sucking pests like Hemiptera (e.g. aphids and whiteflies) feed. This is a highly desirable property of an insecticide as it allows long distance translocation in plants and an even distribution of the compound within the leaf lamina compensating for uneven spray deposition on the foliage (*3*). In opposite, "contact" insecticides control their targets either by direct contact spray and/or by residual contact with spray deposits on leaf surfaces. But not by feeding on untreated leaf undersides (or areas in spray shadow on e.g. vertically exposed leaves).

In this paper we share our insights obtained with a new class of systemic insecticides (*4*) and extend the knowledge to general requirements for chemicals targeting sucking pests.

Original Problem: Weak Correlation between Lab and Field Efficacy

The research compounds of the 'imidazoline' (IM) class (Table 1) demonstrated in a standard screening assay (Table 2) efficacy comparable to the commercial standard thiamethoxam (THMX). Young pepper plants infested with a mixed population of *Myzus persicae* were curatively sprayed with test solutions. The aphid control was measured as percent mortality one and seven days after application (DAA).

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Table 1. Imidazoline type research compounds. Data source: Syngenta (*5*, *6*).

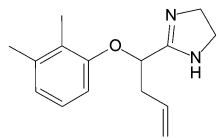
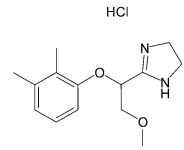
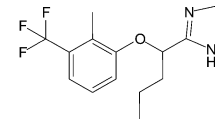
Abbreviation	Imidazoline 1	Imidazoline 2 (HCl salt)	Imidazoline 3
			
Solubility (ppm)	776.0	2071.5	34.8
log P	2.8	1.9	4.3
pKa	9.4	9.1	9.4
MW	244	285	300
Valency	+1	+1	+1

Table 2. Efficacy against *Myzus persicae* on pepper seedlings (*Capsicum annuum*)

	imidazoline 1		*imidazoline 3*		*thiamethoxam*	
mg/L	*1 DAA*[a]	*7 DAA*	*1 DAA*	*7 DAA*	*1 DAA*	*7 DAA*
100	99	100	99	100	99	100
50	98	99	97	100	99	100
25	98	97	95	99	99	100
12.5	95	97	95	98	99	100
3.0	50	95	80	90	80	100
0.8	30	90	50	90	15	93

[a] DAA: days after application.

However this promising performance was not confirmed in field trials. The aphid control on cotton (Figure 1) with imidazoline 1 was much weaker than that found with the standards thiamethoxam (THMX) and pymetrozine (PYME) under field conditions. Even when applied at three times the rate, the imidazoline 1 did not reach the performance of thiamethoxam, despite comparable efficacy in the lab.

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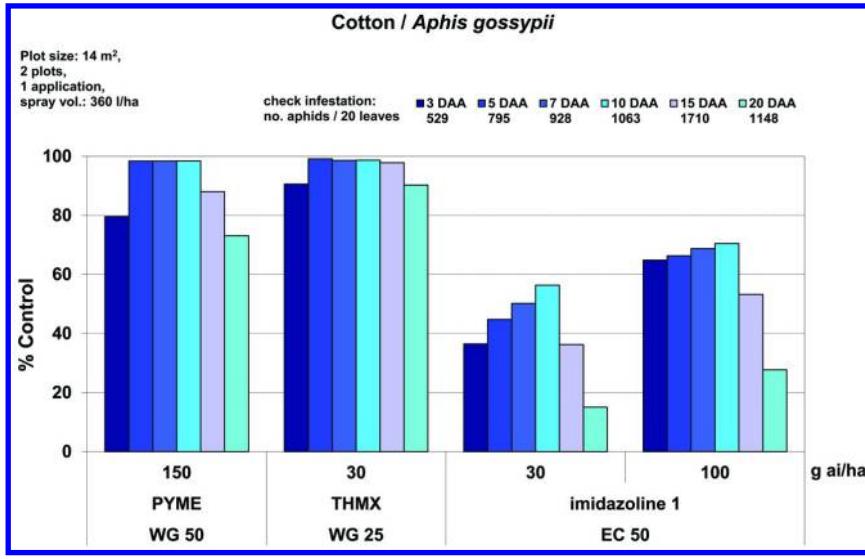

Figure 1. Field efficacy of pymetrozine (PYME), thiamethoxam (THMX) and imidazoline1 against cotton aphid (Aphis gossypii) after foliar spray over time (days after application, DAA)

Lack of Translaminar Control Identified as Cause of Weak Field Performance

Special translaminar bioassays revealed that aphids feeding on the underside of the leaf (abaxial surface) were insufficiently controlled when imidazolines were applied to the upper leaf surface. In this bioassay bean plants (*Phaseolus vulgaris* L.) were standardized to one unifoliate leaf. Aphids (*Aphis craccivora*) were infested to the leaf underside and confined with an insect glue. The application was done with a track sprayer onto plants with horizontally exposed leaves. Standards like thiamethoxam could control aphids very effectively in this bioassay, where they are feeding on the untreated leaf underside and not directly exposed to the spray. In an attempt to enhance leaf uptake a broad range of adjuvants such as commercial blends of uptake enhancing compositions, plasticizers (e.g. Brij 96), humectants (e.g. sorbitol) and buffers were added to the spray solution of imidazoline 1, but the translaminar aphid control was not improved (selected examples shown in Figure 2).

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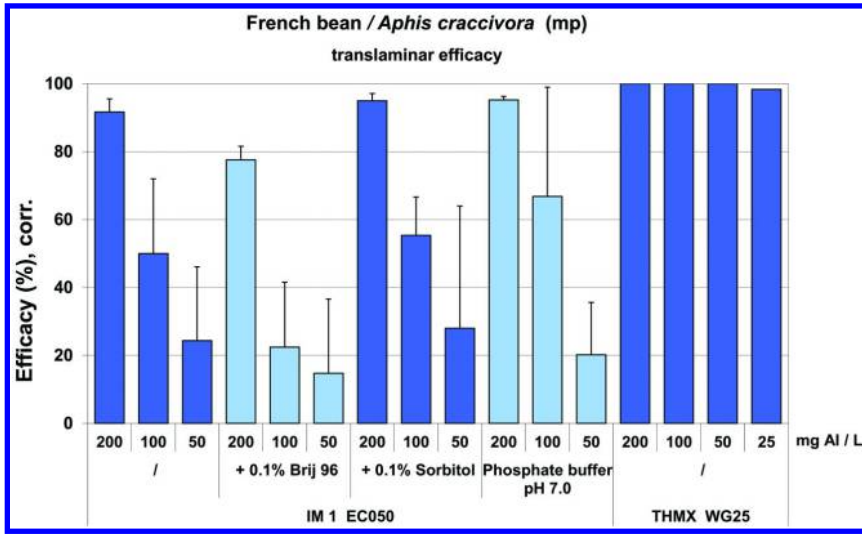

Figure 2. Translaminar efficacy against Aphis craccivora (mixed population, mp) on French bean. Imidazoline 1 was tested in combination with different adjuvant types. Bars represent means of 4 plants (Abbott corrected mortality in %), error bars indicate standard deviation.

The weak translaminar aphid control suggested poor foliar uptake of this chemical class which stimulated us to examine the plant cuticle penetration in more depth. Leaf cuticles can be a very effective penetration barrier for pesticides (*7*, *8*). Without passing this first and critical hurdle the insecticide is not accessible to insects sucking on cellular fluids. Therefore, a new method combining cuticle penetration with an aphid bioassay was developed, which functioned as an as 'artificial leaf' (Figure 3): An apple leaf cuticle (*Malus domestica* L.) enyzmatically isolated from adaxial leaf surfaces (*9*, *10*) was laid above a 15% sucrose solution, which had been placed on a layer of stretched Parafilm™, through which aphids were allowed to feed. A 5 µl droplet of spray solution was applied to the center of the cuticle at time zero. Aphid mortality was recorded frequently, which provided an indirect measure of quantities of the insecticide penetrating across the leaf cuticle and dissolving into the sucrose solution. This gave us the unique opportunity to study physical diffusion kinetics with insect pharmacology in real time.

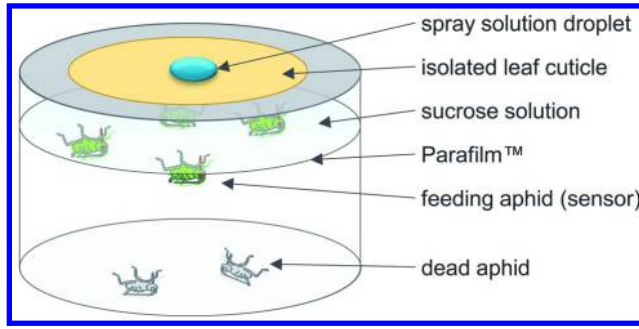

Figure 3. The 'artificial leaf': a method combining leaf cuticle penetration with pharmacokinetics on target pest.

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The method was validated by demonstrating the asymmetry of plant cuticles (*11*) and its effect on the penetration of the insecticide imidazoline 1 (Figure 4). The asymmetric distribution of intracuticular waxes creates two functional layers of the cuticle: the outer transport-limiting skin with a higher density of particularly crystalline waxes, lowering solubility and diffusion, and the inner sorption compartment, the cutin matrix (*8*). The application to the morphological inner side of the cuticle resulted in lower penetration, whereas the application to the outer side which is more relevant to the real foliar application resulted in higher penetration (i.e. earlier aphid mortality). This is due to the increased driving force which is defined as the product of the partition coefficient and concentration gradient (*12*). When the imidazoline containing droplet was added directly to the sucrose solution there was no barrier and the aphids started immediately ingesting the maximum dose. It took them two days to die. This simulated the maximum rate of penetration of the leaf cuticle that is possible, and served as a reference point at the dose tested, and allowed us to calibrate the sensitivity of aphids as an analytical sensor.

The recorded aphid mortality in this method reflects the continuous intoxication of sucking aphids with the insecticide, but it does not necessarily reflect the AI concentration in the sucrose solution at time t. When optimizing for good translaminar aphid control the aim is to reach the maximum effect within the shortest time after application.

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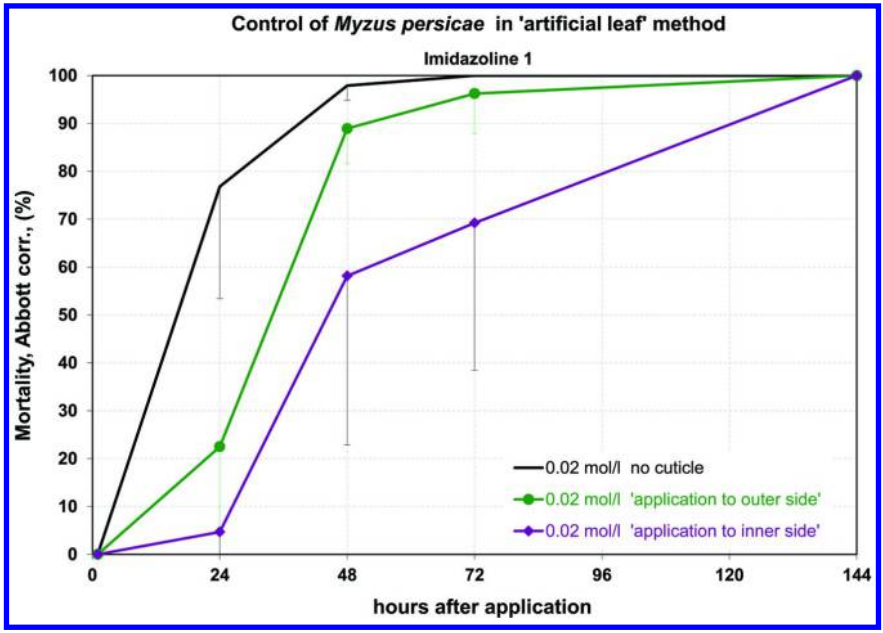

Figure 4. Asymmetry of leaf cuticles affects penetration rates. The amount of imidazoline 1 penetrated over time is manifested by aphid mortality. Each line represents the mean of 5 replicates, error bars indicate standard deviation.

Systematic Formulation Optimization for Increased Foliar Uptake

This 'artificial leaf' method was then used to optimise the formulation composition to improve cuticle penetration. The contribution of individual formulation components to the overall leaf uptake of the research insecticide imidazoline 2 was evaluated (Figure 5). These studies were done with the more stable hydrochloride salt (*4*). This approach helped to design a formulation that was so effective that cuticle penetration caused very little delay in reaching full mortality. When using isolated cuticles from the same batch, the data were highly reproducible, much more so than data from regular plant assays such as that shown in Figure 2. This reliable ranking of treatments together with the parallel screening of individual components allowed us to rapidly find the best formulation recipe.

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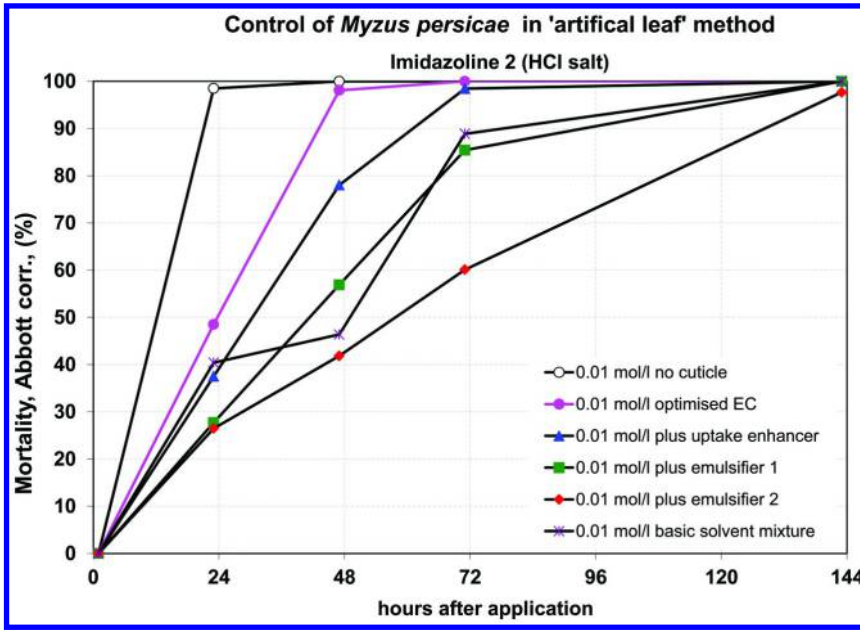

Figure 5. Systematic formulation optimisation by measuring individual effects of formulation components on cuticle penetration of the HCl salt of imidazoline 2

Cuticle penetration of imidazolines is good and therefore cannot account for the large differences between their greenhouse and field performance. It is thus clear that leaf uptake of this imidazoline type chemistry is not the real reason for their weak translaminar (and field) activity against aphids. This triggered a series of further studies encompassing other target pests such as mites and whiteflies.

Translaminar Control of Different Target Pests

The comparative study of translaminar control against different target pests (Figure 6). revealed that imidazoline 3 provided excellent translaminar control of mites. In contrast, there was in effect no translaminar control of aphids and whiteflies at practical application rates. Whereas the mite control confirmed good plant cuticle penetration as demonstrated with the 'artificial leaf' method above. Since assays were done on the same crop (French beans) and in parallel (i.e. same batch of plants) it also confirms that substantial quantities of penetrated imidazoline 3 were present in leaf tissues of plants. Because of the high intrinsic potency against aphids and whitefly we were surprised that these amounts were not sufficient to control them.

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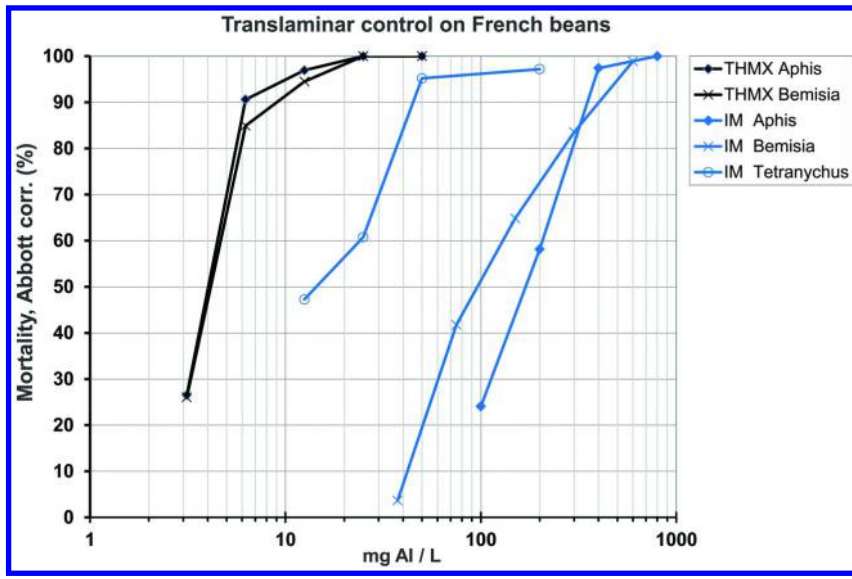

Figure 6. Translaminar efficacy of thiamethoxam (THMX) and imidazoline 3 (IM) on French beans against different pests: aphids (Aphis craccivora), whiteflies (Bemisia tabaci) and spider mites (Tetranychus urticae). Dose (mg AI/L) refers to active ingredient concentration in spray solution.

A systematic evaluation of intrinsic, curative (contact spray) and translaminar efficacies of imidazoline 3 compared with other commercial insecticides against these pests showed a selective weakness of this chemical class in translaminar control of aphids and whiteflies (*13*). This led us to conclude that the basicity of the molecules was causing them to concentrate in the acidic vacuoles of plant cells ('vacuole trapping') (*4*). This inner compartment of plant cells is entirely ingested by 'mesophyll feeders' like mites (*14*), but rarely punctured by aphid stylets on their path towards the vascular bundle (*15*). Whiteflies, on the other hand, find the vascular system on a purely intercellular path, i.e. mesophyll cells do not get punctured (*16*).

To support these qualitative conclusions we turned to a quantitative model of intracellular distribution.

Models Describing Intracellular Active Ingredient Localization

The passive distribution of xenobiotics across cell compartments can be calculated with the Fick-Nernst-Planck equation (*17*, *18*). When applied to both imidazolines and thiamethoxam (Table 3) the basic imidazolines are calculated to concentrate in the acidic vacuole ('ion trapping'), which can explain the weak translaminar activity of these compounds against aphids and whiteflies. Almost the entire amount of imidazoline 3 (pKa 9.1) is expected to be found in the vacuole (96 %) and the surrounding cytosol (4 %). Almost none is calculated to be in the vascular system, which is the main feeding site of Hemiptera. The same

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is calculated for imidazoline 1 (pKa 9.4); here is the expected mass fraction in the acidic vacuole even higher: 98%.

Table 3. Calculated mass distribution (%) in cellular compartments (cytosol, vacuole) and the plant vascular system (phloem, xylem)

	volume (%)	*pH*	*Imidazoline 1*	*Imidazoline 3*	*Thiame-thoxam*
cytosol	10.6	7.2	1.23	4.39	10.55
vacuole	84.8	5.5	98.08	95.54	84.37
phloem	2.3	8.0	0.04	0.03	2.29
xylem	2.3	5.5	0.65	0.04	2.8

The commercial insecticide thiamethoxam, a neutral and polar compound, is distributed approximately homogenously throughout the compartments. The various values in Table 3 reflect the different sizes of the cellular compartments. The equal concentration of thiamethoxam in different fluids (and no particular accumulation in any compartment) provides a substantially higher dose in the vascular system.

The insights obtained by this model of standardized plant cells were so valuable that we created a new model for translaminar pest control (*13*). In addition to the distribution of compounds between different leaf compartments, the velocity of distribution from the upper leaf (source) to the bottom side (sink) was of special interest (Figure 7).

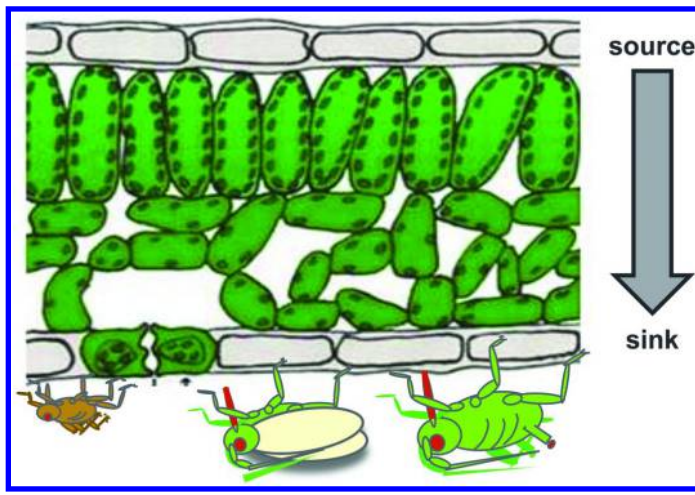

Figure 7. Scheme of the'translaminar model' which takes the upper leaf as source compartment and the bottom side as sink compartment.

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In Table 4 data from several commercial insecticides and acaricides are compiled covering a broad range of physico-chemical properties and chemistries. The parameters shown represent the information required as input for the 'translaminar model'. Table 5 demonstrates the calculated mass distributions of these compounds in leaf compartments. The model predicts the velocity of translaminar movement and discriminates between the apoplastic pathway (i.e. in the intercellular space) and the symplastic pathway (i.e. inside the cells). The pathway with the least resistance to transfer is considered to be the 'preferred pathway' (i.e. the route for fastest compound distribution).

Common Features Regarding Intracellular Localization of Commercial Insecticides and Acaricides

The empirical results and calculations described above for the basic imidazolines revealed that their intracellular localization in plant tissues was not suitable for the desired target spectrum of pests (*4*, *13*). The postulated 'vacuole trapping' depleted bioavailability *in planta* in the compartments appropriate for control of aphids and whiteflies. Despite the high intrinsic activity and excellent performance in curative plant assays, this weakness prevented a good performance under field conditions. This weakness, which is inherent to the active ingredient, cannot be overcome by formulation optimization.

The analysis of several commercial pesticides reveals the correlations between the estimated compartmentalization in leaf tissues and the major target pest orders (Table 5). Very strong aphicides (PYME, FLON, THMX, ACET) show a relative high mass fraction in the apoplast, typically around 40%, which enables both, good access to the xylem as feeding compartment and a channel for rapid translaminar distribution. All compounds which address aphids as major targets are calculated to use the apoplastic pathway as preferred route of distribution.

Compounds which have their strength in mite and or thrips control (ABAM, SPIN, CYEN) are calculated to be absent in the aqueous phase of the apoplast. Their lipophilicity causes then to adsorb onto cellular lipids (e.g. plasmalemma, tonoplast). This gives high mass fractions for both, cytosol and vacuole, since the model considers the fluid and its surrounding membrane as one compartment. The translaminar distribution occurs – if at all – along the symplastic pathway. This localization is favorable for the control of mites and thrips, which ingest whole cell contents.

The two diamides, CTPR and CYNT, are used for a different spectrum of target pests. CTPR is used for the control of chewing pests (Lepidoptera, Coleoptera). CYNT, which shows in addition activity against aphids and whiteflies (amongst others), is predicted, in contrast to CTPR, to distribute in the apoplast. The apoplast (continuum of cell walls) is in direct exchange with xylem vessels. The main determinant of distribution is the lower log P of CYNT.

Figure 8 illustrates the classification of sucking pests into cell (mites and thrips) and phloem (aphids and whiteflies) feeders. Chewing pests like Lepidoptera and Coleoptera ingest entire pieces of foliage so intracellular localization of the insecticide used to combat them is irrelevant.

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Table 4. Physico-chemical characteristics of commercial insecticides and acaricides. Data source: (*19*) and Syngenta

	Cyantraniliprole	*Chlorantraniliprole*	*Cyenopyrafen*	*Pymetrozine*	*Flonicamid*	*Abamectin*	*Spinosyn A*	*Thiamethoxam*	*Acetamiprid*
Abbreviation	CYNT	CTPR	CYEN	PYME	FLON	ABAM	SPIN	THMX	ACET
IRAC class	28	28	25	9B	9C	6	5	4A	4A
Log Kow neutral	1.94	2.76	5.6	-0.23	0.3	4.4	5.2	-0.13	0.8
Log Kow ion[a]	-1.56	-0.74	2.10	-3.73	-3.20	0.9	2.8	-3.63	-2.7
pKa	9.1	10.88	0.6	4.1	11.6	/	8.1	/	0.7
Valency	-1	-1	1	1	-1	0	1	0	1
MW	474	483	394	217	229	873	732	292	223

[a] Log Kow ion = log Kow (neutral) - 3.5 (*17*).

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Table 5. Calculated mass distribution (%) in a leaf cell, preferred pathway (least resistance in transfer) and major target pest orders of selected insecticides and acaricides[a]

		CYNT	*CTPR*	*CYEN*	*IM 1*	*IM 3*	*PYME*	*FLON*	*ABAM*	*SPIN*	*THMX*	*ACET*
Calculated mass distribution (%)	cytosol diss.	1.5	0.3	0.0	0.9	0.7	5.7	5.7	0.0	0.0	6.0	5.0
	cytosol total	9.9	10.9	11.1	1.1	4.4	6.0	6.5	11.1	10.3	6.3	7.0
	vacuole	79.1	87.0	88.9	85.7	94.7	50.7	51.6	88.8	89.7	50.0	56.3
	apoplast	11.0	2.1	0.0	13.2	1.0	43.4	41.9	0.1	0.0	43.7	36.7
preferred pathway		apoplast	symplast	symplast	apoplast	apoplast	apoplast	apoplast	symplast	symplast	apoplast	apoplast
major target pest orders	Aphids & WF	x			(x)	(x)	x	x			x	x
	Mites			x		x			x			
	Thrips							x	x	x	x	
	Lepidoptera	x	x							x		
	Coleoptera	x	x				x			x	x	x

[a] Apoplast volume: 30%. WF is whiteflies; Coleoptera: beetles; Lepidoptera: moths and butterflies.

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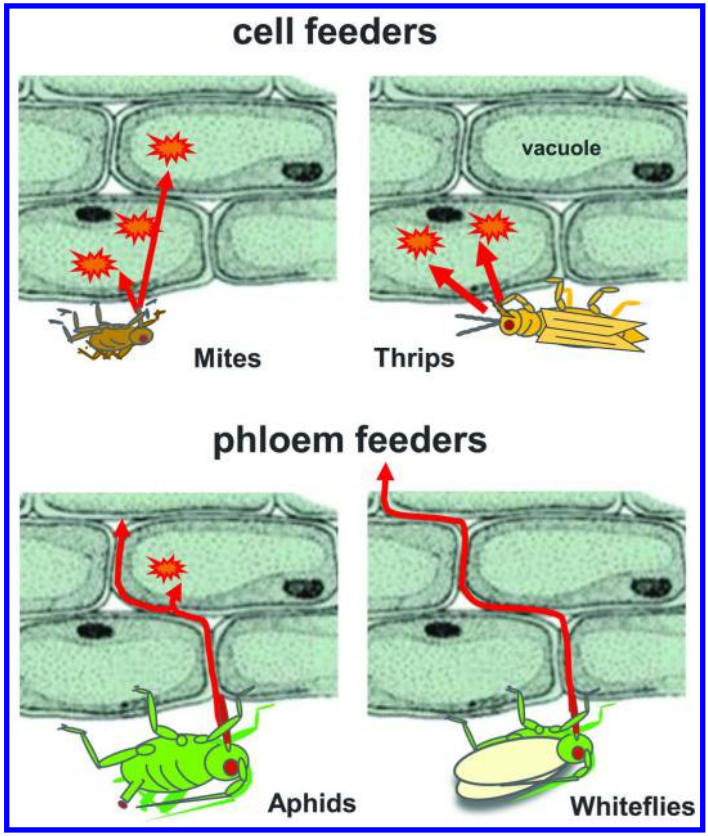

Figure 8. Classification of sucking pests into cell (mites and thrips) and phloem feeders (aphids and whiteflies) according to their feeding sites in foliar tissues. The arrows describe the probing pathway of the stylet reaching the feeding compartment where an insecticide (marker) would be best placed to kill the pest.

This knowledge of estimated active ingredient localization in leaf tissues can be applied to pesticide design. Figure 9 shows a simple visualization of the 'translaminar model' describing the mass distribution in relation to log P. This can be used as a rough guideline to determine physico-chemical properties useful for the control of defined target pests. Phloem feeders are better controlled by compounds distributing into the apoplast (but distribution into phloem is also a model output), and cell feeders by compounds in the symplast.

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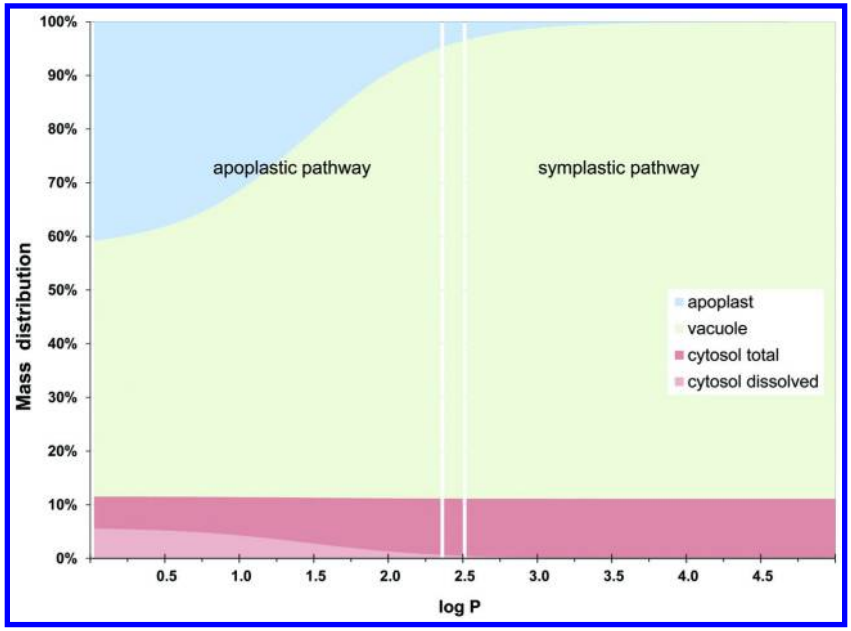

Figure 9. Calculated mass distribution (%) in plant cell compartments of a hypothetical uncharged compound with molecular weight of 350 and denoted log P

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Chapter 9

Plant- or Fungal-Produced Conophthorin as an Important Component of Host Plant Volatile-Based Attractants for Agricultural Lepidopteran Insect Pests

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Conophthorin (7-methyl-1,6-dioxaspiro[4.5]decane) is a semiochemical common to numerous coleopteran and hymenopteran insects, and exhibits varying semiochemical behavior among these species. Conophthorin has recently gained attention as a semiochemical for navel orangeworm, *Amyelois transitella* (Lepidoptera: Pyralidae); yet, other lepidopterans also have reported semiochemical behavior to conophthorin. Sources of conophthorin are diverse and include plants, insects, fungi, and bacteria. Until recently, conophthorin production was generally believed to be from plants and insects. The recent inclusion of microbes as a source of conophthorin has expanded its current role as mediator of plant-insect interactions to include mediating discrete insect-microbe interactions. Recent investigations into a host plant-based attractant for navel orangeworm have indicated a mutualism between conophthorin-producing fungal spores and navel orangeworm, specifically when considering almond orchards as the host plant for both. The diverse role of conophthorin as a semiochemical for such a wide range of insects suggests

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that conophthorin is a critical chemical cue. These studies also suggest that the bioactivity of conophthorin is enhanced or defined by the associated background odors of the relevant host plant. Provided herein is an overview of the source and role of conophthorin as it relates to lepidopteran insect pests. Also included are data on a blend of volatiles that contains conophthorin and the blend's efficacy to attract both male and female navel orangeworm in mating disruption environments.

Introduction

Conophthorin, chemical name 7-methyl-1,6-dioxaspiro[4.5]decane, is a small organic molecule ($C_6H_{16}O_2$, molecular weight 156 g/mole) with two chiral carbons and has been classified as both a spiroacetal and a spiroketal. The use of both class names is generally accepted in the scientific literature for conophthorin, its associated structural isomer, chalcogran, as well as other similar compounds. Conophthorin can exist as the four possible stereoisomers shown in Figure 1. The (*E*)- forms, (5*S*,7*S*) and (5*R*,7*R*), appear to be the predominant isomers produced by plants and insects, and both (*E*)-enantiomers elicit the most electrophysiological and behavioral responses (*1*).

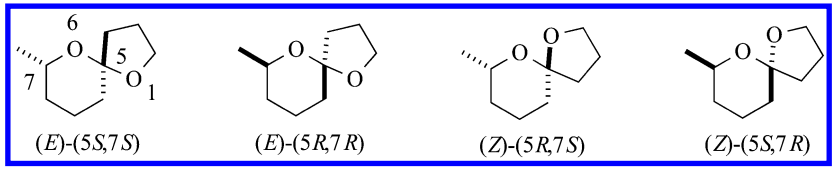

Figure 1. The spiroketal conophthorin, its numbering, and corresponding stereoisomers.

Conophthorin was first isolated from the common wasp (*Vespula vulgaris*) (*2*), and since that time has shown a rich history as a semiochemical of numerous wasp, bee, beetle, and fly species. Table 1 lists examples of the various insects that use and/or produce conophthorin as a chemical cue. The reader is directed to some informative publications that provide specific information regarding conophthorin from non-lepidopteran species. Focus has primarily been on coleopterans (*1–3*), with a vast amount of literature on bark beetles (Coleoptera) and conophthorin. This is due to the severe impact this insect pest has had on conifer forests and the potential of conophthorin to repel the bark beetle away from their targeted conifers. The focus and emphasis of the present report is conophthorin as a semiochemical of lepidopterans and their related plant or fungal hosts that produce it.

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Table 1. Examples of non-lepidopterans that produce or utilize conophthorin as a chemical cue

Common Name	*Scientific Name*	*Insect Order*	*Source or Role of Conophthorin*	*Ref.*
Pacific fruit fly	*Bactrocera xanthodes*	Diptera	Rectal gland secretion	(*4*)
Large earth bumblebee	*Bombus terrestris*	Hymenoptera	Mandibular	(*4*)
Stingless bee	*Partamona cupira*	Hymenoptera	Cephalic secretion	(*4*)
Common paper wasp	*Polistes olivaceus*	Hymenoptera	Venom volatile	(*5*)
Common wasp	*Vespula vulgaris*	Hymenoptera	Sting gland, venom sack	(*2*)
Camphor shot borer	*Cnestus mutilates*	Coleoptera	Attractant	(*6*)
Fir bark beetle	*Cryphalus piceae*	Coleoptera	Repellant	(*7*)
Poplar and willow borer	*Cryptorhynchus lapathi*	Coleoptera	Semiochemical	(*8*)
Sap beetle	*Epuraea variegate*	Coleoptera	Attractant	(*7*)
Southern pine beetle	*Dendroctonus frontalis*	Coleoptera	Repellant	(*9*)
Larch elm bark beetle	*Hylesinus crenatus*	Coleoptera	Pheromone	(*1*)
Coffee berry borer	*Hypothenemus hampei*	Coleoptera	Attractant	(*10*)
Double-spined bark beetle	*Ips duplicatus*	Coleoptera	Attractant	(*1*)
Ash bark beetle	*Leperisinus varius*	Coleoptera	Repellant	(*7*)
Checkered beetle	*Madoniella dislocates*	Coleoptera	Attractant	(*11*)
-	*Phloephthorus rhododactylus*	Coleoptera	Pheromone	(*1*)
Twig beetle	*Pityophthorus pubescens*	Coleoptera	Repellant	(*12*)
Granulate ambrosia beetle	*Xylosandrus crassiusculus*	Coleoptera	Attractant	(*6*)

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Conophthorin and Lepidopterans

The first report of lepidopteran response to conophthorin (Table 2) was in 2002 by Bedard *et al.* (*13*). They reported that conophthorin and other nonhost angiosperm volatiles inhibited the capture of spruce seed moths (*Cydia strobilella,* Lepidoptera: Tortricidae) when the spiroketal was added to the pheromone-baited traps. The addition of conophthorin to nonhost angiosperm volatiles was a common practice for researchers investigating methods to repel bark beetles from conifers. However, this was the first application of conophthorin to repel a lepidopteran, whose larvae feed on the seeds of North American spruces (*Picea* spp.). The authors of this report identified and bioassayed the volatiles emitted by the host plant (*Picea*), which included primarily terpenoids, among others. To evaluate repellency affects, the authors also bioassayed conophthorin, many individual non-host volatiles, and blends of volatiles combined with the pheromone of the spruce seed moth, (*E*)-8-dodecenyl acetate. Conophthorin, when combined with the moth pheromone significantly diminished the number of male moth captures. While the authors evaluated the electrophysiological response of both male and female antennae to individual conophthorin isomers, and demonstrated a decreased response to the pheromone, the paper did not determine whether conophthorin on its own would inhibit the moth from being attracted to the actual host itself. A query for this particular plant-insect system would be what affects other ambient host or nonhost volatiles have on the insect's response to conophthorin.

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Table 2. Lepidopterans that utilize or are suspected of utilizing conophthorin as a chemical cue

Common Name	*Scientific Name*	*Role of Conophthorin*	*Ref.*
Spruce see moths	*Cydia strobilella*	Repellant	(*13*)
Peachtree borer	*Synanthedon exitosa*	Ovipositional attractant component	(*14*)
Navel orangeworm	*Amyelois transitella*	Attractant component in host plant volatile blend, attracts male and female	(*15*)
Cactus moth	*Cactoblastis cactorum*	Possible semiochemical	(*16*)

The first report of conophthorin as a possible attractant of a lepidopteran (Table 2) occurred in 2007 when Derksen *et al.* described semiochemical-mediated ovipositional behavior of the agricultural insect pest, peachtree borer (*Synanthedon exitiosa*, Lepidoptera: Sesiidae) (*14*). In their report, the authors

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showed that gravid female peachtree borer moths preferentially oviposited on traps containing gum frass. Upon evaluation of the headspace of the gum frass, it was determined that conophthorin was a component among 20 other candidate volatiles. In separate top-down ovipositional experiments where classes of compounds were removed from the synthetic blend, the results suggested that conophthorin was one of the important components for ovipositional activity. The specific role of conophthorin was not demonstrated via the ovipositional bioassays; however, electrophysiological experiments of the individual blend components showed conophthorin as eliciting a large antennal response.

An important aspect of the Derksen *et al.* report (*14*) was that the authors noted that the peachtree borer frass and gum on the tree was what attracted the female peachtree moths. In a sense, this infestation may be signaling a particular vulnerability of the host plant to the insect pest. This topic of plant vulnerability will be discussed further in relation to other Lepidoptera, but it should be noted that it is thought that infestations of insects (e.g. bark beetles) and microbes render a host plant vulnerable to further attack, and that volatiles may play a role in signaling this vulnerability (*3*). Additionally, microbes within frass are known to produce semiochemicals that convey host suitability and ovipositional preferences (*17*).

In 2012, our laboratory reported a blend of host plant volatiles that attracted both male and female navel orangeworm moths (*15*) (Table 2). The blend comprised (±)-1-octen-3-ol, ethyl benzoate, methyl salicylate, acetophenone, and (±)-(*E*)-conophthorin in a 12:4:4:1:1 ratio, using ethyl acetate as the solvent. The origin of conophthorin was initially thought to be damaged or hull split almonds as evidenced by earlier investigations (*15*, *18*). Soon after, our laboratory was able to definitively show that developing fungal spores, transitioning from the resting state to germination and placed on fatty acids, produced conophthorin, among other volatiles (*19*). Conophthorin was proven to be a key component of the attractant blend for navel orangeworm by field trapping studies that varied the relative ratios of components as well as electrophysiological studies (*20*). Interestingly, when tested as an individual component, conophthorin did not attract navel orangeworm or elicit strong antennal responses from male and female navel orangeworm. Studies using the (*Z*)-isomer of conophthorin in the blend suggested that the (*E*)-isomer was the bioactive stereoisomer. This sensitivity to stereoisomerism agrees with reports of coleopteran and hymenopteran preference for the (*E*)-isomer (*1*). A history of the discovery of conophthorin for navel orangeworm is provided in previous publications (*21*, *22*).

A final instance of conophthorin presence from a plant tissue/fungal spore matrix and as a possible semiochemical for a lepidopteran was a recent study (*16*) that investigated the volatile headspace of cactus tissue from prickly pear (*Opuntia humifusa*) (Table 2). In our laboratory's report (*16*), abiotically stressed *ex situ* cactus tissue produced conophthorin, among other volatiles. Though not fully replicated with sufficient samples, initial headspace trials of the tissues (Beck, unpublished) provided a volatile profile that very closely mimicked that of the attractant blend for navel orangeworm. In addition to the five components detected in the cactus tissue that were also present in the attractive orangeworm blend, there were varying classes of compounds such as green leaf volatiles and terpenoids.

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The monoterpenes [R]-limonene and [1S]-α-pinene have elicited strong antennal responses from male navel orangeworm moths (*20*); however, the identities of the specific stereoisomers of these two compounds were not determined in the cactus tissue study. An interesting result from the cactus study was the detection of the common monoterpenes limonene and pinene, in addition to key semiochemicals for navel orangeworm. Monoterpenes are ubiquitous volatiles emitted by pistachio orchards. This may become an issue if the current spread of the invasive cactus moth, *Cactoblastis cactorum* (Lepidoptera: Pyralidae) (*23*), currently along the Atlantic and Gulf coast states from Florida north to South Carolina and West to Louisiana, spreads to the California Central Valley where a large percentage of U.S. pistachios are grown. Though not a general rule, it is not uncommon for certain classes of compounds to elicit similar behavioral or electrophysiological responses from insects in the same family (*20*, *24*, *25*).

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Host Plant Volatiles and Conophthorin

Generalist insect scavengers such as the navel orangeworm (*26*) are thought to respond to several host plant volatile cues, and not just one or two specific semiochemicals (*27*). An example is the navel orangeworm's attraction to the synthetic host plant volatile blend, which comprises five components (*15*). Further support of this idea was evidence of the influence of background odor, such as the ambient odor of almond orchards, on the navel orangeworm's discrimination of acceptable semiochemicals (*28*). In Beck et al. (*28*), the navel orangeworm's preference for the same synthetic blend of host plant volatiles was evaluated in two different commercial tree nut orchards, almond and pistachio, in relatively close proximity and at identical trapping dates. Figure 2 illustrates the disparity of total male and female navel orangeworm moths captured by the host plant volatile blend (HPV) in the two orchards in 2011 and 2012. The capture numbers were compared to the current attractant, almond meal. The host plant volatile blend captured 3-5 times more moths in almond orchards than it did in pistachio orchards.

In terms of insect attractancy to a host plant by using emitted plant volatiles, there are two broadly accepted theories. One theory states that phytophagous insects are attracted to the host plant by specific ubiquitous volatiles but the volatiles vary in ratios depending on the host plant (*29*). The other theory states that certain semiochemicals are enhanced by background host plant volatiles, e.g., ambient odors associated with a particular orchard may enhance the detection of a more orchard-specific semiochemical or semiochemicals (*30*).

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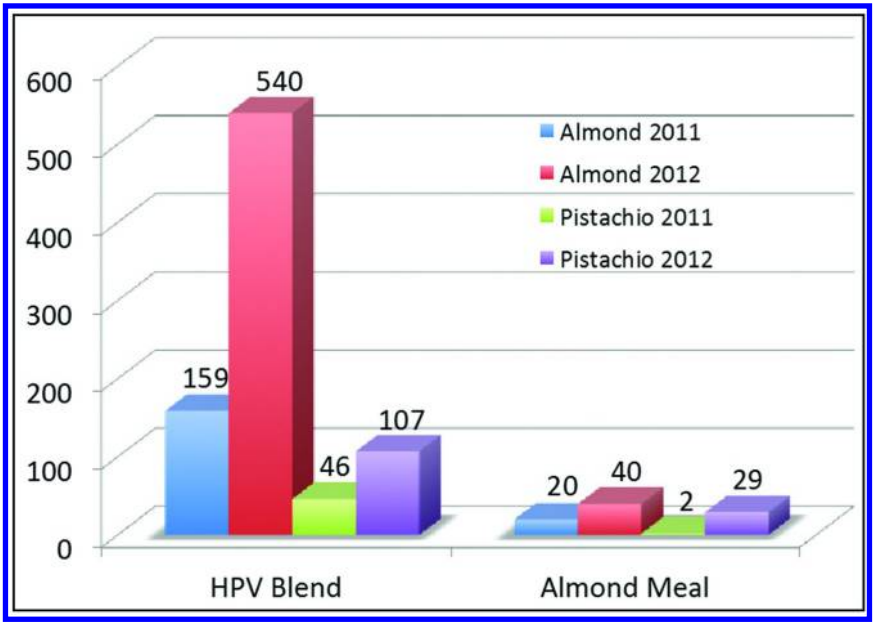

Figure 2. Total number of male and female navel orangeworm moths captured by the host plant volatile (HPV) blend in almond and pistachio orchards. Trap capture numbers of the HPV blend were compared to almond meal, the current industry attractant for navel orangeworm monitoring.

Given the trap capture numbers shown in Figure 2, and considering the relative complexity of the host plant volatile blend (three orchard-specific benzenoids, an alkenol, and the spiroketal), it is reasonable to consider that conophthorin may be an important semiochemical, but is not the only odor that navel orangeworm are aiming toward when searching for a host in almond orchards. This idea was supported by the result that conophthorin on its own was not attractive to navel orangeworm, and that the blend without conophthorin did not consistently or in high numbers attract the moths (*15*, *22*, *28*). Work to date on an effective attractant for the navel orangeworm in pistachio orchards has revealed that the fungal-derived compounds (±)-1-octen-3-ol and (±)-(*E*)-conophthorin, when combined with a third orchard-specific volatile, attracts the navel orangeworm in pistachio orchards, but this formulation has not been consistent in its attractancy (Beck and Higbee, unpublished observations). These results may support the theory that for navel orangeworm certain semiochemicals are enhanced by background host plant volatiles (*30*).

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Regarding the lepidopteran species discussed earlier, the contribution of other host and nonhost plant volatiles/semiochemicals appeared to influence host plant selection by the spruce seed moth, where conophthorin and other host plant volatiles inhibited male response to sex pheromone (*13*). The same was true for the peachtree borer, where conophthorin, in addition to the other volatiles from the peachtree borer frass, were required in a blend formulation to stimulate an ovipositional response by the female moth (*14*). This apparent semiochemical behavior of conophthorin in conjunction with associated host plant volatiles being necessary for a specific response of the corresponding lepidopteran is illustrated in Figure 3. It should be noted that the semiochemical activity of conophthorin for the cactus moth is only speculative at this time.

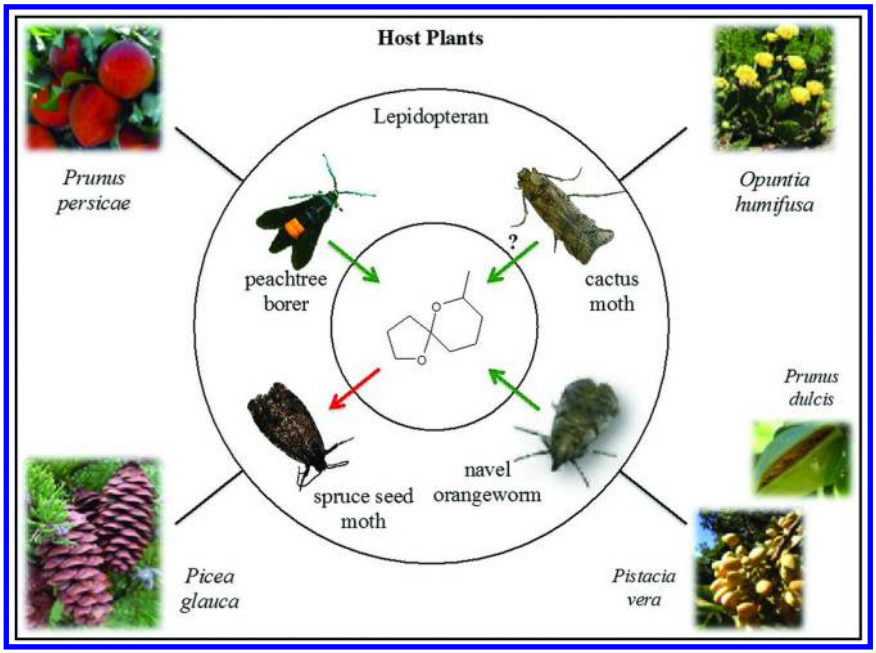

Figure 3. Associations of conophthorin (innermost circle), lepidopteran that are attracted to (green arrows, top left, top right, bottom right) or repelled by (red arrow, bottom left) conophthorin, and the host plants that are associated with each lepidopteran (outside corners). The chemical cue relationship between the cactus moth and conophthorin are hypothesized at this time.

Sources of Conophthorin

Table 1 includes some examples of insect sources of conophthorin. Plants associated with bark beetles and wasps have the longest history for emission of

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conophthorin (*2*, *3*, *31*) with reports dating back to the late 1970's. Over the last 25 years there have been numerous other non-insect sources reported. Table 3 provides examples of reports that describe the detection of conophthorin, and include agricultural and non-agricultural plants, insect frass, fungi, and bacteria. Because of the vast literature on coleopteran-based host sources, Table 3 primarily focuses on other sources of conophthorin. Surprisingly, there are a large number of plants that have been reported to produce the spiroketal.

In 2006 Knudsen and co-workers provided a summary of the numerous reports of flowers that produced conophthorin (*32*). In their summary they list 13 different families of flowers in which conophthorin was detected. Further examples include a palm flower (*33*) and toxic perennial (*34*).

In 2007, Derksen and co-workers (*14*) reported the detection of conophthorin from the gum frass of the peachtree borer (*Synanthedon exitiosa*). In their paper, the authors describe gum frass as "a mixture of tree phloem particles, tree sap (gum), and larval feces (frass)". Hence, though a new source of conophthorin was identified, the exact organismal origin was not specifically demarcated and could have been the host tree tissue (wood particles or sap), frass, or perhaps the microbes on either of these materials. Soon after the 2007 Derksen investigation (*14*) Cottrell and co-workers (*35*) reported the attraction of the lesser peachtree borer (*Synanthedon pictipes*) to damaged peach tissue sans lesser peachtree borer larvae (thus, no larval frass). This observation presented the possibility that an attractant was produced by host tree tissue, and thus indirectly implies that conophthorin was not emitted by the insect frass. This possibility would benefit from a more thorough investigation into the exact origin of conophthorin in this particular insect-plant system.

In 2008, our laboratory detected conophthorin from *ex situ* intact and damaged almonds (*18*); however, the exact source of the spiroketal was not definitively demonstrated. Despite only whole almonds (hull, shell, and kernel) being evaluated, it was not known at the time if noted fungal growth played a role in the detected volatile emission profiles. Later in 2008 (but not reported until 2012) an *in situ* volatile collection system was utilized to evaluate hull split almonds, a natural damage event of almonds. In our 2012 article (*15*) it was reported that conophthorin was detected from the *in situ* almonds and that no fungal growth was noted. This finding suggested a plant source of conophthorin. However, a follow-up investigation subsequently demonstrated developing fungal spores as a definitive source of conophthorin (*19*). A total of five fungal species/strains were investigated for their ability to produce conophthorin, in which all five did, but to a varying degree. A review of the development of the navel orangeworm attractant blend discussed this in more detail (*21*).

In 2012 Citron and co-workers (*38*) reported the first detection of conophthorin from *Streptomyces* spp. grown on artificial media. This report, in addition to the finding of conophthorin produced by fungal spores, highlights an important area of research that warrants further in-depth investigation, particularly with respect to conophthorin and lepidopteran insects. For instance, this phenomenon could help explain the presence of conophthorin in insect frass (*14*) and direct researchers to evaluating damaged peach tree tissue for conophthorin (*35*).

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Table 3. Examples of organisms that reportedly produce conophthorin: agricultural commodities (ag), plants (p), gum frass (gf), or microbes (fungal spores = fs; bacteria = bac)

Scientific Name	*Common Name/ Organism*	*Tissue/Conditions*	*Ref.*
Prunus dulcis	Almond (ag)	Hull, shell, kernel; *ex situ*	(*18*, *36*)
		Hull split; *in situ*	(*15*)
		Mummies	(*37*)
Pistacia vera	Pistachio (ag)	Leaves; extracted, *ex situ*	Beck, unpub.
Opuntia humifusa	Prickly pear (ag, p)	Pad tissues; biotically stressed	(*16*)
-	Gum frass (gf)	Frass *et al.* on peach trees	(*14*)
Aspergillus spp.	(fs)	Spore on fatty acid	(*19*)
Penicillium glabrum	(fs)	Spore on fatty acid	(*19*)
Rhizopus stolonifer	(fs)	Spore on fatty acid	(*19*)
Streptomyces spp.	(bac)	Soja flour medium	(*38*)
Colepteran-Related Plant Examples (see also ref (*3*))			
Betula pendula	Silver birch (p)	Bark, branches, stems, leaves	(*1*)
Dracaena fragrans	Cornstalk dracaena (p)	Bark	(*1*)
Populus tremula	Aspen (p)	Bark	(*1*)
Quercus suber	Cork oak (p)	Bark	(*12*)
Other Specific Examples			
Chelyocarpus ulei	Palm (p)	Flower	(*33*)
Dorstenia turnerifolia	Toxic perennial (p)	Flower	(*34*)
Families of Reported Flowers			(*32*)
Arecaceae		Asteraceae	
Fabaceae		Hydrangeaceae	
Lecythidaceae		Malvaceae	
Moraceae		Orchidaceae	
Passifloraeae		Rubiaceae	
Ruscaceae		Rutaceae	
Solanaceae			

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Another investigation where conophthorin was detected, but not fully defined as a lepidopteran attractant, was published by our laboratory in 2014 (*16*). In the study, cold-stressed *ex situ* cactus tissue produced conophthorin in which fungi were implicated as the source, but not definitively proven. An interesting result of the study was the implication that stressed plant tissue, and/or the microbiome, are signaling a vulnerability to associated herbivores. Finally, conophthorin was detected from the headspace of pistachio leaf extract (Beck, unpublished). The pistachio leaves were fresh, cut up, and extracted with pentane. The implications of this finding are under investigation.

It should be noted that the structural isomer chalcogran (Figure 4) and its corresponding diastereomer, are occasionally detected in many non-insect sources. One hypothesized biosynthetic pathways for conophthorin (*19*) from fatty acids included a similar route for chalcograns, which may explain the co-occurrence of the two isomeric structures. While chalcogran has important semiochemical behaviors for many non-lepidopteran insects, this same reported activity has not yet been noted in lepidopterans toward the chalcograns.

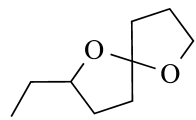

Figure 4. The structurally similar spiroketal chalcogran.

Navel Orangeworm and Fungal Spore Mutualism?

Bark beetles have a long and established history of mutualistic relationships with various fungi (distinct benefit to both organisms) (*39*, *40*). This type of relationship has recently been proposed between navel orangeworm and aflatoxin-producing fungi (*21*, *28*). The navel orangeworm has been shown to not only metabolize aflatoxin (*41*), but it has also been demonstrated that the larvae can develop without apparent side effects in environments with high amounts of aflatoxins (*42*). With the production of conophthorin by developing fungal spores (including aflatoxigenic aspergilli) recently established (*19*), it is conceivable that there exists a mutualism between navel orangeworm and fungal spores. Once a relatively few number of fungal spores locate a host plant with the appropriate conditions (nutrients, water activity), they can signal to the navel orangeworm moths that a suitable host plant (*17*) is vulnerable to further infestation by both the insect larvae and the resident microbes on the larvae (*43*). Investigation by researchers with expertise in the field of insect-microbe mutualisms is needed to fully explore this phenomenon.

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Application of Host Plant- and Fungal Spore-Based Attractant Blends

Just as researchers have used conophthorin and other semiochemicals to protect conifers from bark beetle infestations (*44*), there has been considerable progress toward the implementation of a synthetic blend of host plant volatiles, with conophthorin included as a key ingredient, for the monitoring and control of navel orangeworm (*15*, *22*). The navel orangeworm moth is the major insect pest of almonds and pistachios grown in California. It incurs millions of dollars of damage to the tree nut industry through direct damage to fruit and by vectoring aflatoxigenic fungi (*15*, *22*).

Our laboratories have recently begun evaluating the trapping efficacy of the synthetic host plant volatile (HPV) blend, which contains conophthorin, in mating disruption treated almond orchards. Mating disruption for navel orangeworm entails the use of a female sex pheromone component dispersed throughout an orchard to confuse the male moth and inhibit mating (*45*). Current monitoring of navel orangeworm populations in mating disruption treated orchards is typically done with the use of almond meal, an almond kernel-based tissue matrix that is inconsistent and has low attractancy. In the 2014 growing season study, the HPV blend's trapping efficacy in both conventional and mating disruption treated almond orchards was compared to that of almond meal. Figure 5 illustrates some preliminary results from the first year of investigation. These results are offered as an example of the application of a HPV blend that contains conophthorin as a critical component for attraction.

As the data show in Figure 5, the HPV blend provided a more sensitive monitoring tool to keep track of navel orangeworm population dynamics information in mating disruption and conventional environments when compared to the almond meal-based attractants. These preliminary data are from the first year of a three-year study. Correlations to almond damage in both the conventional and mating disruption orchards will be analyzed and compared to years two and three.

The data from the HPV blend in conjunction with the other material presented in this book chapter are meant to convey the expanding field of fungal spore-derived semiochemicals (*17*), particularly as they may relate to discrete insect-microbe interactions that signal host plant vulnerability. In each case outlined above, there was an implied association of plant damage to the emission of conophthorin. While in some instances there is not definitive proof of the origin or purpose of conophthorin (was it produced by the plant or fungus and is it a true semiochemical for the associated lepidopteran?), it is hoped that the material presented provides enough insight to alert other researchers as to the possibility of fungal (or microbe) involvement. Moreover, the production of conophthorin from a stressed plant/fungal tissue opens other lines of possibilities in terms of volatile emissions. If the conophthorin and other volatiles were produced from stressed plant tissue, were the resultant emissions signaling plant vulnerability? Or, if the resultant volatiles were from fungi (or microbes), were they signaling other microbes?

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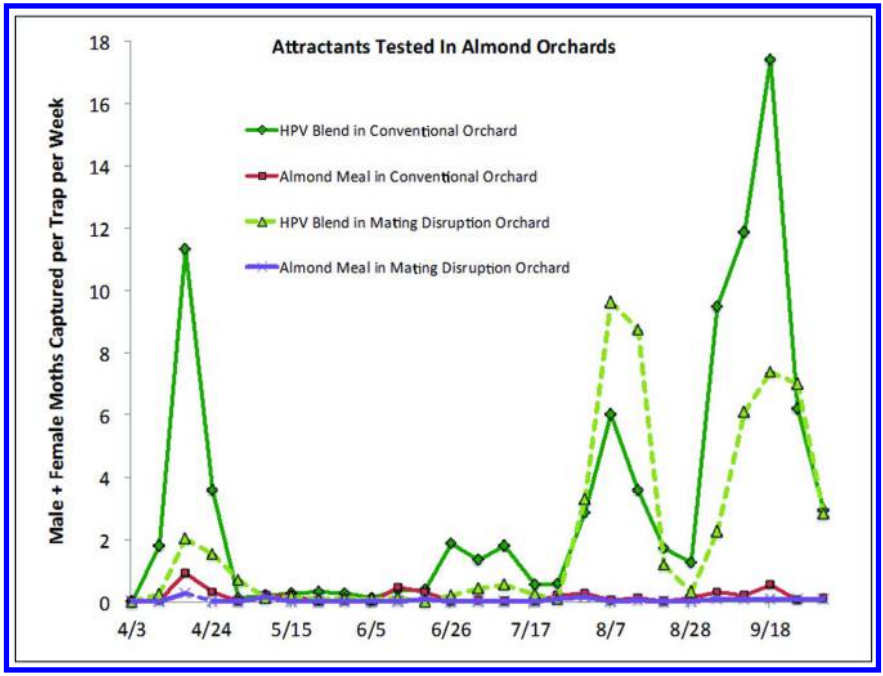

Figure 5. Trap capture data from the 2014 almond orchard growing season of navel orangeworm moths attracted to and trapped by the synthetic host plant volatile (HPV) blend that contains conophthorin. Trap captures are compared to almond meal. The HPV blend was evaluated simultaneously in conventional almond orchards and orchards undergoing mating disruption treatments.

Whatever the answers to these inquiries will be, conophthorin appears to be playing an important signaling role as a component in a bouquet of host plant volatiles. As Figure 3 illustrates, conophthorin is a central character in several plant-insect systems involving lepidopterans, with the primary difference among them being either the identities of other key semiochemicals, or the difference of the ambient odors that are typical of that particular host plant.

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Chapter 10

Tioxazafen: A New Broad-Spectrum Seed Treatment Nematicide

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Tioxazafen is Monsanto's new seed treatment nematicide designed to provide consistent broad-spectrum control of nematodes in corn, soy, and cotton. This new compound exhibits excellent activity against soybean cyst, root knot, and reniform nematodes in soy; lesion, root knot, and needle nematodes in corn; as well as reniform and root knot nematodes in cotton. Tioxazafen is a disubstituted oxadiazole, which represents a new class of nematicidal chemistry demonstrating equal or better performance in greenhouse and field trial evaluations when compared with commercial treatments. This mini-review will cover the discovery process of tioxazafen and its control of nematodes infesting major row crops.

Introduction

Nematodes (roundworms) are ubiquitous and important pathogens of plants (*1*). Plant nematodes globally cause significant crop injury and yield loss for growers (*2*). Among nematode-infested crops with major economic losses are soybeans, potatoes, cotton, corn, citrus, strawberries, tomatoes, carrots, peppers,

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and sugar beets (*3–5*). Plant parasitic nematodes including root knot, cyst, reniform, lesion, and others infest all parts of plants, especially the roots. The most economically damaging are sedentary endoparasitic nematodes (e.g., root knot and cyst) which establish root feeding sites. Roots suffer from nutrient loss and diminished capacity to survive drought and stress. Yield losses in soybean can range from 5 to 80%, depending on rainfall, soil fertility, presence of other diseases and the nematode population. Nematode invasion also facilitates secondary infection with fungal pathogens. For example sudden death syndrome (SDS) is more severe when the soybean cyst nematode (SCN) is also present in a field (*6*) and invasion of the roots by root knot nematode (RKN) enhanced infection by *Fusarium oxysporum* (*7*). For tuber crops, nematodes cause loss of harvest quality as well as yields.

There are currently a limited number of pesticides registered to control nematodes. At the end of the last century, pre-plant soil fumigants were commonly utilized for this purpose (*8–11*), but many are now restricted or face significant regulatory pressure (*12*, *13*). The use of non-fumigant nematicides is also restricted, with the vast majority of compounds (organophosphates, carbamates) relying upon a single mode of action (*14*). For the past 25 years, development of new compound classes that confer broad-spectrum nematode control has been rare (*15–17*).

In the broader context, achieving safe and effective nematode control is a key part of the move toward environmentally sustainable agriculture. Therefore, chemical seed treatments are gaining traction in the marketplace because of their reduced environmental footprint, economical advantage due to lower use rates, and ease of use for the farmer. Seed treatment applications reduce the chances for off-target movement and reduce the amount of product required because of specific placement. Here we report on the discovery of tioxazafen (3-phenyl-5-(thiophen-2-yl)-1,2,4-oxadiazole) - a new broad-spectrum seed treatment nematicide that is designed to provide consistent broad-spectrum control of nematodes in corn, soy, and cotton.

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Discovery of Tioxazafen with the Aid of Molecular Field Models

The early nematicide discovery effort that led to tioxazafen's discovery began with a series of compounds that included stilbenes, chalcones, and azobenzene derivatives (SCA series). These series displayed potent and broad-spectrum nematicidal activity against plant and animal parasitic nematodes as well as the model species *C. elegans*. In addition, assays using parasites resistant to common pesticides such as macrocyclic lactones and benzamidazoles suggested that these compounds have a distinct and novel nematicidal mode of action.

However, these scaffolds had deficiencies that prevented further development as pesticides, including loss of efficacy in soil-based assays. Extensive analoging suggested these deficiencies would be difficult to overcome without altering the core chemical structure. Therefore we investigated approaches to "scaffold hop" to a new, structurally distinct chemical series (*18*). To avoid the time

and expense of screening tens of thousands of compounds in whole-organism assays, a computational screening approach was used (*19*, *20*). Such approaches typically attempt to dock chemical structures into the active site of a characterized protein structure. In this case, however, no crystal structure of the molecular target was available for *in silico* docking. Therefore, a ligand-based model was required. After evaluating several cheminformatic methods, we found success using Cresset's eXtended Electron Distribution (XED) field model along with its suite of screening software (*21*).

The XED field model simulates the asymmetric distribution of electrons around atoms in a manner compatible with quantum mechanics, yielding a more accurate representation of a molecule's electronic field than atom-centered models (*22*, *23*). The force field generated by the XED model, which captures positive and negative charges, Van der Waals forces, and hydrophobicity, is a sophisticated representation of what the binding target "sees" when it interacts with the ligand. Two molecules which are structurally dissimilar in traditional 2-D structure may bind to the same target site if their field patterns are similar—the basis for scaffold hopping.

XED-based comparison of field patterns by Cresset's XedeX algorithm provides a robust, structure-independent method of aligning distinct pharmacophores. Accompanying computational modules allow selection of multiple low energy conformations for searching, visualization of field comparisons, and field-based virtual screening of large compound databases. This software has been validated against a wide variety of ligand classes, including inhibitors of HIV reverse transcriptase, thrombin, and G-protein coupled receptors (*24–26*). To apply the XED model to virtual screening, a database of over four million commercially available compounds has been converted into searchable field patterns (FieldPrints). Searching this database with the XED field of an active molecule extracts a new series of compounds with diverse 2-D structures but comparable fields and potentially similar biological activity (Figure 1).

Using Cresset's software, the SCA series' field patterns were analyzed along with each compound's biological activity, creating a field-based SAR (FSAR) independent of each compound's 2D structure (Figure 2).

This FSAR was further reduced to eight field patterns that were considered most representative of the series' activity, called Field Templates, which were then used as the basis for virtual screening against Cresset's FieldScreen library.

The virtual screening approach is essentially a ranking: each compound conformation in the library is scored by its similarity to each Field Template, and then the four million compounds in the library are ranked by the similarity scores of each compound's top-scoring conformation. The top-scoring 200 compounds for each template (fewer than 1600 total, because some were found in multiple screens) were selected for further clustering and filtering. This resulted in the assembly and commercial acquisition of a focused 477-compound library representing significant scaffold diversity. Compared to the leads in the original SCA series used as templates (Figure 3), the 2D structures of this library were dissimilar (the majority had Tanimoto coefficient less than 0.4) but their molecular fields were highly similar to the template (XED similarity > 0.77).

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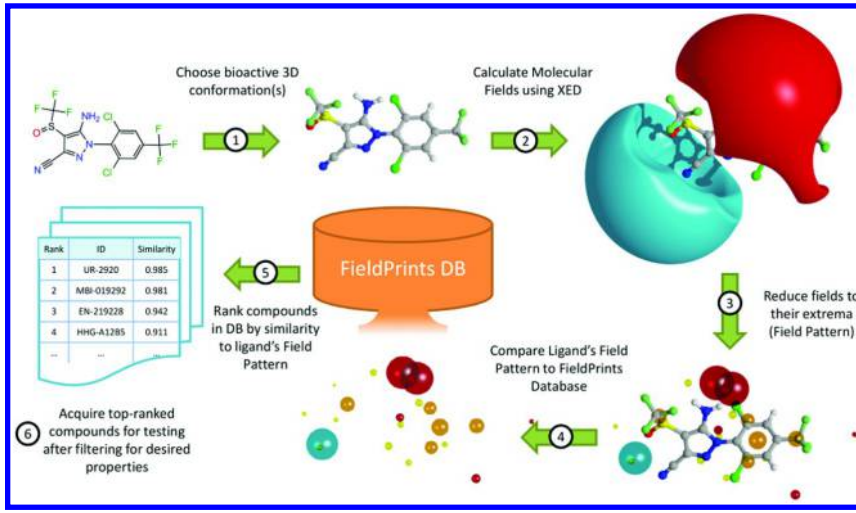

Figure 1. A diagram of the molecular field screening approach, using the phenylpyrazole insecticide fipronil as an example. In this field representation blue = electronegative, red = electropositive, yellow = Van der Waals interactions, orange = hydrophobic interaction. (see color insert)

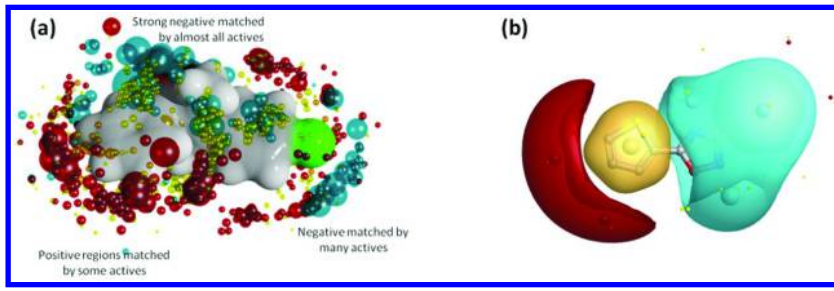

Figure 2. a) the field points and FSAR of the original SCA consensus model, and b) the field pattern for tioxazafen. Colors are described in Figure 1. (see color insert)

At this stage, compounds were evaluated for intrinsic nematicidal activity in both a standard *C. elegans* larval motility assay and against the plant parasitic nematode *Meloidogyne incognita* (Root Knot Nematode or RKN) in a rapid biological assay called *QuickSand* (QS). In the QS assay, cucumber seedlings are planted in sand in test tubes or small pots and inoculated with RKN eggs, typically with 3 replicates per condition or dose. Chemistry is applied as a drench in solution (usually acetone) at the appropriate dose measured in ppm (µg/mL), and root galling is assessed at 12 days. Roots are washed free of sand and root galling is scored visually on a 0-3 scale, with 0 = no galling (good RKN control) and 3 = severe galling. Phytotoxic phenotypes such as chlorosis and root stunting are also assessed visually. Unless specified otherwise, "active" indicates an average QS score of 1.5 or lower.

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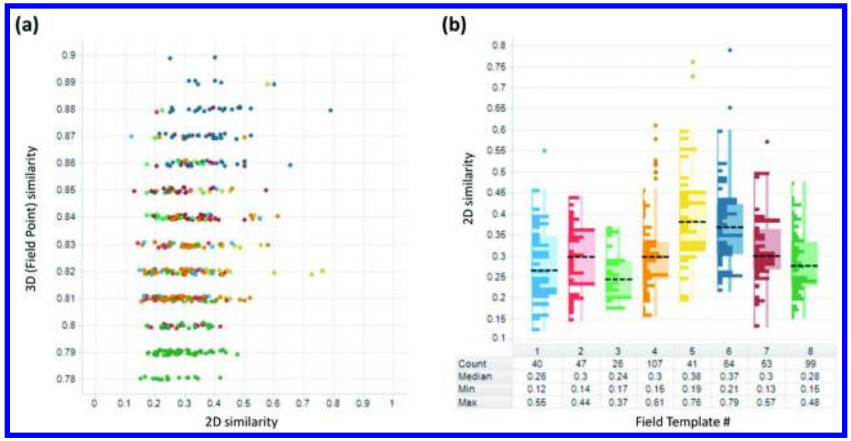

Figure 3. (a) 3D (XED) similarity scores vs. 2D (Tanimoto) similarity for tested compounds, colored by search template. For each structure, similarities are shown as the maximum similarity to the eight field models or structures chosen as search templates. This illustrates that field similarity does not necessarily correlate with 2D structure similarity. (b) Distribution of 2D similarity for compounds tested from each search template, showing that most compounds tested were structurally dissimilar from the template. The Tanimoto Coefficient (T) is a commonly-used measure of the similarity between two chemical structures, ranging from 0-1, where identical structures will have T=1. Calculations of T were performed using Pipeline Pilot v9.1.0 with FCFP4 fingerprints. (see color insert)

17% of the compounds (81/477) demonstrated activity at 6.3µg/ml or less against *C. elegans* larvae in a standard motility assay, a hit rate much higher than the background hit rate observed in a chemical collection that was not targeted for nematode control (1-3%, data not shown). 14% of the compounds (most of them overlapping with the *C. elegans* hits) were active at 40ppm or lower against RKN in the QS assay (Table 1).

Molecules active in Quicksand with favorable physicochemical properties were tested in the greenhouse on cucumber plants growing in a mixture of sand and field soil. By inoculating cucumber with RKN eggs at 0 or 7 days after chemical drench and then measuring percentage of roots with galls after 3 weeks, compounds that are rapidly degraded or subject to strong soil binding can be identified. Among the 67 initial hits advanced to the greenhouse, 23 were active (greater than 50% control of galls relative to standard) at an equivalent of 2 kg/hectare or less. These tests were complemented by other second-tier assays for spectrum and longevity, including drench and seed treatment assays on *Heterodera glycines* (soybean cyst nematode, SCN), treatment on soils inoculated with live juvenile RKN, and pre-plant soil incorporation tests on RKN measured up to eight weeks after soil preparation.

Table 1. Hit rates of each Field Template at 40 and 8 ppm in the QuickSand assay."Active" is defined as a QS rating ≤ 1.5 (see text)

Field Template Number	# Tested	# Active at 40 ppm QuickSand	% Active at 40 ppm QuickSand	# Active at 8 ppm QuickSand	% Active at 8 ppm QuickSand
1	42	4	9.5%	0	0.0%
2	51	3	5.9%	1	2.0%
3	25	3	12.0%	2	8.0%
4	103	19	18.4%	3	2.9%
5	37	6	16.2%	3	8.1%
6	63	8	12.7%	1	1.6%
7	52	8	15.4%	4	7.7%
8	104	16	15.4%	6	5.8%
Totals	**477**	**67**	**14.0%**	**20**	**4.2%**

Because soil longevity was a significant detriment to the original SCA series, this soil activity was considered an important milestone to continue investigation of this approach. However, the efficacy observed among most compounds in these primary screens was still several-fold lower than commercial standards tested in the same assays, and by design many of the compounds were structurally diverse. To improve efficacy and validate chemical series, hits were clustered to identify several core chemical scaffolds, providing a basis for traditional analoging with functional groups to complement the field-based approach (Figure 4).

Over a series of iterations and refinements to the model, hundreds of additional analogs of these compounds were acquired from commercial vendors or synthesized for testing in these assays, and a cluster of highly active oxadiazole analogs was identified (Figure 5).

Structural modifications around the oxadiazole core were evaluated for nematicidal activity (Figure 5). Structural features favored are phenyl or substituted phenyl (ortho or ortho/para substitution pattern with Cl, Me, Br and F as preferable substituents) at the 3-position and 2-thiophenyl or 2-furanyl at the 5-position of the oxadiazole ring. Several compounds in this series demonstrated strong efficacy (comparable to commercial standards) in greenhouse assays against a spectrum of parasitic nematodes, including RKNSCN, reniform, and lesion nematode. As these leads advanced through microplot testing and early toxicity screens, tioxazafen emerged as a lead candidate for its combination of intrinsic efficacy, soil longevity, and synthetic accessibility.

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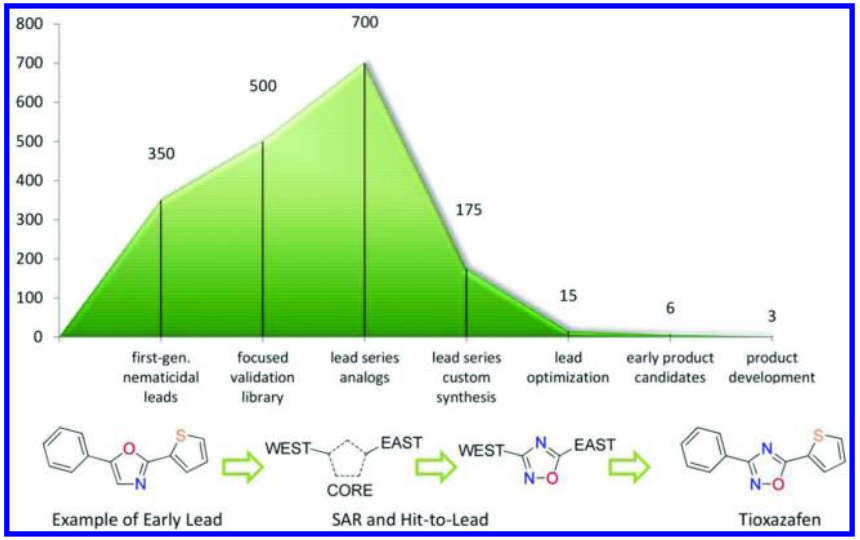

Figure 4. Approximate number of compounds under evaluation at each stage of the nematicide discovery project. (see color insert)

Efficacy Assessment of Tioxazafen

Tioxazafen efficacy was assessed using *in vitro* bioassays, growth chamber and green house studies, and field microplot studies, which utilize plants grown in confined small plots under normal field conditions. These studies assessed direct nematicidal activity against larvae and eggs, as well as the ability of the nematodes to reproduce on plants.

In vitro bioassay results indicate that tioxazafen has direct nematicidal activity against RKN *(M. incognita)* and SCN *(H. glycines*) and is as effective as nematicidal active ingredients of commercial standards in causing nematode mortality. At the lower concentrations, tioxazafen out-performed the commercial standards (Table 2 and 3).

Phenotypic characterization of plant-parasitic and the free-living nematode *Caenorhabditis elegans* in the presence of tioxazafen indicate a mode of action that is distinct from other commercial nematicides. Classical genetic as well as biochemical studies to further elucidate the molecular target(s) of this compound have been initiated.

In early growth chamber studies, tioxazafen showed excellent efficacy when applied as a drench. Subsequent improvements in formulation and seed application gave increased efficacy when using tioxazafen as a seed treatment.

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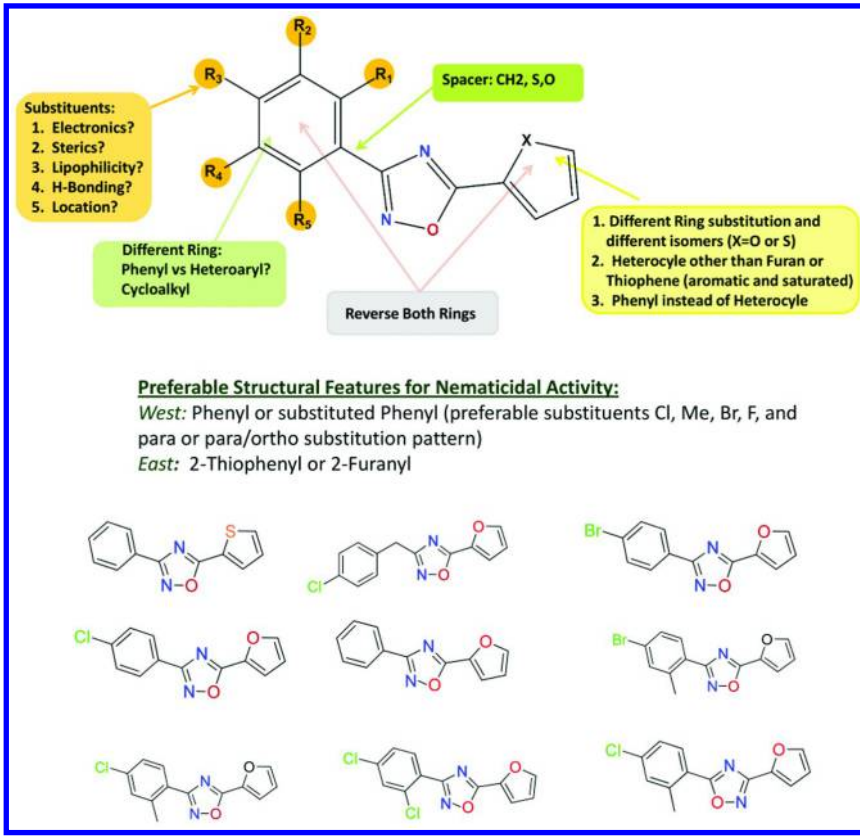

Figure 5. Some structural modifications of the 1,2,4-oxadiazole series, and examples of compounds active in the RKN QuickSand assay (EC_{50} ~0.5-2.0 ppm). Green House efficacy RKN, SCN 0.25- 2.0 kg/ha (drench). (see color insert)

Table 2. Root Knot Nematode (M. incognita) in vitro bioassay. Percentage of nematodes immobilized or killed after soaking in nematicide solutions[a].

Treatment	*Rate*			
	0.1 ppm	*1 ppm*	*10 ppm*	
Nematicide 1	22 b	78 ab	100 a	*% Mortality*
Nematicide 2	37 ab	77 ab	93 ab	
Nematicide 3	27 b	32 c	36 c	
Tioxazafen	46 a	86 a	95 ab	
Untreated Control	27 b	32 c	21 d	

[a] Treatment means with a letter in common are not significantly different at a 0.05 significance level. Nematcide 1, 2 and 3 are active ingredients of commercial standards.

Table 3. Soybean Cyst Nematode (*H. glycines) in vitro* bioassay. Percentage of nematodes immobilized or killed after soaking in nematicide solutions[a]

Treatment	*Rate*			
	1ppm	*5 ppm*	*10 ppm*	
Nematicide 1	24 b	88 b	97 a	*% Mortality*
Nematicide 2	57 a	93 ab	99 a	
Nematicide 3	25 b	33 d	54 c	
Tioxazafen	55 a	75 c	87 b	
Untreated Control	7 c	34 d	12 d	

[a] Treatment means with a letter in common are not significantly different at a 0.05 significance level. Nematcide 1, 2 and 3 are active ingredients of commercial standards.

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Based on the physical properties of tioxazafen ($LogK_{ow}$ 4.13, aqueous solubility 1.24 mg/L) and its intended use as a seed treatment, our formulation efforts focused on developing a stable, high loading suspension concentrate. Initial prototype formulations demonstrated modest efficacy against SCN as a seed treatment compared to the high levels of efficacy observed as a soil amendment (Figure 6a). We hypothesized that availability of tioxazafen was limited by its low aqueous solubility and investigated formulation technologies to lower the use rate needed to produce improved efficacy relative to the initial prototypes. Through an iterative process we identified formulations that demonstrated a 20-fold rate reduction while maintaining acceptable loading and physical stability targets. Tioxazafen formulations were incorporated into Acceleron, a fungicide/insecticide seed treatment package available from Monsanto Company. An example of tioxazafen performance against SCN as a result of improved seed treatment formulations is shown in Figure 6b.

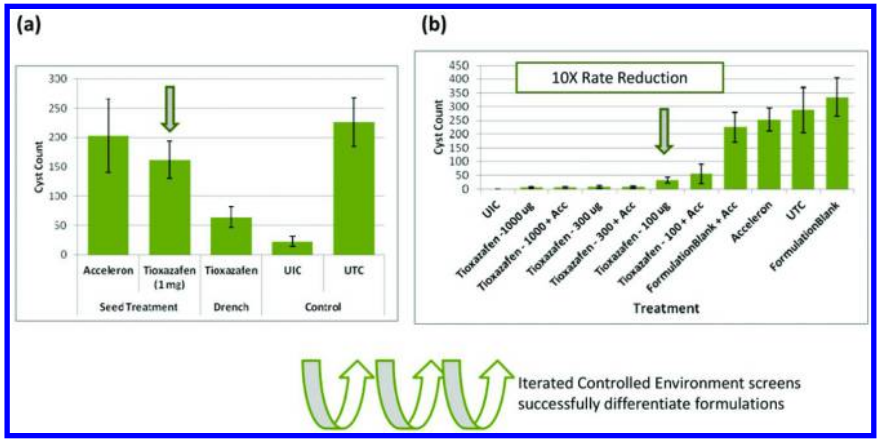

Figure 6. Tioxazafen performance as a seed treatment against SCN (Controlled environment): (a) Early results (b) Improved formulations; UIC = Uninoculated Control; UTC =Untreated Control; Acc = Acceleron = Fungicide/Insecticide base. (see color insert)

An uptake study with soybean seed treated with radiolabeled tioxazafen indicates that the compound is predominantly distributed to the root zone and is not upwardly mobile to the vegetative tissue (radiogram in Figure 7). Retention within the root zone is ideal for nematode management.

Extensive growth chamber and green house studies demonstrate excellent tioxazafen efficacy as a seed treatment against several key nematodes (soy cyst nematode, root knot nematode and lesion) in common row crops (Figure 8).

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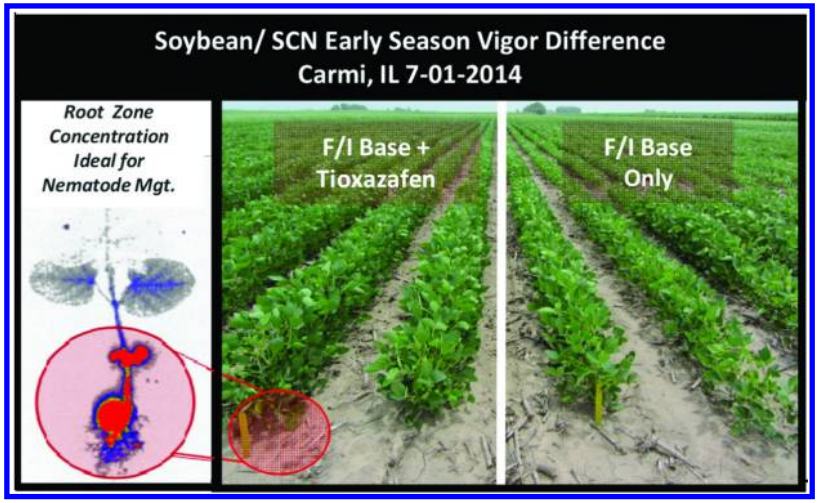

Figure 7. Tioxazafen radiogram in soybean followed uptake from the seed indicating compound concentration in a root zone; F/I = Fungicide/Insecticide. (see color insert)

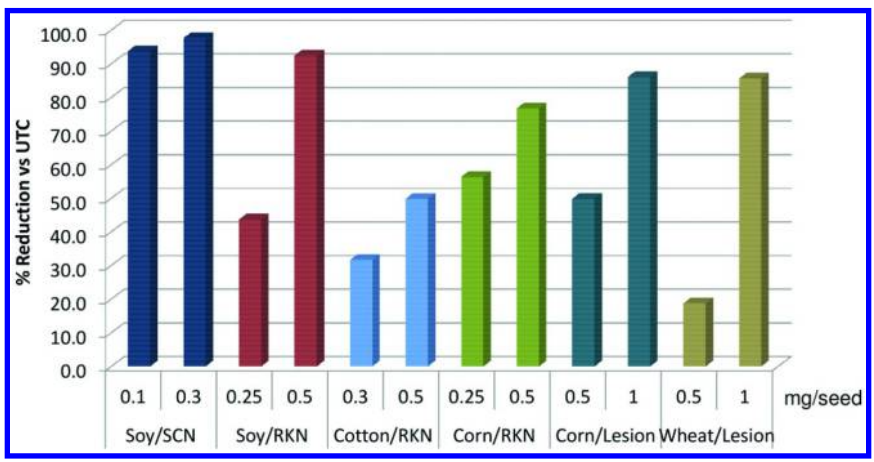

Figure 8. Tioxazafen protects key row crops from damaging nematodes. Data shown are the percentage reduction of cysts, galls, and nematodes on plants grown from treated seed in controlled environment. UTC=Untreated Control. (see color insert)

Tioxazafen has demonstrated effective soybean cyst and root knot nematode control in microplots. Three years of microplot testing with key row crops in multiple environments and against different nematodes show consistent efficacy results, confirming controlled environment data (Figure 9).

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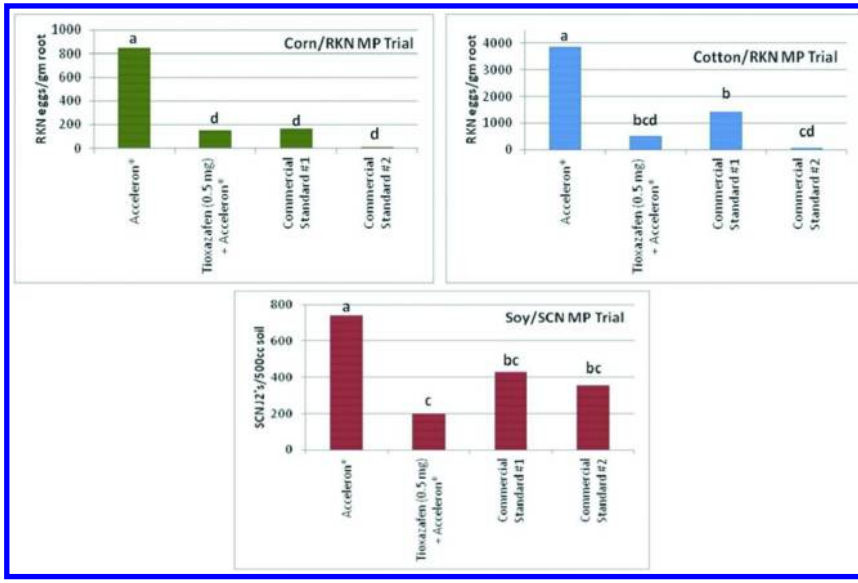

Figure 9. Tioxazafen seed treatment provided effective RKN and SCN control in microplots. Units are mg/seed; Treatment means with a letter in common are not significantly different at a 0.05 significance level. (see color insert)

Microplot studies were conducted on Monsanto research farms as well as in blinded studies with independent academic testers. Significant RKN pressure was obtained in each microplot study as evidenced by the high number of eggs and juveniles recovered from plants receiving only fungicide and insecticide seed treatment. Tioxazafen provided significant reductions in the total egg and juveniles stages parasitizing the plants with efficacy that was equal to the commercial standards. Commercial standards 1 and 2 used in microplot and large scale field trials were the current market leading nematicidal seed treatment products and applied according to the respective labels.

Tioxazafen provides consistent broad-spectrum nematicidal control (soy cyst, root knot and reniform) in soybean field trials that is equal or better than commercial standards. A field trial design using paired plots was employed to address nematode spatial population variability and provide more precise yield data. Performance evaluation is based on differences between treatments and paired control plots and includes stand, vigor, percent germination, nematode counts and yield. Tioxazafen provided plant health benefit in early season vigor and increased yield as demonstrated in small plot (2 row X 20 ft length) field trials in soybean infested with reniform nematodes (Figure 10).

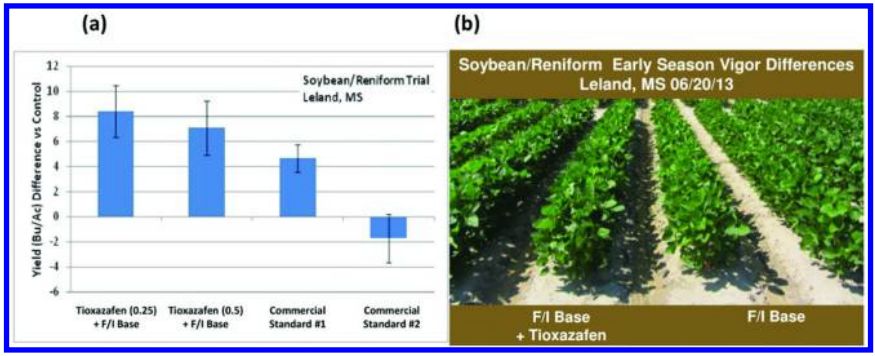

Figure 10. Tioxazafen seed treatment at 0.5 and 0.25 mg/seed in soybean infested Reniform nematodes provided (a) Yield benefit (b) Plant health benefit (Vigor); Units are mg/seed; F/I=Fungicide/Insecticide. (see color insert)

As shown in Figure 10a, tioxazafen provided 7 to 8 bushels per acre yield increase and was superior to both commercial standards.

Tioxazafen was found to provide consistent broad-spectrum nematode (root knot, needle and lesion nematodes) control in corn demonstrated in growth chambers and field trials with representative examples shown on Figure 11 and 12.

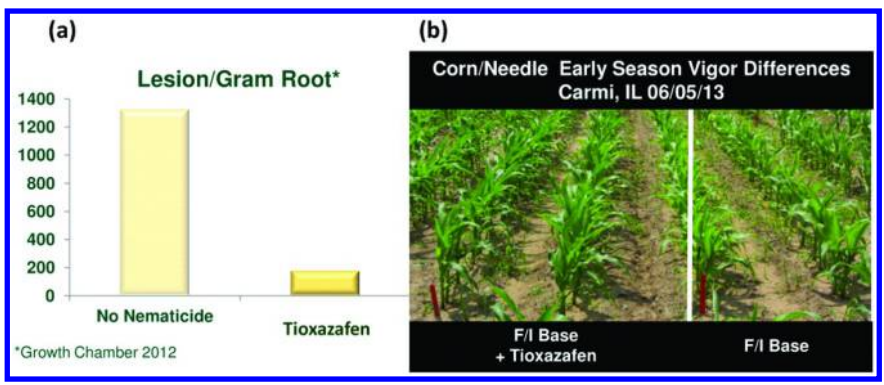

Figure 11. Tioxazafen performance as seed treatment for nematode control in corn (a) Lesion nematode reduction in growth chamber (b) Early season health plant benefit (vigor) in field trial; F/I=Fumgicide/Insecticide. (see color insert)

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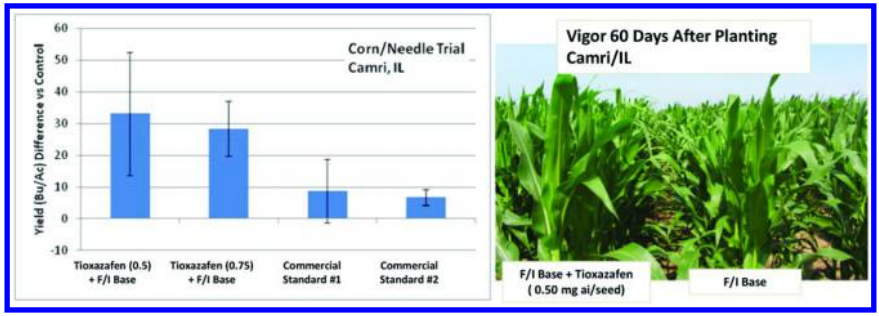

Figure 12. Tioxazafen as a seed treatment provided health benefit and increased yield in corn infested with needle nematodes; Units are mg/seed; F/I = Fungicide/Insecticide. (see color insert)

Tioxazafen as a seed treatment was found to provide consistent broad-spectrum nematode (root knot and reniform) control in cotton demonstrated in growth chambers and microplot field trials with representative examples shown on Figure 13 and 14. The ability of tioxazafen seed treatment to provide season-long benefit from a seed treatment is shown in Figure 13. Over the course of the season nematode soil populations increased 20X in nontreated plots but only 5X in treated plots. The reproductive factor (Rf) is calculated as ratio of Final Population (Pf) divided by Initial Population (Pi) and a reduction in Rf is a measure of nematode control.

Figure 13. RKN reproductive factor reduction in RKN infested cotton microplot trials; Reproductive Factor (Rf)=Final population(Pf)/Initial Population (Pi). (see color insert)

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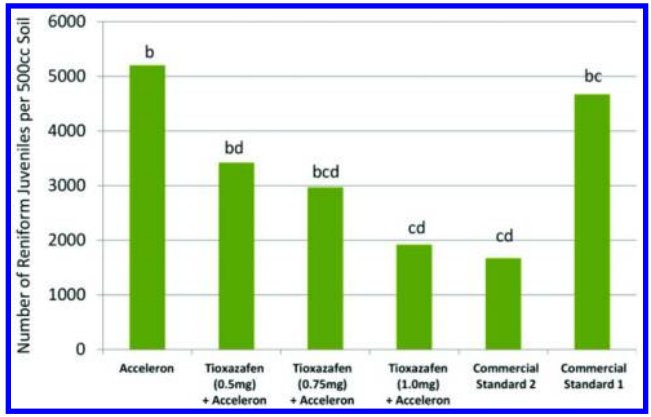

Figure 14. Tioxazafen vs commercial standards reniform nematode control in cotton microplots trials. Units are mg/seed; Treatment means with a letter in common are not significantly different at a 0.05 significance level. (see color insert)

Large scale field trials in corn and soybean were conducted under a USA EUP (Experimental Use Permit). Up to 40 locations with nematode populations above the economical damage threshold were used to plant 8 row x 300-400ft length strip plots. These large scale trials have demonstrated increased yield in corn and soybean that is equal to or better than commercial standards (Figure 15 and 16).

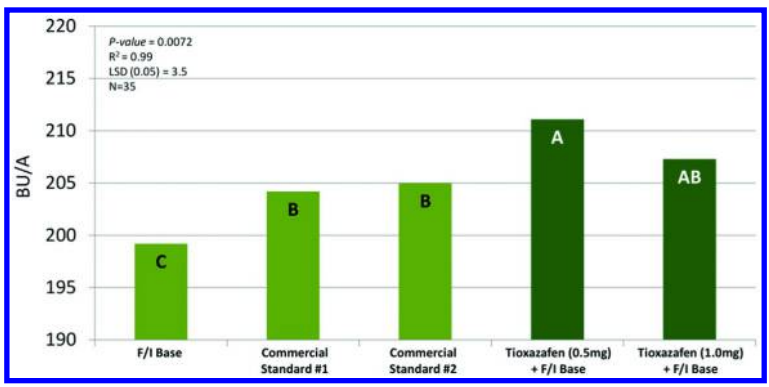

Figure 15. Tioxazafen seed treatment increased corn yield in large scale field trials. All sites contain moderate to high nematode pressure. Treatment means with a letter in common are not significantly different at a 0.05 significance level; Units are mg/seed; F/I=Fungicide/Insecticide.. (see color insert)

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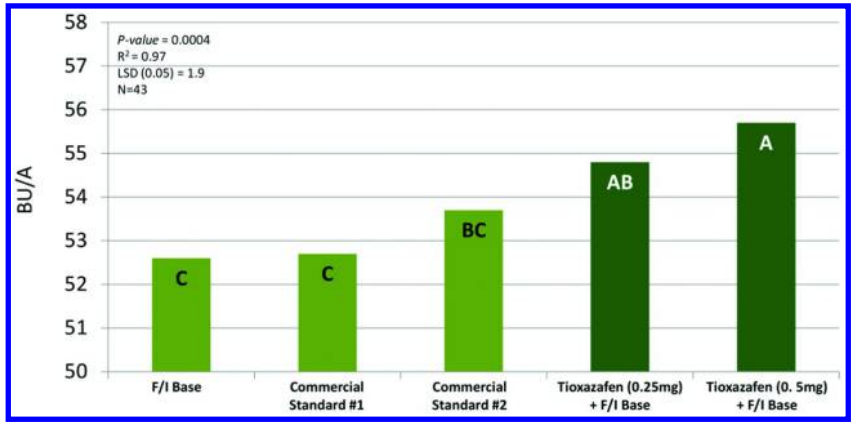

Figure 16. Tioxazafen seed treatment increased soybean yield in large scale field trials. All sites contain moderate to high nematode pressure. Treatment means with a letter in common are not significantly different at a 0.05 significance level; Units are mg/seed; F/I=Fungicide/Insecticide. (see color insert)

Tioxazafen Synthesis

3,5-Disubstituted 1,2,4-oxadiazoles are a class of biologically important heterocycles that can be prepared in a number of ways (*27*). Generally, 1,2,4-oxadiazoles are synthesized by cyclodehydration of O-acylamidoximes that can be promoted by either heat and/or bases. The O-acylamidoximes are synthetically available via reaction of amidoximes with activated carboxylic acid derivatives or with carboxylic acids in the presence of a coupling reagent. The literature reported synthesis of 3-phenyl-5-thiophen-2-yl-[1,2,4]oxadiazole involved an acylation of benzamidoxime with thiophene-2-carbonyl chloride in pyridine and subsequent cyclization at an elevated temperature (*28*). Other methods involving a one-pot conversion of carboxylic acid derivatives and amidoxime to the corresponding 1,2,4-oxadiazole under milder conditions are more attractive due to ease of synthesis, product isolation and potential for scale-up (*29*, *30*).

An example of the method that was used for production of larger quantities of tioxazafen is depicted in Figure 17. The one-step ring closure procedure involves an acylation of benzamidoxime **2** with methyl thiophene-2-carboxylate **3** followed by instant cyclodehydration at 140 °C in xylene in the presence of potassium carbonate and features an easy final product isolation.

The benzaminodoxime **2** was prepared from benzonitrile and hydroxyl amine (release in situ from its salt by sodium hydroxide) in methanol. This method was used for production of larger quantities of tioxazafen in high yields (in a range of 85-87%) and high purity.

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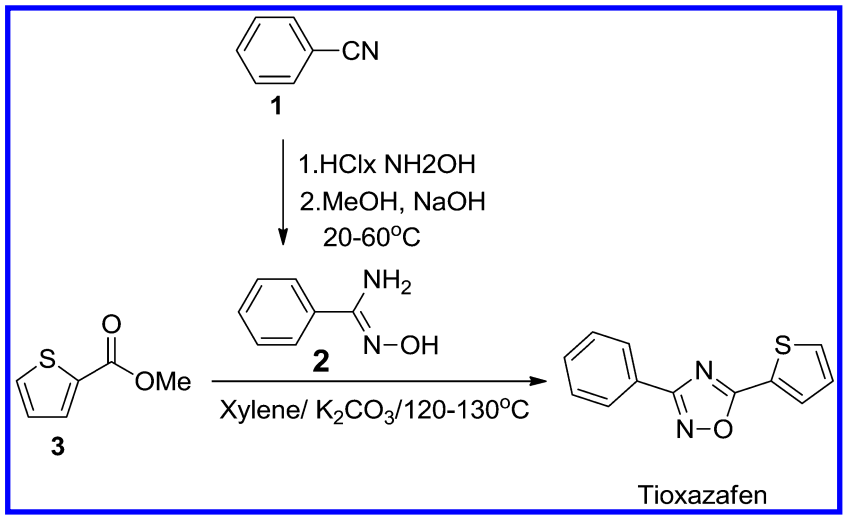

Figure 17. Tioxazafen synthetic route amenable for scale-up.

Summary

In summary, tioxazafen is Monsanto's new seed treatment nematicide for row crops that provides consistent and broad spectrum control of key nematode pests in corn, soy, and cotton. This new nematicide has been found to exhibit excellent field activity against cyst, root knot, reniform, lesion, and needle nematodes. Tioxazafen has demonstrated equal or better performance in greenhouse and field trials compared to commercial nematicide products. It offers nematode control via a mode of action that is distinct from current commercial nematicides. Regulatory studies and large scale field efficacy testing are progressing to support a commercial launch.

Acknowledgments

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Chapter 11

The Discovery of Oxathiapiprolin: A New, Highly-Active Oomycete Fungicide with a Novel Site of Action

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Oxathiapiprolin is the first member of a new class of highly-active oomycete fungicides, the piperidinyl thiazole isoxazolines. It acts via a novel fungal target, an oxysterol binding protein, resulting in excellent preventative, curative and residual efficacy against key diseases of grapes, potatoes and vegetables. Oxathiapiprolin is being developed globally as DuPont™ Zorvec™ disease control with first registration and sales anticipated in 2015. The discovery, optimization and efficacy of this new chemical class will be reviewed.

Hit Identification and Activity

The discovery of oxathiapiprolin began with the purchase of a diverse compound library from Tripos Associates built around a piperidine-thiazole-carbonyl core using a diverse set of acid and amine moieties, as detailed in Figure 1. Compound **1** was the only member of this library where a phenyl acetic acid moiety had been combined with a benzyl amine moiety and showed modest preventative fungicidal activity against *Phytophthora infestans* on tomatoes (tomato late blight; TLB) and *Pseudoperonospora cubensis* on cucumbers (cucumber downy mildew; CDM) and weak curative activity against *Plasmopara viticola* on grapes (grape downy mildew; GDM) in early stage screens. This hint of curative activity, an important, but rarely seen attribute for oomycete

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fungicides, caught our attention and an optimization program was initiated which led to the discovery of the piperidinyl thiazole isoxazoline class of oomycete fungicides (*1*, *2*).

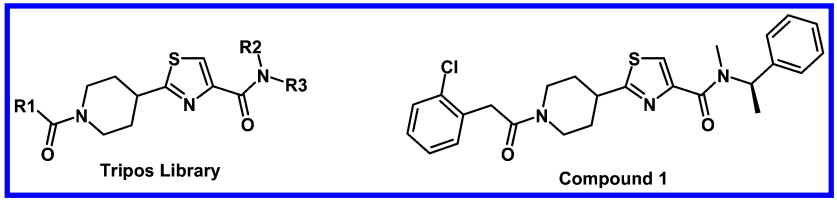

*Figure 1. Purchased Tripos library details: R1 and R3 are aryl, heteroaryl and benzyl; R2 is hydrogen or methyl. Compound **1** is a library member*

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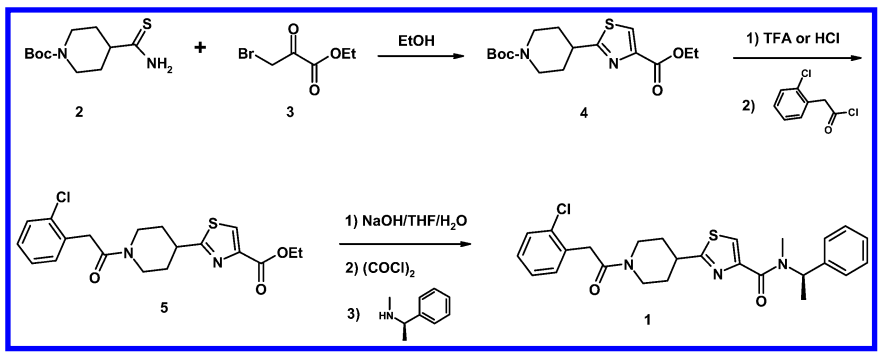
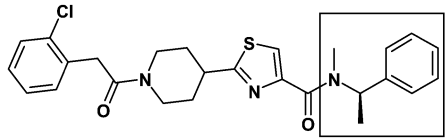
Chemistry and Initial SAR

The synthesis of Compound **1** is outlined in Scheme 1 and begins with condensation of the commercially available Boc-protected piperidine thioamide **2** with ethyl bromopyruvate **3** to give the orthogonally protected piperidinyl thiazole **4** (*3*). Boc-deprotection, followed by acylation affords ester **5**, which upon base hydrolysis and amide coupling provides Compound **1**. These steps can be reversed where ester hydrolysis and amide coupling precedes Boc-deprotection and acylation.



*Scheme 1. Synthesis of Compound **1***

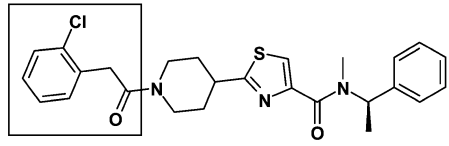
Using the method of Scheme 1, a positional scanning approach was used to assess the initial SAR for the amine component by holding the 2-chlorophenyl acetic acid moiety constant. As summarized in Figure 2, numerous amine variations were explored, but did not lead to significant improvement over the original *N*,*α*-dimethyl benzyl amine moiety. Key observations were the requirement for a methyl substituent at nitrogen and a small alkyl substituent on the benzylic carbon, with the (*R*)-enantiomer being preferred.



- (*R*) >>> (*S*)
- *N*-Methyl required for high activity: Me >> H ~ Et >> Pr
- α-Substituted benzylic required for activity:
  Et > Me > CN > Pr > *i*-Pr > vinyl > allyl > Bu > *i*-Bu > CF_3 > H
- Phenyl substituents do not appear to enhance activity
- Replacing benzylic carbon with *N*-Me, C=O or O eliminates activity
- Ph, alkyl, phenethyl or reduced α-methylbenzyl amides are inactive
- Heteroaryl in place of phenyl have poor to moderate activity

Figure 2. Initial amine moiety SAR

Variation of the acid moiety, holding the (*R*)-*N*,*α*-dimethylbenzyl amine moiety constant, is summarized in Figure 3. Key observations were the requirement of a carbonyl linked to a 2,5-disubstituted phenyl ring by either an unsubstituted methylene or nitrogen atom, with small alkyl, haloalkyl and halogen substituents being optimal. The 2,5-dimethyl analog, Compound **6**, showed a 5-fold increase in activity vs. Compound **1**, with > 90% control of preventative and curative TLB and curative GDM observed at an application rate of 40 ppm. This became the lead compound.



- Substituted aryl acetic acids required for activity
- Benzoyl and lower alkoyl are inactive
- Unsubstituted methylene is best: CH_2 > NH > O >>> bond
- Carbonyl required for activity: desoxy and sulfonyl inactive
- Meta > ortho >>> para
- CH_3, CF_3, OCH_3, Cl, Br are best substituents
- 2,5-disubstituted the best: Me/Me ~ Cl/Cl ~ Cl/CF_3 ~ OCH_3/Br

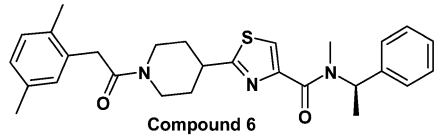


*Figure 3. Initial acid moiety SAR; Compound **6**: unbroken activity at 40 ppm*

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Compound **6** showed rapid leaf penetration but did not move systemically in the plant, most likely due to its high logP and low water solubility. As summarized in Figure 4, replacing the dimethyl phenyl ring with a 3,5-disubstituted *N*-linked pyrazole gave a significant boost in activity and systemicity. Thus, Compound **7** showed a 20-fold increase in activity vs. Compound **1**, with > 90% control of preventative and curative TLB and curative GDM observed at an application rate of 10 ppm.

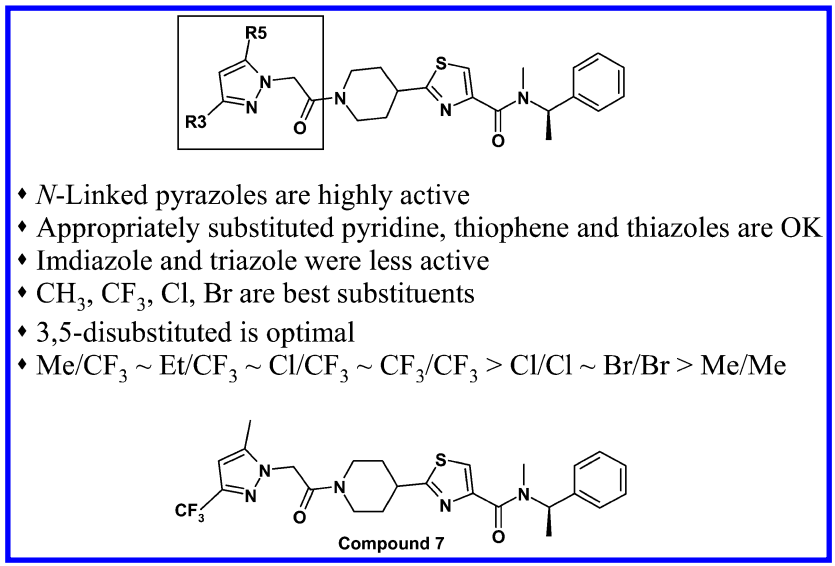

Figure 4. Heterocyclic acid moieties. Compound 7: unbroken activity at 10 ppm

Modification of the piperidinyl thiazole core was carried out while keeping the acid and amine moieties as shown in Compound **7**. A wide variety of chemical methods were needed to explore changes to this portion of molecule and elaborating them here is beyond the scope of this review. The SAR developed is summarized in Figure 5 and revealed that an unsubstituted piperidine ring was best. Replacing the piperidine ring with a piperazine ring gave analogs with good activity, but poor photostability, most likely due to extended conjugation of the extra nitrogen lone pair.

High activity could be retained by replacing the thiazole ring with other 5-membered ring heterocycles, as shown in Figure 6, with the original thiazole or corresponding oxazole being the best. Substitution on the 5-position of the thiazole ring or replacing the thiazole ring with a phenyl, pyridine or pyrimidine ring resulted in loss of activity.

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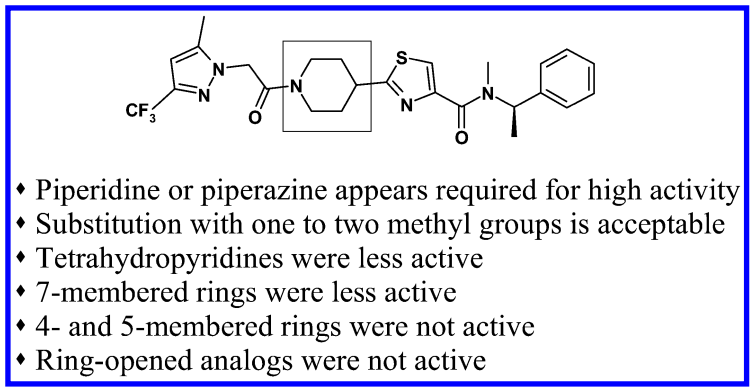

Figure 5. Piperidine SAR. Unsubstituted piperidine appears optimal

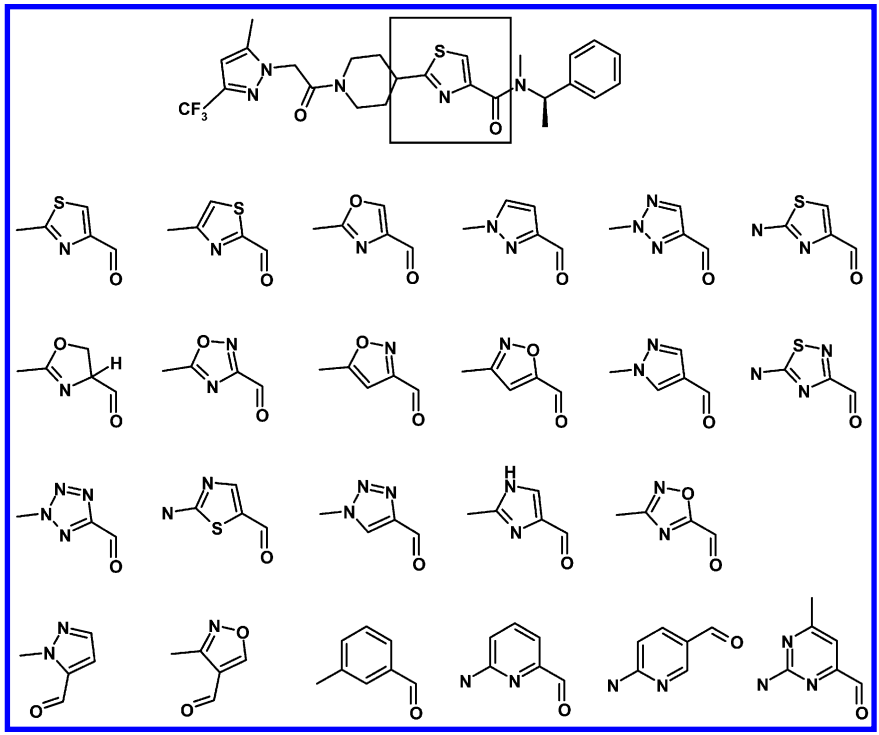

Figure 6. Thiazole moiety SAR. Row 1 and row 2 variations were highly active, row 3 variations were moderately active and row 4 variations were inactive

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Restricting Conformations

We had explored the three key regions of the molecule and an activity plateau appeared to have been reached. Compound **7** had excellent preventative and curative oomycete control at 10 ppm, but needed to be active at lower rates to be commercially viable. Inspection of Compound **7** shows that it has eight rotatable bonds allowing for a wide variety of accessible low energy conformations. Flexible molecules may require more energy to adopt a needed binding conformation, reducing potency. If one could pre-organize a molecule into the appropriate binding conformation, less energy would be required and potency should increase. A program to conformationally restrict Compound **7** by forming new rings near the chiral center region of the molecule was initiated by tying-back the *N*-methyl onto the *a*-methyl, the *N*-methyl onto the phenyl and the *α*-methyl onto the phenyl as outlined in Figure 7. The first two modifications were unproductive, but the third resulted in a remarkable boost in activity.

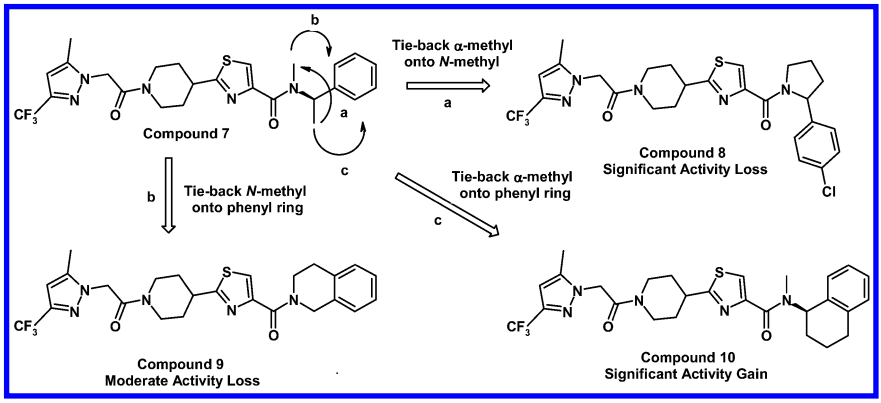

Figure 7. Restricting conformations near the chiral center

Compound **10** showed a 1000-fold increase in activity vs. Compound **1**, with > 90% control of preventative and curative TLB and curative GDM observed at an application rate of 0.4 ppm. Compound **10** is the (*R*)-enantiomer; it's corresponding (*S*)-enantiomer is significantly less active. Field tests in the United States and Europe showed excellent preventative, curative and residual efficacy against *Phytophthora infestans* on potatoes (potato late blight; PLB) and GDM at 100 gai/ha, outperforming commercial fungicides (*4*).

With this approach validated, we turned our attention to the amide bond. Compound **7** exists as a mixture of two rotamers, clearly observable in its NMR spectrum and requiring a temperature of 110 °C for signal coalescence. We envisioned preparing the two isomeric imidazolines **11** and **12** as our best probe compounds, but also prepared the isoxazoline **13** (see Figure 8). This was fortuitous, since both Compounds **11** and **12** were essentially inactive, while Compound **13** again resulted in a remarkable boost in activity.

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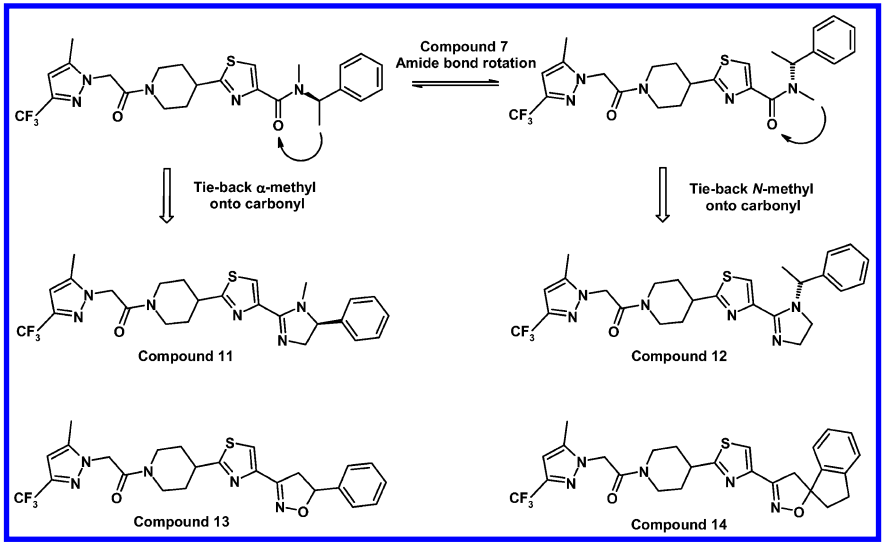

*Figure 8. Amide bond bioisosteres. Compound **13** and **14** have unbroken activity at 0.4 and 0.08 ppm, respectively.*

Like Compound **10**, racemic Compound **13** was 1000-fold more active than starting Compound **1**. The (*R*)-enantiomer of Compound **13** was more active than the (*S*)-enantiomer. Restricting the rotation of the phenyl ring by tying it back onto the 5-position of the isoxazoline ring gave Compound **14** and another significant boost in activity. Compound **14** showed a 2500-fold increase in activity vs. Compound **1**, with > 90% control of preventative and curative TLB and curative GDM observed at an application rate of 0.08 ppm. Field tests demonstrated its superior performance vs. the leading commercial oomycete fungicides (*1*, *2*).

The Challenge: Selecting the Optimal Candidate

An intensive effort looking at a wide range of alternate amide bioisosteres led to many active chemotypes (*5*, *6*), but none were more active than the isoxazoline chemotype, as summarized in Figure 9. The corresponding isoxazole and the homologous dihydrooxazine were also quite active.

The piperidinyl thiazole isoxazolines can be readily prepared by either a linear or a convergent synthesis route shown in Scheme 2 for Compound **13**. Nitrosation of dichloroacetone gives the bench stable dichloroketooxime **16** which undergoes a [3+2] cycloaddition with styrene to form the chloroketoisoxazoline **17**. Condensation with the Boc-piperdine thioamide **2** forms the thiazole ring followed by Boc-deprotection and coupling with the pyrazole acetic acid **19** to afford Compound **13**. The more advanced thioamide intermediate **21** can also be used and provides crystalline product directly from the reaction.

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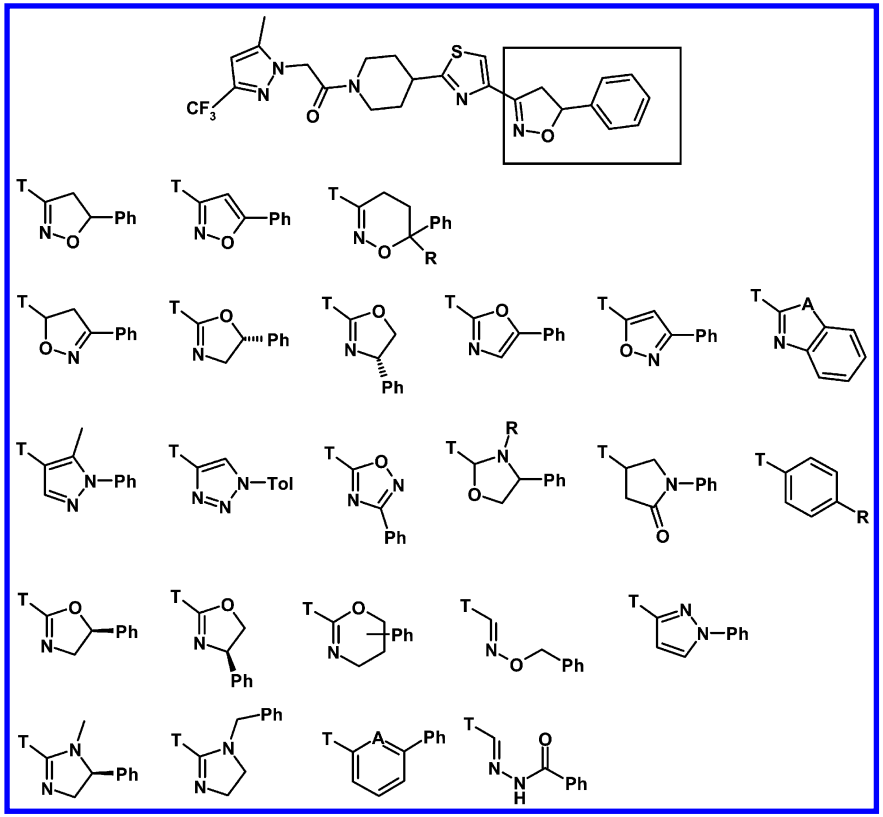

Figure 9. Isoxazoline moiety SAR. Row 1 variations were highly active, row 2 and row 3 variations were moderately active and row 4 and row 5 variations had low or no activity

Exploring the SAR of the isoxazoline moiety showed that 5-substitution was essential for high activity, with a wide variety of aryl, heteroaryl and other groups being explored. A second 5-substituent was also tolerated with 5,5-spiro analogs such as Compound **14** being among the best. The phenyl ring of Compound **14** is twisted out of plane of the isoxazoline ring by the spiro ring fusion. This twist can also be forced by adding substituents in the 2- and 6-positions of the isoxazoline phenyl ring of Compound **13** to give analogs with unprecedented levels of activity. For example, Figure 10 depicts the 2,6-difluoro analog Compound **22**, which showed a 10,000-fold increase in activity vs. Compound **1**, with > 90% control of preventative and curative TLB and curative GDM observed at an application rate of 0.02 ppm (*1*).

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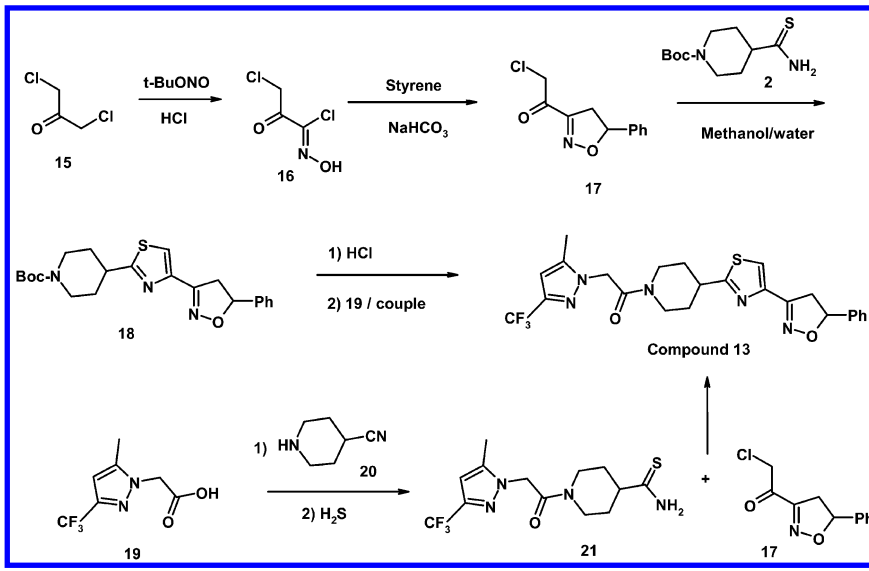

Scheme 2. Linear and convergent synthesis routes

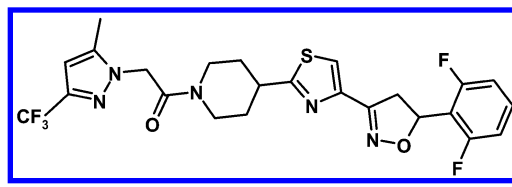

*Figure 10. Compound **22**: unbroken activity at 0.02 ppm*

In parallel with the isoxazoline SAR development, work continued on exploring alternatives to the pyrazole acetic acid moiety. Acyclic structures were explored and found to be highly active provided that they were linear chains of 5-7 atoms preferably terminated with a trifluoromethyl group (*7*). The piperidine could also be replaced with a 1,2-oxazinane, a hexahydropyridazine or a 1,5-diazabicyclo[3.3.0]octane ring system to give compounds with high levels of activity (*8*).

With so many chemotypes with high intrinsic activity, it soon became apparent that the key challenge would now be finding the best of the best to move forward into development. Figure 11 shows a selection of optimized acid and isoxazoline moieties, the combination of which all produced viable candidates. An added complication was the observation of polymorphism in many of these candidates (*9*) which necessitated finding the most stable polymorph for each one to assess formulatability and biological efficacy.

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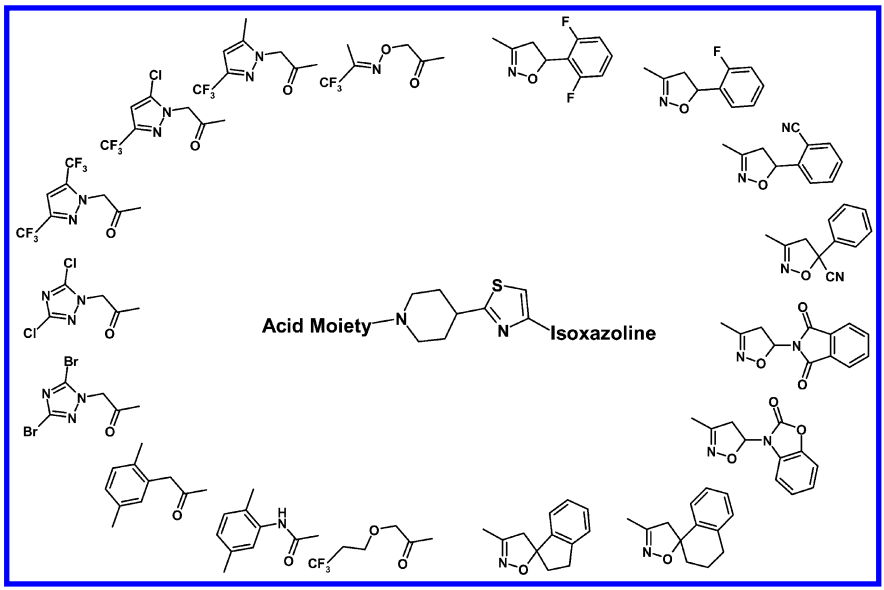

Figure 11. Selection of functionalities which impart high intrinsic activity

This effort led to the selection of a set of forty top analogs. These "final forty" underwent extensive characterization and greenhouse testing to produce our "final four" candidates for broad scale tox and field testing. All of the candidates performed well in the field with Compound **22** (Figure 10) being selected as the commercial development candidate, oxathiapiprolin.

Biological Activity of Oxathiapiprolin

Oxathiapiprolin was evaluated in the field for control of the major oomycete diseases of grapes, potatoes and vegetables. Oxathiapiprolin demonstrated outstanding control of potato late blight at rates as low as 12-30 gai/ha in preventative and curative trials, along with residual control of 7-10 days. Control of grape downy mildew was also impressive with both fruit and foliar protection at rates as low as 20-30 gai/ha. Cucumber downy mildew and crown and root rot of peppers were also controlled at rates as low as 12-30 gai/ha. It consistently outperformed all of the commercial disease control standards.

Oxathiapiprolin acts at multiple stages of the pathogen's life cycle at extremely low concentrations. Preventatively, it inhibits zoospore release and stops zoospore and sporangia germination; curatively, it stops mycelial growth within the host plant before visible lesions occur, offering protection at 1, 2 and 3 days; post-infection, it stops mycelial growth and inhibits further lesion expansion; and as an antisporulant, it inhibits spore production and viability. Oxathiapiprolin is rapidly absorbed into the epicuticular waxy layer of the plant making it extremely resistant to wash-off. Once inside the plant, it shows translaminar and acropetal systemic movement, protecting treated leaves as they

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grow and expand and new leaves as they emerge and grow. Oxathiapiprolin has an excellent toxicity profile, with low oral, neuro, developmental, dermal, aquatic, avian and bee toxicity.

Mechanism of Action

Initial work to determine the mechanism of action of oxathiapiprolin focused on comparisons with known fungicides. Tests to determine if oxathiapiprolin acted by known mechanisms were all negative. A fluorescent tagged version of Compound **10** showed localization to the nucleus, golgi and endoplasmic reticulum using confocal microscopy. Staining could be reversed by high concentrations of active analogs, but not by other known fungal classes.

A bis-tritiated version of Compound **13** was found to bind to a single, low abundance target contained in a soluble protein extract from *P. infestans*. Displacement experiments with analogs of varying potencies showed that the ability to bind to this target mirrored the observed biological activity. These radio ligand binding assay were used to develop purification schemes that allowed for a five-fold enrichment of activity compared to crude extracts.

At this point two parallel efforts were undertaken to identify the target protein using the model oomycete *Phytophthora capsici*. A biochemical approach used a tethered version of Compound **13** to isolate binding proteins by affinity chromatography. One of these was identified by mass spectrometry as an oxysterol binding protein (OSBP). A molecular genetics approach provided definitive data that OSBP was a critical component in the mechanism of action. *P. capsici* zoospores were irradiated with UV light and resistant mutants were selected with growth media infused with Compound **14** or **22**. Numerous independent resistant mutants were isolated and were determined by sequencing to have a single nucleotide polymorphism in the *P. capsici* OSBP gene. Changes at 9 different amino acid positions, all located within the oxysterol binding domain, were observed (*10*, *11*). Additionally, transformation of *P. capsici* cells with a plasmid encoding a mutated OSBP gene conferred resistance to oxathiapiprolin.

OSBP represents a novel target for disease control. The cellular role of OSBPs is not well understood (*12*). A flag-tagged OSPB protein has been expressed in *Phytophthora* and purified by affinity chromatography. The flag-purified protein showed a 10,000 fold increase in binding activity and it exhibits binding kinetics similar to wild type *P. capsisi* OSBP. Studies to provide definitive evidence for the cellular role of OSBP in *Phytophthora* and the exact mechanism of oxathiapiprolin cytotoxicity are still underway.

Conclusion

Oxathiapiprolin is the first member of a new class of highly-active oomycete fungicides, the piperidinyl thiazole isoxazolines. It is effective at extremely low use rates and shows excellent preventative and curative efficacy, excellent antisporulant properties and long residual control with protection of new growth. It was discovered using a high throughput screening approach with

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diverse structural classes and optimized to this high potency chemotype by conformationally restricting the benzyl amide moiety using an isoxazoline ring as an amide bioisostere. Oxathiapiprolin has a novel site of action, binding strongly to an oxysterol binding protein with an, as yet, unknown cellular function. Oxathiapiprolin has the perfect combination of attributes to provide outstanding oomycete disease control and is being developed globally as DuPont™ Zorvec™ disease control with first registration and sales anticipated in 2015. We expect that products powered by oxathiapiprolin will become a valuable tool for growers in their effort to combat these diseases.

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Chapter 12

Novel Substituted Anilinopyrimidine Compounds: Design, Synthesis, and Fungicidal Activity

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Anilinopyrimidinones exhibited moderate fungicidal activity against cucumber downy mildew (CDM) or wheat powdery mildew (WPM) in previous research. To improve their bioactivity, a series of novel anilinopyrimidine analogs were designed and synthesized using 'Intermediate Derivatization Method'. Preliminary bioassays demonstrated that most of the compounds exhibited weak to moderate activities against downy mildew. But surprisingly, some compounds synthesized showed moderate to good fungicidal activities against powdery mildew. The relationship between structure and fungicidal activity was also discussed. Compounds 7a-4 exhibited potent fungicidal activity against powdery mildew and effective in the control of powdery mildew on wheat, strawberry, tomato, cucumbers at 250-300 mg/L in field trials.

Pyriminostrobin (SYP-11277) is a novel acaricide of methoxyacrylate class developed by our group with terminal group replacement method (TRM) which was one important type of Intermediate Derivatization Method (IDM) (*1*–*8*). TRM focuses on novel key intermediates that have the potential to replace terminal moieties of known agrochemicals, pharmaceuticals, or natural products. These unique intermediates are synthesized through chemical reactions starting

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from raw chemicals. So a number of anilinopyrimidinone compounds 1 were synthesized in the process of discovery of pyriminostrobin. Bioassay indicated that compounds 1 showed moderate fungicidal activities against cucumber downy mildew (CDM) or wheat powdery mildew (WPM), especially compound 2 provided 60% control of CDM at 25 mg/L (*3*, *4*, *9*). To improve its bioactivity, a series of novel anilinopyrimidine analogs 3 were designed and synthesized using IDM based on the structural features of commercial fungicides dimethirimol, ethirimol and bupirimate, which are major fungicides in control of powdery mildew (Figure 1). Many of the compounds represented by 3 provided more than 90% control of WPM or CDM at 100 mg/L. Several sufficiently active compounds were evaluated in field tests to assess the potential for commercial development.

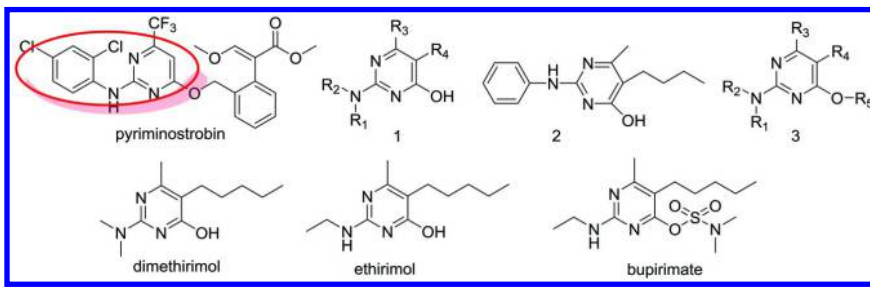

Figure 1. Structure Design and Some Known Fungicides

Synthesis

Synthesis of different sub-classes of compounds of generic structure 3 will be discussed. When R_3 was CH_3, anilinopyrimidinones 6 were prepared in 88–95% yield by the reaction of substituted guanidines 5 with substituted ß-keto esters 4 in refluxing toluene for 8–12 h. The substituted guanidines 5 were purchased from commercial sources or prepared by treating corresponding amine hydrochlorides with cyanamide and Na_2CO_3. ß-Keto esters 4 were prepared by condensation of ethyl acetoacetate with R_4X (haloalkanes, halobenzenes or benzyl halides) in 85–90% yield. For further optimization, compounds 7 were synthesized by the reaction of anilinopyrimidinones 6 with R_5X such as haloalkanes, propargylic halides, ethyl chloroacetate, methyl chlorofonmate, ethyl chloroformate, isopropyl chlorocarbonate, dimethylsulfamoyl chloride, and dimethylcarbamyl chloride under basic conditions (Scheme 1).

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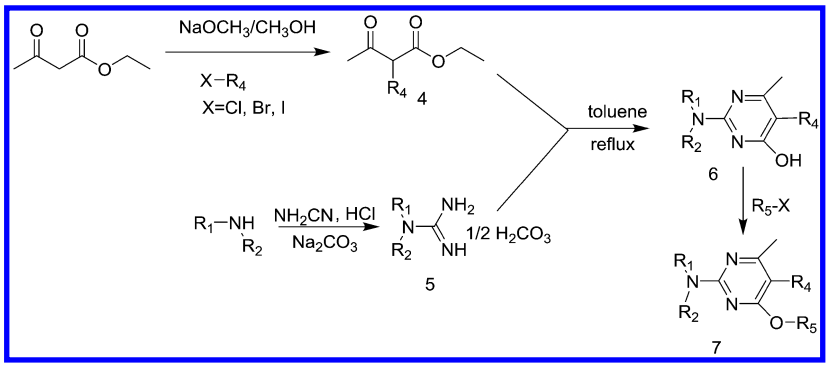
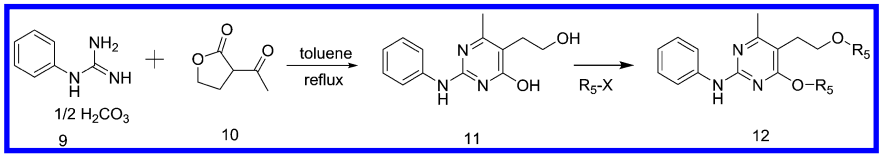


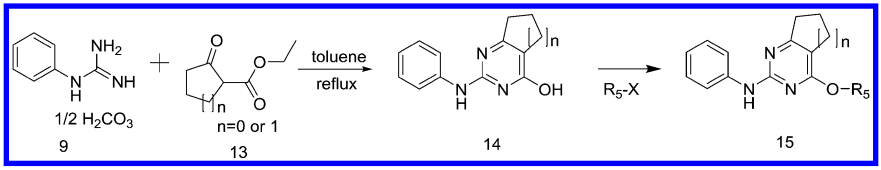
Scheme 1. General Synthesis Methods for Compounds 7

Compound 11 was prepared by condensation of the phenylguanidine carbonate 9 with 2-acetylbutyrolactone 10 in toluene in 85% yield, and compounds 12 were obtained by treating of 11 with R_5X as the same procedure as compounds 7 (Scheme 2).



Scheme 2. General Synthesis Methods for Compounds 12

Compounds 14 with 5 or 6- membered rings were prepared by condensation of the phenylguanidine carbonate 9 with 2-carbethoxycyclopentanone or 2-carboethoxycyclohexanone 13 in toluene in 80% and 85% yields respectively, and compounds 15 were obtained by the reaction of 14 with R_5X with the same procedure as compounds 7 (Scheme 3).



Scheme 3. General Synthesis Methods for Compounds 15

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Biological Data

The fungicidal activities of the title compounds are shown in Tables 1-4. Most compounds showed moderate fungicidal activities against cucumber downy mildew (CDM) or wheat powdery mildew (WPM). By changing the A, B, and C moieties of anilinopyrimidine compounds (Figure 2), and analyzing the greenhouse disease control data we were able to develop structure activity relationships.

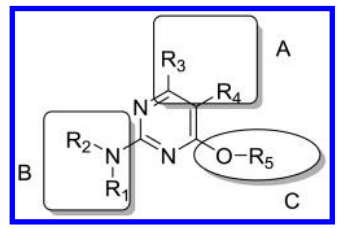

Figure 2. General Formulae of Anilinopyrimidine Compounds

Previous studies demonstrated that compound 2 showed 60% control of CDM at 25 mg/L. First, moiety B (R_1 = Ph, R_2 = H) and C (R_5 = H) were maintained, moiety A was changed (Table 1), but all compounds, except compound 6a-3, did not show good fungicidal activities against CDM, which were significantly inferior to compound 2. Some compounds showed some control of WPM. As the result shown in Table 1, we found that only the compounds 6a-3 and 2, of which each R_3 was CH_3 and R_4 was CH_2CH_2OH or $CH_2(CH_2)_2CH_3$, showed good fungicidal activity against CDM.

Then moiety A (R_3 = CH_3, R_4 = $CH_2(CH_2)_2CH_3$) and C (R_5 = H) were maintained, moiety B was changed (Table 2), and all R_1 was substituted phenyl and R_2 was maintained as H. The electron-withdrawing group and electron-donating group all be lead into phenyl ring. But the regularity of fungicidal activity still not been found. Only compound 6b-2 (R_1 = 4-CF_3O-Ph) had 90% control of CDM at 25 mg/L, which was superior to compound 2. As shown in Table 2, we found that the compounds 6b-2 and 2, of which R_1 was 4-CF_3O-Ph or Ph, showed good fungicidal activity against CDM at 25 mg/L.

The Moiety C was changed for further studying structure activity relationships, while moieties A (R_3 = CH_3, R_4 = $CH_2(CH_2)_2CH_3$) and B (R_1 = Ph, R_2 = H) were maintained. With cost and process considerations being taken into account, we found compound 2 with a simple phenyl substituent as the best group to maintain in the subsequent optimization. Bupirimate was generally used for control of powdery mildew of fruits and crops, which has $SO_2N(CH_3)_2$ in the 4 position of structure. So compound 7a-1 was prepared by introducing the group $SO_2N(CH_3)_2$ at 4-OH of compound 2, but the fungicidal activity was poor against CDM and WPM. The fungicidal activity improved when the group $SO_2N(CH_3)_2$ was replaced by $CON(CH_3)_2$ (compound 7a-2), and especially

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when replaced by $CO_2CH(CH_3)_2$ (compound 7a-3), compound 7a-3 had better control of CDM and WPM than compound 7a-1, and it had superior control of WPM as expected, despite showing better control of WPM than compound 2, but worse control of CDM than compound 2. So a series of compound 7a-3 analogs were synthesized, that is, R_5 was changed from the group $CO_2CH(CH_3)_2$ to chloroformate derivatives, alkyl, propargyl, ethyl acetate, etc. Most compounds showed better control of WPM than CDM, except compound 7a-8 showed 80% control of CDM at 25 mg/L, better than compound 2. Especially compound 7a-4 (R_5 = CO_2CH_3) displayed more outstanding control of WPM than Compound 7a-3, with 100% control of WPM at 6.25 mg/L.

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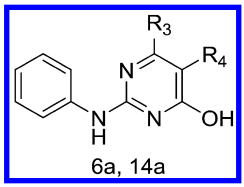
Table 1. Biological Data of Compounds 6a and 14a[a]



6a, 14a

#	R_3	R_4	*CDM (% control at the given concentration in mg/L)*			*WPM (% control at the given concentration in mg/L)*		
			400	*100*	*50*	*400*	*100*	*50*
6a-1	CH_3	H	0	NT	NT	80	NT	NT
6a-2	CH_3	CH_3	0	NT	NT	40	NT	NT
6a-3	CH_3	CH_2CH_2OH	100	0	NT	NT	NT	NT
6a-4	CH_3	CH_2-Ph	0	NT	NT	0	NT	NT
6a-5	CH_3	Ph	0	NT	NT	0	NT	NT
6a-6	CH_3	CH_2CH_2Br	0	NT	NT	30	NT	NT
6a-7	CH_3	$CH_2(CH_2)_6CH_3$	0	NT	NT	0	NT	NT
14a-1	$CH_2CH_2CH_2$		0	NT	NT	20	NT	NT
14a-2	$CH_2CH_2CH_2CH_2$		0	NT	NT	20	NT	NT
2	CH_3	$CH_2(CH_2)_2CH_3$	100	90	80	0	NT	NT

[a] CDM-cucumber downy mildew (*Pseudoperonospora cubensis*); WPM-wheat powdery mildew (*(Erysiphe graminis*); NT-Not Tested. the same as following tables.

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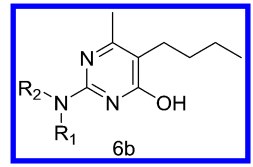
Table 2. Biological Data of Compounds 6b[a]



6b

#	R_1	R_2	*CDM (% control at the given concentration in mg/L)*			*WPM (% control at the given concentration in mg/L)*		
			400	*100*	*50*	*400*	*100*	*50*
6b-1	3,5-di-Cl-Ph	H	100	0	0	100	0	0
6b-2	4-CF_3O-Ph	H	100	100	90*	0	NT	NT
6b-3	4-F-Ph	H	0	NT	NT	0	NT	NT
6b-4	4-CF_3-Ph	H	0	NT	NT	0	NT	NT
6b-5	4-CH_3O-Ph	H	0	NT	NT	30	NT	NT
6b-6	4-CN-Ph	H	0	NT	NT	30	NT	NT
2	Ph	H	100	90	80	0	NT	NT

[a] * indicates 25mg/L. the same as following tables.

It could be concluded that the general trend in WPM activity for R_5 of moiety A (7a) was that : CO_2CH_3 > $CO_2CH(CH_3)_2$ > $CO_2C_2H_5$, $CO_2CH_2CH(CH_3)_2$, $CO_2CH_2CH_2CH_3$, $CO_2CH_2CH_2OCH_3$, CO_2CH_2-(tetrahydrofuran-2-yl), $COC(CH_3)_3$, $CH_2C{\equiv}CH$, COC_2H_5, $COC(CH_3)_3$ > $CO_2CH(CH_3)$-Ph, CO_2CH_2-Ph > SO_2(4-CH_3-Ph), $CO_2SCH_2CH{=}CH_2$, $CON(CH_3)_2$ > $SO_2N(CH_3)_2$, CH_2COCH_3, CO-Ph, CH_3 >> CO_2(4-NO_2-Ph), CO_2(2-CH_3O-Ph), $CSNH(CH_2)_2CH_3$, H.

Compounds 7b-1 and 7b-2 were prepared in order to optimize the WPM activity, but with disappointing results as 7a-4 still was the best compound.

The fungicidal activities of compound 7a-4 were shown in Figure 3. Compound 7a-4 showed more than 50% inhibition effect to *Rhizoctonia solani*, *Clttihmoloru ecgooprodslesoiie*, *Cytospora*, *Fulvia fulva*, *Alternaria solani*, *Phytophthora megasperma*, *Drechslera SoFokiniana*, *Fusarium oxysporumf*, *Fusarium oxysporum*, *Pythium aphanidermatum* at 10 mg/L. Field trials were carried out on wheats, strawberries, tomatos and cucumbers separately, compound 7a-4 as a 20% emulsifiable concentrate (EC) was effective in the control of powdery mildew at 250-300 mg/L (Tables 5-8). Compound 7a-4 showed slightly higher control than the structurally related powdery mildew standard ethirimol at an equivalent dose.

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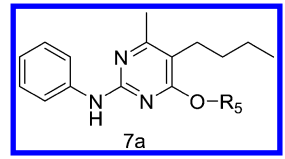
Table 3. Biological Data of Compounds 7a[a]



7a

#	R_5	*CDM (% control at the given concentration in mg/L)*			*WPM (% control at the given concentration in mg/L)*		
		400	*100*	*50*	*400*	*100*	*50*
7a-1	$SO_2N(CH_3)_2$	30	NT	NT	60	NT	NT
7a-2	$CON(CH_3)_2$	98	0	0	100	0	0
7a-3	$CO_2CH(CH_3)_2$	95	10	0	100	100	98
7a-4	CO_2CH_3	95	20	0	100	100	100**
7a-5	$CO_2C_2H_5$	95	20	0	95	98	50
7a-6	$CO_2CH_2CH(CH_3)_2$	95	0	0	100	90	25
7a-7	$CO_2CH_2CH_2CH_3$	70	NT	NT	100	95	70
7a-8	$CO_2CH_2CH_2OCH_3$	100	100	80*	100	100	70
7a-9	CO_2CH_2-(tetrahydrofuran-2-yl)	98	95	0	100	100	20
7a-10	CO_2CH_2-Ph	30	NT	NT	100	80	0
7a-11	$CO_2CH(CH_3)$-Ph	50	NT	NT	100	80	30
7a-12	SO_2(4-CH_3-Ph)	0	NT	NT	100	0	NT
7a-13	CO_2(4-NO_2-Ph)	0	NT	NT	0	NT	NT
7a-14	CO_2(2-CH_3O-Ph)	50	NT	NT	0	NT	NT
7a-15	CO_2-Ph	100	98	85	100	0	0
7a-16	$CO_2SCH_2CH{=}CH_2$	0	NT	NT	100	0	0
7a-17	COC_2H_5	30	NT	NT	100	50	30
7a-18	$COC(CH_3)_3$	50	NT	NT	100	98	40
7a-19	CO-Ph	100	15	0	70	NT	NT
7a-20	$CSNH(CH_2)_2CH_3$	0	NT	NT	0	NT	NT
7a-21	$CH_2C{\equiv}CH$	100	0	0	100	100	80
7a-22	CH_2COCH_3	98	30	10	40	NT	NT
7a-23	$CH_2CO_2C_2H_5$	0	NT	NT	0	NT	NT
7a-24	CH_3	0	NT	NT	95	0	NT
2	H	100	90	80	0	NT	NT

[a] ** indicates 6.25mg/L. the same as following tables.

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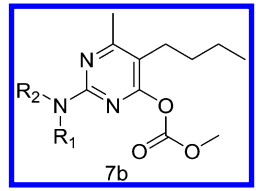
Table 4. Biological Data of Compounds 7b



#	R_1	R_2	*WPM (% control at the given concentration in mg/L)*		
			400	*100*	*50*
7b-1	3,5-di-Cl-Ph	H	100	0	0
7b-2	4-CF_3O-Ph	H	95	10	0
7a-4	Ph	H	100	100	100**

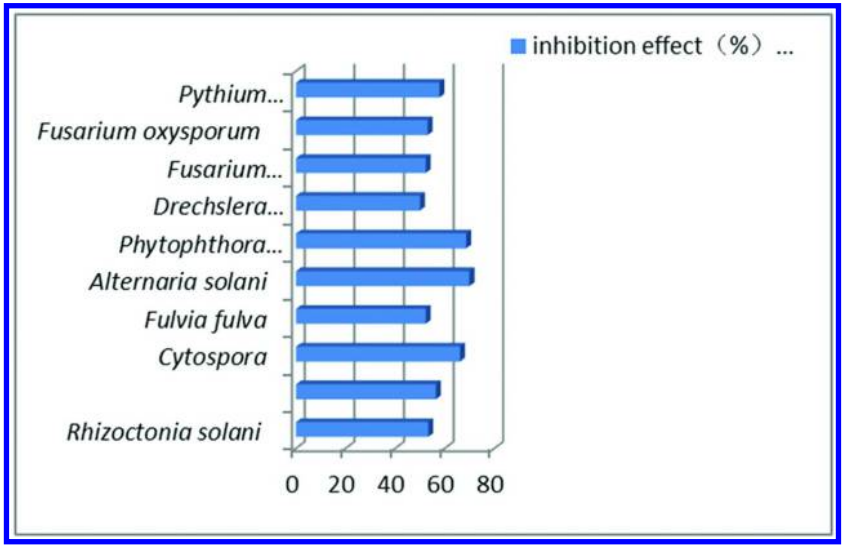


Figure 3. Fungicidal Activities of Compound 7a-4

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Table 5. Field Trials Results for Compound 7a-4 Control of Wheat Powdery Mildew[a]

compound	*concentration (8mg/L)*	*control after spraying (%)*			
		I	*II*	*III*	*average*
compound 7a-4 20% EC	250	66.7	70.1	69.4	68.8
	125	61.6	54.2	52.0	55.9
	62.5	42.7	39.5	36.0	39.4
ethirimol 25% SC	250	60.0	60.5	63.7	61.4
triadimefon 25% WP	125	92.5	91.2	93.9	92.5

[a] EC -- emulsifiable concentrate, SC -- Suspension concentrate, WP -- Wettable Powder. the same as following tables.

Table 6. Field Trials Results for Compound 7a-4 Control of Strawberry Powdery Mildew[a]

compound	*concentration (mg/L)*	*control after spraying (%)*			
		I	*II*	*III*	*average*
compound 7a-4 20% EC	600	90.6	90.2	89.6	90.1
	300	84.9	75.5	75.4	79.3
	150	73.3	72.7	63.1	70.4
ethirimol 25% SC	300	61.4	61.5	66.2	62.7
difenoconazole 10% SL	150	59.6	58.3	63.9	60.4
kresoxim-methyl 30% SC	150	92.1	92.1	86.1	90.5

[a] SL -- soluble concentrate, the same as following tables.

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Table 7. Field Trials Results for Compound 7a-4 Control of Tomato Powdery Mildew

compound	*concentration (8mg/L)*	*control after spraying (%)*			
		I	*II*	*III*	*average*
compound 7a-4 20% EC	450	79.6	83.6	86.7	83.3
	300	70.2	81.9	82.1	78.2
	150	63.4	66.6	81.1	70.5
difenoconazole 10% SL	150	70.6	80.9	78.1	76.5
azoxystrobin 25% SC	150	82.0	80.0	84.6	82.3

Table 8. Field Trials Results for Compound 7a-4 Control of Cucumbers Powdery Mildew

compound	*concentration (8mg/L)*	*control after spraying (%)*			
		I	*II*	*III*	*average*
	600	93.1	96.3	97.3	95.6
compound 7a-4 20% EC	300	85.4	89.3	85.0	86.6
	150	79.6	84.1	82.0	81.9
ethirimol 25% SC	300	79.9	80.6	76.7	79.1
pyraclostrobin 25% EC	150	63.4	65.3	62.6	63.7
tebuconazole 43% SC	150	68.7	68.2	69.0	68.6

Conclusions

The anilinopyrimidine compounds showed good fungicidal activity against CDM or WPM. Compounds had more better control of WPM with the general structure 3 substituted by R_1 = Ph, R_2 = H, R_3 = CH_3, R_4 = $CH_2(CH_2)_2CH_3$ and OR_5 = carbonates, especially compound 7a-4 showed 100% control of WPM at 6.25 mg/L and had good inhibition effect on a variety of diseases at 10 mg/L, as a 20% emulsifiable concentrate (EC) was effective in the control of powdery mildew on wheat, strawberry, tomato, cucumbers at 250-300 mg/L in field trials, little more potent than ethirimol at equivalent dose. The potential for commercial development is being assessed.

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Chapter 13

Succinate Dehydrogenase: An Ideal Target for Fungicide Discovery

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The mitochondrial respiratory complex II or succinate dehydrogenase (SDH), an essential component of cellular respiratory chain and tricarboxylic acid (or Krebs) cycle, is an ideal target for fungicide development. SDH inhibitors (SDHIs) displayed a broad-spectrum activity against various fungal species. Over the last two decades, great achievements have been made in revealing the function and structure of complex II. Currently, 18 SDHIs have been developed as agricultural fungicides. Although SDHIs as agricultural fungicides have been applied for near fifty years, in order to prevent or delay the onset of resistance to SDHIs, we continue to uncover exciting future prospects for novel fungicides targeting SDH. In this review, we have summarized the structures of complex II of different species, the interactions between complex II and inhibitors, the progress of SDHIs discovery and the resistance to SDHI fungicides.

Introduction

Succinate dehydrogenase (SDH, complex II) or succinate:ubiquinone oxidoreductase (SQR) is an enzyme complex, bound to the inner mitochondrial membrane of mammalian mitochondria and many bacterial cells, which is the only enzyme involved in both respiratory chain and tricarboxylic acid (TCA) or

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Krebs cycle. In the inner mitochondrial membrane, SDH catalyzes the oxidation of succinate to fumarate, coupled with the reduction of ubiquinone to ubiquinol (*1–4*).

In agrochemical research, SDH was identified as a significant target for structurally diverse fungicides and acaricides. The fungicidal effect of all SDH inhibitors relies on the disruption of the TCA cycle, while the novel acaricide cyflumetofen (*5*) was reported to act *via* a potent inhibition of SDH after hydrolysis of the *t*-butyl ester. There are also some other kinds of SDH inhibitors such as the natural product Atpenin A5 (Figure 1) (*6*).

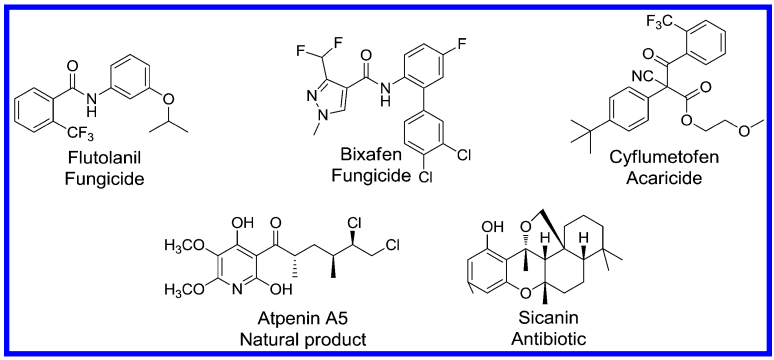

Figure 1. Selected inhibitors of succinate dehydrogenase

In this review, we present the applications of SDH inhibitors (SDHIs) on fungicidal research, including the structures of SDHs from different species; the interactions between SDH and inhibitors; the progress of SDHIs; the resistance to SDHI fungicides.

Structures of Complex IIs

With the development of protein crystallization technique, the protein components of SDH from different species are confirmed by X-ray diffraction at different resolutions, revealing physicochemical properties of complex II and its inhibition mechanism.

SDH is composed of four distinct subunits. In mitochondrial matrix, hydrophilic flavoprotein subunit (SdhA or Fp), where electron transport chain of SDH starts in, including a constant flavin adenine dinucleotide (FAD) near the dicarboxylate site (*1*, *3*). Substrate succinate is catalyzed to be fumarate after transfering two electrons to FAD. Iron-sulfur subunit (SdhB), which contains

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three Fe–S clusters: [2Fe–2S], [4Fe–4S] and [4Fe–3S], forming a linear electron transport chain, connects dicarboxylate site with quinone-binding site (Q_p-site). Noteworthy, quinone-binding site, where quinone reduction/quinol oxidation occurs, is surrounded by three domains of the iron-sulfur subunit and the cytochome b_L (SdhC) and cytochome bS (SdhD). A heme b links cytochome b_L and cytochome bS also receives electrons from [4Fe–3S]. As for quinol:fumarate oxidoreductase (QFR), the structure is similar with SQR, except for the lack of heme and the difference in cytochome b_L and cytochome bS (Figure 2a) (*1*, *3*, *7*).

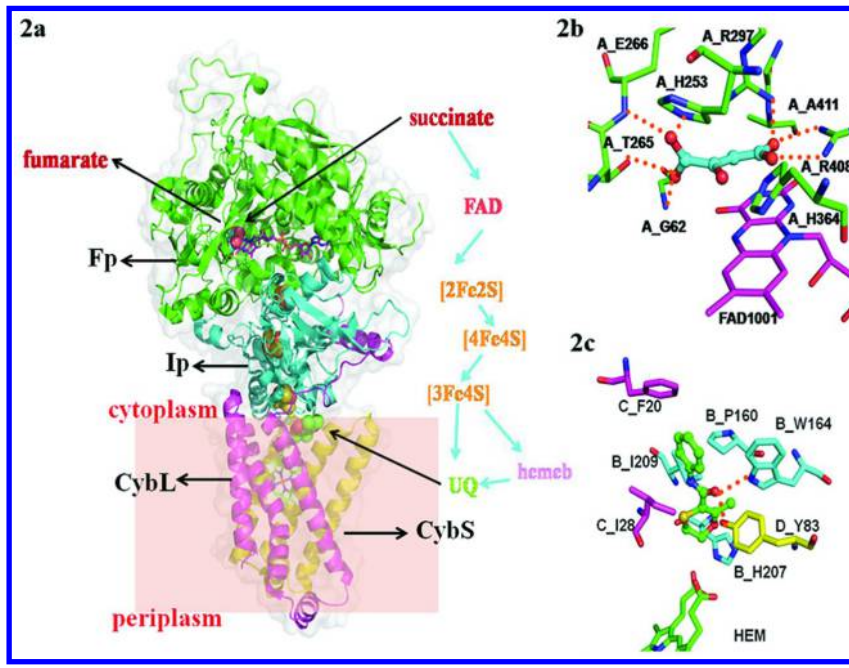

Figure 2. Overall structure of E.coli SQR (pdb 1NEK). (2a) SQR catalyzes the oxidation of succinate to fumarate and transfers electrons from succinate to ubiquinone pass by FAD of flavoprotein (Fp) and 2Fe-2S,4Fe-4S,3Fe-4S of iron-sulfur protein (Ip). (2c) Description of the Qp site with carboxin. (2b) Description of the dicarboxylate site with OAA. The oxygens of OAA have interaction with residues The carbonyl oxygen of carboxin binds to Trp-B164 and Tyr-D83 through hydrogen-bond

The first *E. coli* QFR (*8*) and *E. coli* SDH (*2*) structure were obtained at 3.3 Å (PDB: 1FUM) in 1999 and at 2.7 Å (PDB: 1NEK) in 2003, respectively. Until now, SDH structures from three species, including *E coli*, porcine and avian (*9*), have been determined (Table 1). It has revealed that the identified SDHs were characterized with similar spacial architectures, the subunit arrangement, the electron transfer pathway, and even the quinone-binding site in prokaryotes and eukaryotes (*10*).

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Table 1. Crystal structures of SDH

Source	*Protein Data Bank code*	*Resolution*	*Relevance*	*Reference*
E.coli	1NEK (2003)	2.7 Å	with bound UQ	(*2*)
	2ACZ (2006)	2.6 Å	with Atpenin A5 (AA5)	(*11*)
	2WDR (2009)	3.2 Å	with pentachlorophenol (PCP)13	(*12*)
	2WDQ(2009)	2.4 Å	with carboxin	(*12*)
	2WDV (2009)	3.2 Å	with an empty UQ-binding site	(*12*)
Pig	1ZOY (2005)	2.4 Å	with bound UQ	(*13*)
	1ZP0 (2005)	3.5 Å	Crystallized with 3-NP and TTFA	(*13*)
	2H88 (2005)	2.4 Å	with bound UQ	(*14*)
Chicken	1YQ3 (2006)	2.2 Å	Crystallized with OAA	(*15*)
	1YQ4 (2006)	2.4 Å	Crystallized with 3-NP	(*16*)
	2FBW (2006)	2.1 Å	Crystal soaked with carboxin	(*16*)

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The co-crystals for SDH of *E.coli* with quinone and inhibitors by Iwata and coworkers, showed the binding modes of inhibitors in the catalytic site. The combination of structural, computational, and kinetic studies of succinate dehydrogenase has proposed the corresponding binding site distribution and Q-site-dependent working mechanism. Ubiquinone (UQ) is located in a pocket composed of residues derived from three subunits, SdhB, SdhC, and SdhD, which are close to the [3Fe-4S] cluster (*2*). Furthermore, unbiquinone binds to the entry site Q1-site where just a carbonyl oxygene has strong interaction with Trp-B164 and Tyr-D83 forming H-bonds initially. Because of transferred electrons resulting in the movement of UQ into catalytic site, Q2-site, another H-bond is formed between Ser-C27 and another ketonic oxygen atom, His B207 binds to the 3-methoxy group of ubiquinone. The 2-methoxy group binds to Arg-C31 and Asp-D82 through a water molecule, respectively. As a result, UQ receives two electrons from iron-sulfur proteins to form the phenolate dianion to get protons from HOH39, where H-bonds were finally broken to release QH_2 (*4*, *11*).

The mechanism explains enzyme kinetic differences between SDH mixed-type inhibitor and competitive inhibitor for quinone, focusing on binding in Q1-site or Q2-site. The co-crystal structures of the specific quinone-binding site inhibitor carboxin with chicken SQR at 2.1 Å (*16*) (PDB: 2FBW) and *E.coli* SDH at 2.4

Å (*12*) (PDB:2WDQ) are presented to reveal how carboxin inhibits the Qp-site, respectively. Berry and coworkers also found that the carbonyl oxygen binds to Trp-B173 and Tyr-D58 through hydrogen-bond as the position of quinone carbonyl oxygen based on the co-crystal structure of chicken SDH with carboxin. The methyl-oxathiin ring of carboxin is buried into the bottom of Q2-site (*16*), called the cavity (Asp-D57 and His-B216) (Figure 2c).

SDH was co-crystallized with Atpenin A5 (AA5), also revealing that the binding position and additional structural details of the Q-site (*11*). AA5 is located in the same hydrophobic pocket as ubiquinone binds, but at a different position within the same pocket. Compared with carboxin, two H-bonds with Tyr D83 and His-B207 were observed in *E.coli* SDH with PCP (*12*) and pig SDH with TTFA (*13*). Another two H-bonds between Ser-C27/Arg-C31 still exists. AA5 was inserted deeper in Q2-site, where the side chains of a serine and histidine residue are suitably positioned to provide hydrogen bonding partners to the carbonyl and methoxy groups of ubiquinone.

Moreover, there is a certain difference between the quinone binding site of *E. coli* SDH and QFR for the counterpart residues in this region are poorly conserved in QFR. As seen, TTFA and carboxin can inhibit ubiquinone reduction activity in mitochondrial SDH, but show weak inhibition on *E. coli* QFR. Another kind of inhibitors, like HQNO, is strongly effective on QFR, but not inhibitory to SDH. The third class of compounds inhibit both SDH and QFR, such as pentachlorophenol (PCP) and 2-(4-thiazolyl) benzimidazole (TBZ) (*10*).

On the other hand, co-crystal for *E. coli* QFR at 3.3 Å (*8*) with menaquinone indicates that two menaquinone-binding sites include one site on the cytoplasmic side (Qp site) and the other distal quinone binding site on the periplasmic side (Qd site), but Qd-site is empty. It has been demonstrated fully that TTFA inhibitor binds both two quinone-binding sites of Qp site and Qd site through the analysis of the QFR structure at 3.5 Å resolution determined by Sun and coworkers in 2005 (PDB: 1ZPO) (*13*). However, just one site Qd has not been detected in chicken and *E.coli* SDH structures based on enzymatic kinetic studies. Until now, it is widely accepted that Qp-site is the unique catalytic site. Hence, TTFA might bind to Qd-site non-specifically at high concentration.

The other class of competitive inhibitors of SDH is targeted at the dicarboxylate site such as 3-nitropropionic acid (3-NP) and oxaloacetate (OAA), for succinate is oxidized at the same site near by FAD in the Fp. Irreversible inhibitor 3-NP is structurally similar to the substrate succinate. In 2005, Berry reported the structure of chicken SDH with 3-NP at 2.4 Å resolution. It found that inhibitor 3-NP forms a covalent bond with the side chain of Arg297, assuming that the guanidino group of Arg297 reacts with C-3 and N of 3-NP to lose two oxygens to make a ring (*16*). For OAA, as the initial substrate and the end product of the Krebs cycle, is an endogenous and classical competitive inhibitor, with low dissociation rate K_d. However, complex II not only oxidizes succinate, but also oxidizes D- or L-malate into oxaloacetate. Comparing cocrystals of SDH with OAA and empty succinate-site, the carboxylate site includes a malate-like ligand in a well-ordered state, allowing reassignment of the other residues. Moreover, Arg297 is well positioned for the role of general acid-base catalyst abstracting a proton during conversion of succinate into fumarate (Figure 2b) (*15*).

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Kinetics study indicates that carboxin is a noncompetitive inhibitor when succinate is the substrate and the dye dichlorodiisopropylether (DCIP) receives the electron from ubiquinol lastly. But when succinate and unbiquinone(UQ) analogue are substrates, carboxin is a mixed-type inhibitor for Q analog. *In vivo*, it was also determined in 1974 that carboxin inhibited *Micrococcus denitrificans* with K_i = 16 μM. Oxycarboxin shows less inhibitory activity in the study. When the concentration is 1 mM, carboxin and oxycarboxin exhibited 96% and 54% inhibition rate, respectively. Inhibition constant is dependent on the sources of SDH, 0.32 μM for the fungus *Ustilago maydis*, 270 μM for the yeast *Saccharomyces cerevisiae* and 40 μM for rat liver (*17*). The later introduced flutolanil is better than carboxin and IC_{50} is 0.5 μM for *R. Solani* (*18*).

Beyond fungicidal carboxanilides, the atpenins, which are naturally occurring ubiquinone analogs, show potent inhibitory activity (*19*). For example, IC_{50} of AA4 is 0.22 μM for both nematode *A. suum* SDH and QFR, 0.011 μM for bovine SDH, and 0.024 μM for rat liver SDH. AA5 is also effective, with 0.012 μM IC_{50} for *A. suum* QFR, 0.032 μM for *A. suum* SDH, 0.0036 μM for bovine SDH, and 0.0037 μM for rat liver SDH. In fact, atpenins are significantly more effective than the other complex II inhibitors. Carboxin (IC_{50} = 1.1 μM) and TTFA (IC_{50} = 5.8 μM) showed 300-fold and 1,600-fold higher activity than AA5 for bovine SDH, respectively.

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SDH Inhibitors

Commercial SDH Inhibiting Fungicides

Due to its critical role in the respiratory chain, SDH has been identified as one of the most significant targets for agrochemicals, especially for the discovery of new fungicides. Until recently, commercial SDHI fungicides were consisted of structurally diverse framworks, including oxathiin-carboxamides, phenyl-benzamides, furan-carboxamides, thiazole-carboxamides, pyridine-carboxamides, pyridinyl-ethyl benzamides, phenyl-oxo-ethyl thiophene amide and pyrazole-4-carboxamides (Figure 3) (*20*).

Early SDH Inhibitors

In 1966, the first SDHI carboxin was introduced to the market by Uniroyal Chemical Co., Inc. (now Chemtura Corp.), which was used for the control of smuts and bunts (*21*). Fenfuram and mebenil, which had similar biological properties, were discovered by Shell Research Ltd. and BASF, by replacing the oxathiin ring with a furan ring and a phenyl ring, respectively. Thereafter, flutolanil and mepronil, derived from benzoic acid, were invented and used as agricultural fungicides (*22*).

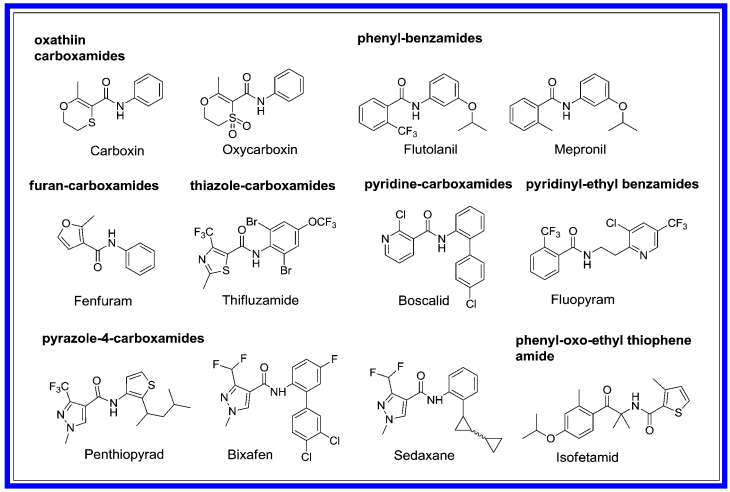

Figure 3. Chemical structures of representative SDH-inhibiting fungicides

Thifluzamide and Boscalid

In 1994, thifluzamide was initially developed by Monsanto Co. and displayed broad spectrum fungicidal activity on *Basidiomycetes*, particular for diseases caused by *Rhizoctonia spp*., on rice, potatoes, maize, and amenity grass (*23*). Boscalid, which was discovered by BASF, has been used as a broad-spectrum fungicide against mainly on *Alternaria atternata, A. Solani, Botrytis cinerea* and *Sclerotinia sclerotiorum* in the fruit and vegetable segments. The discovery of boscalid can be considered as an optimum of the lead F-427 by the replacement of the oxathiine with the pyridine group (*24*).

Pyrazole-4-carboxamides

Among these existing commercial SDHIs, pyrazole-4-carboxamides have gained a considerable attention in the field of fungicide chemistry in recent years (*25*). So far, eight pyrazole-4-carboxamides derivatives have been introduced to the market as agricultural fungicides (*20*). For instance, furametpyr, the first commercial fungicide of this class, was developed by Sumitomo in 1996 (*26*). A few years later, penflufen was invented by Bayer CropScience as a commercial fungicide (*27*). Mitsui chemists introduced penthiopyrad in 2003, which contains a trifluoromethyl substituent on the pyrazole ring. It provides a good control of gray mold, powdery mildew and apple scab (*28*). The latest generation of inhibitors, including isopyrazam (Syngenta), sedaxane (Syngenta), bixafen (Bayer), fluxapyroxad (BASF), and benzovindiflupyr (Syngenta), contain a difluoromethyl group at the 3-position of the pyrazole ring, they were introduced to the market in the past five years. These pesticides have already reflected a good market outlook because of broad-spectrum fungicidal activities (*25*).

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Fluopyram and Isofetamid

Fluopyram, a new broad-spectrum fungicide, was discovered by Bayer CropScience. It is biologically active against all the stages of fungal growth, from spore germination to spore production, and its activity spectrum includes several pathogens belonging to Ascomycetes and Deuteromycetes, such as *Botrytis spp., Sclerotinia spp*. and *Monilinia spp*., on vegetable, pome and stone fruit crops (*29*). As the latest example of SDHI, isofetamid is a thiophene-carboxamide fungicide from Ishihara Sangyo Kaisha with the research code name of IKF-5411 (*30*).

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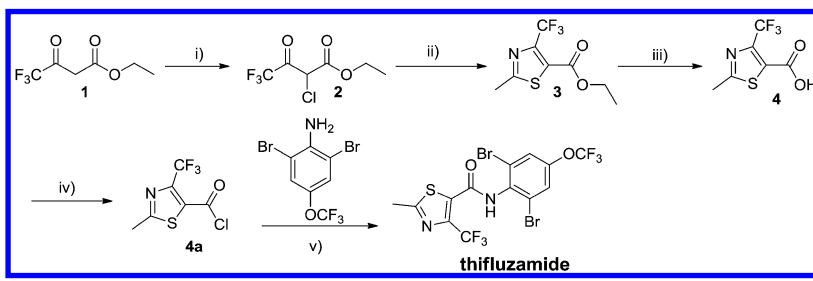
Synthesis of Typical SDH Inhibiting Fungicides

Synthesis of Thifluzamide

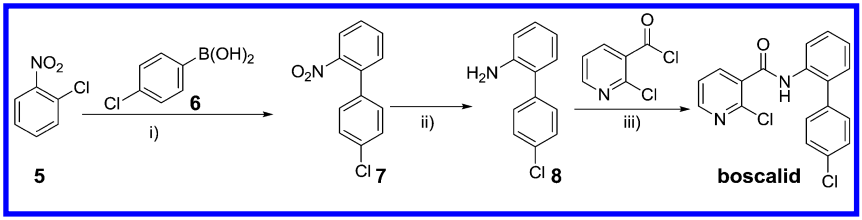
The synthetic route of thifluzamide was shown in Scheme 1. Ethyl trifluoroacetoacetate was chlorinated to afford intermediate **2** which could be cyclized to the thiazole by reaction with the thioacetamide **3**. After hydrolysis and chlorination, the key intermediate chloride **4a** was synthesized. Treatment of **4a** with 2,6-dibromo-4-(trifluoro-methoxy)aniline afforded the target molecule (*23*).



Scheme 1. Synthetic route to thifluzamide (Reagents and conditions: i) SO_2Cl_2, r.t.; ii) thioacetamide, Et_3N; iii) NaOH, THF, H_2O, reflux, HCl; iv) $SOCl_2$, reflux; v) Toluene, reflux.)

Synthesis of Boscalid

There are two distinct parts of the compound boscalid in which the pyridyl component is connected to the biphenyl ring *via* an amide linker. The biphenyl part **7** of boscalid was successfully synthesized through Suzuki coupling reaction by BASF chemists. This is the first example of applying a palladium-catalyzed coupling reaction to agrochemical industrialization (Scheme 2) (*31*).



Scheme 2. Synthetic route to boscalid (Reagents and conditions: i) $Pd(OAc)_2$, PPh_3, NaOH, toluene, reflux; ii) H_2, Pd/C; iii) 2-chloronicotinoyl chloride, Et_3N, r.t.)

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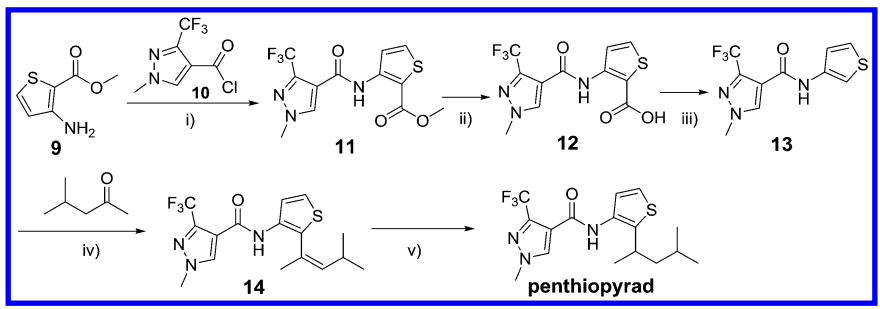
Synthesis of Penthiopyrad

Penthiopyrad was synthesized starting from the reaction of methyl 3-aminothiophene-2-carboxylate **9** with 1-methyl-3-(trifluoromethyl)-1*H*-pyrazole-4-carbonyl chloride **10**, the resulting intermediate **11** was hydrolyzed and decarboxylated to yield **13**, which reacted with 4-methylpentan-2-one in the presence of catalytic TsOH to give compound **14**. The hydrogenation of the double bond in the alkenyl side chain of **14** yielded the target penthiopyrad (Scheme 3) (*32*).

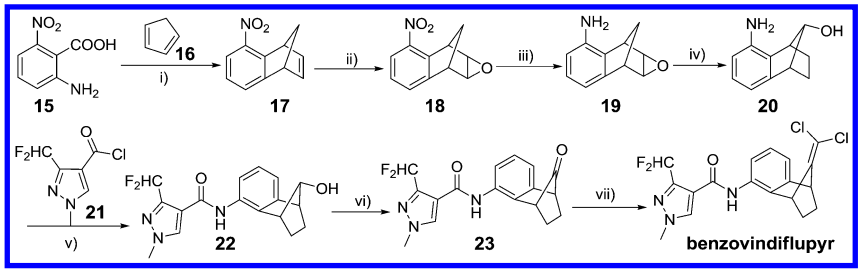


Scheme 3. Synthetic route to penthiopyrad (Reagents and conditions: i) Et_3N, r.t; ii) NaOH, 50 °C, HCl; iii) oxalic acid; iv) 4-methylpentan-2-one, TsOH; v) H_2, Pd/C.)

Synthesis of Benzovindiflupyr

Benzovindiflupyr is the latest pyrazole-4-carboxamides SDH inhibitor, and its discovery could be considered as the structure modification of isopyrazam. Synthetic approaches to the aniline moiety of benzovindiflupyr began from 2-amino-6-nitrobenzoic acid **15**. Treatment of **15** with *t*-BuONO yielded nitrobenzyne, which reacted with cyclopenta-1, 3-diene to give compound **17**. Oxidation of **17** with *m*CPBA afforded **18**, and reduction of **18** yielded **19**. This intermediate was hydrolyzed and transformed into **20**, which reacted with the pyrazole chloride **21** to result in formation of intermediate **22**. Finally, after

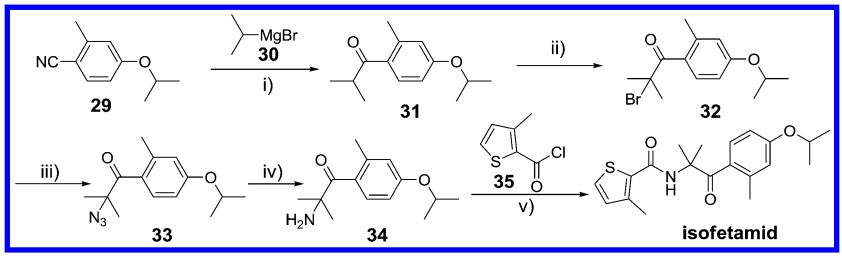
oxidation with DMSO and the Witting reaction, benzovindiflupyr was prepared (Scheme 4) (*33*).



Scheme 4. Synthetic route to benzovindiflupyr (Reagents and conditions: i) t-BuONO, cyclopenta-1,3-diene; ii) mCPBA; iii) H_2, Rh/C; iv) HBr, Zn; v) Et_3N, r.t; vi) DMSO; vii) PPh_3, CCl_4.)

Synthesis of Isofetamid

Synthesis of isofetamid was described in Scheme 5. Addition of 4-isopropoxy-2-methylbenzonitrile **29** with isopropyl magnesium bromide **30** afforded intermediate **31.** Bromination of **31** gave the compound **32**, which reacted with sodium azide to yield intermediate **33**. Finally, reduction of **33** with H_2 yielded intermediate **34**, followed by a coupling reaction with **35** to give the title product (*30*).



Scheme 5. Synthetic route to isofetamid (Reagents and conditions: i) isopropylmagnesium bromide; ii) phenyltrimethyl-ammonium tribromide; iii) NaN_3; iv) H_2; v) 3-methylthiophene-2-carbonyl chloride, Et_3N, r.t.)

Progress of Pyrazole Carboxamides Derivatives as New SDHIs

As mentioned before, the discovery of new SDHIs remains highly attractive. Among these reported fungicides, pyrazole carboxamides were regarded as the privileged pharmacophore because this moiety was emerged in all inhibitors which showed high fungicidal activities. Therefore, these inhibitors are termed as pyrazole carboxamide derivatives in this section and the recent progress of new SDHIs based on pyrazole carboxamides are presented (*34*).

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In the latest few years, the pyrazole carboxamides are one of the most representative types of fungicides among the reported SDHIs (Figure 4). Chemists from Syngenta synthesized a series of novel pyrazole carboxamides derivatives bearing cyclopropyl substituent, including the trimethylsilyl cyclopropyl analog **36** displaying wide spectrum fungicidal activities (*35*). Based on the previous work, Dunkel and his coworkers from Bayer reported optically active carboxamides. For example, the compound **37** showed better fungicidal activity than its corresponding isomer. Compound **38** was developed by Isagro-Ricerca, the amide group of this compound is similar to furametpyr, showed good fungicidal activity against *Erysiphe graminis* (*36*). Compounds **39**, **40** and **41** were reported in recent years, they are the analogues of commercial SDHIs, which also displayed good fungicidal activities (*37–39*).

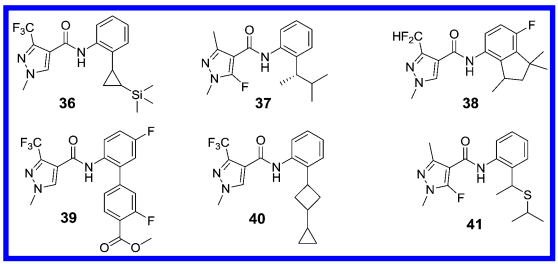

Figure 4. SDHIs with novel amine moieties

As mentioned above, all commercial SDHIs contain the amide bond, which is very important to define the orientation of the molecule (*9*). Substitution of the amide bond –NHCO- while maintaining good activity may be a challenge, but the thioamide (NHCS) group seen in compound **42** can deliver good levels of activity (*40*). Another modification of –CONH- is the introduction of substituents to the nitrogen atom including methyl (**45**) (*39*), methoxyl (**44**) (*41*) and cyclopropyl (**43**) (*42*). These compounds also displayed good fungicidal activities. In fact, modifications of the amide bond did not result in better activity in most cases (Figure 5).

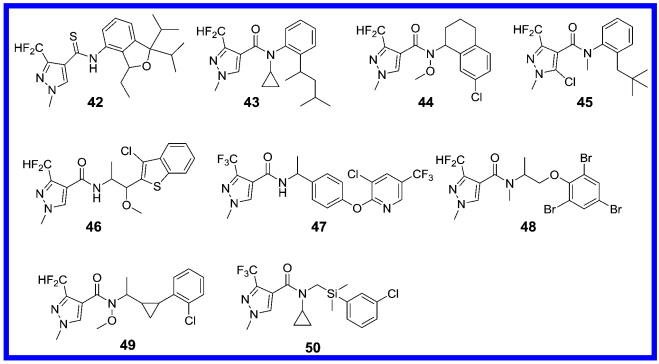

Figure 5. Chemical structures of SDHIs with promising fungicidal activity

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On the amine side of the amide bond, the moiety can be considered as two main parts, the first one is the linker. The most frequent linker is a phenyl group or a heterocycle such as a thiophene ring, which is normally substituted in the ortho position with a hydrophobic group (*9*). In recent studies, different linkers were developed. As showed in Figure 5, compounds **46** to **50**, which bear different substituted groups, were reported as fungicidal molecule (*40*, *43–46*).

Other New SDHIs

Nicotinamide Derivatives as SDHIs

Ye and coworkers reported a series of nicotinamide derivatives (*47*). These compounds were obtained by introducing the methylthio or sulfur benzyl to replace the chlorine atom on the ortho position of pyridine. Most of the compounds showed moderate activity against *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. Of all the synthesized initial hits, compound **52** was the most potent candidate (Figure 6). According to the molecular modeling study, the introduction of a methylthio group make the inhibitor interact better with SDH.

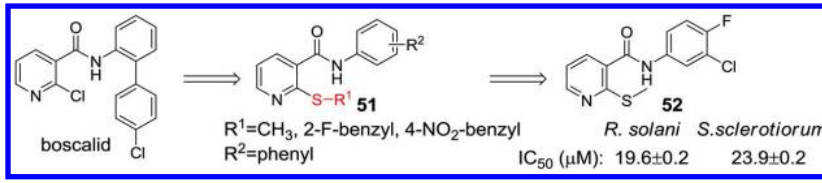

Figure 6. Structures of nicotinamide derivatives

Novel 5-Methyl-1H-1,2,3-trizole-4-carboxyl Amide and Ester Analogues

Novel SDHIs containing a bioactive 1,2,3-triazole moiety have been designed and synthesized by Wang and coworkers (*48*). Most of these compounds showed good fungicidal activities (Figure 7). Meanwhile, replacing the amide group with ester group, the fungicidal activity could be maintained and some samples showed even higher activity than the corresponding amide analogues. Interestingly, some of them displayed good insecticidal activities against *Culex pipiens pallens*.

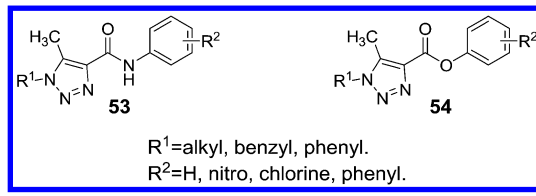

Figure 7. Structures of 5-methyl-1H-1,2,3-trizole-4-carboxyl amide and ester analogues

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Based on the structures of natural SDHIs atpenins and harzianopyridone, some derivatives were reported (Figure 8). For instance, chemists from Bayer Crop Protection reported the preparation of 2-pyridones and 2-prydinols (*49*). Compound **55** showed 100% efficacy against *Sphaerotheca fuliginea* at 100 g/ha, while compound **56** showed 78% efficacy against *Phytophthora infestans* at 500 g/ha. Selby and coworkers from DuPont Crop Protection reported a series of synthetic Atpenin analogs (*50*). These newly synthesized compounds generally showed excellent activity against both mammalian and fungal SDH. It should be pointed out that compound **58** is so far the most potent SDHI with IC_{50} values of 0.003 and 0.004 μM against bovine and *Septoria n*. SDH, respectively. However, unfortunately, none of these compounds showed good antifungal activity *in vivo*. The pesticide-likeness properties of these compounds need to be further optimized.

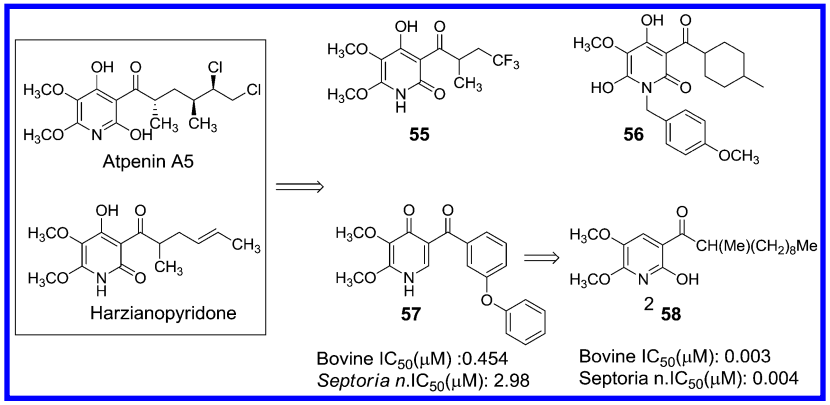

Figure 8. Structures of atpenin analogs

Structure–Activity Relationships of SDHIs

To summarize the structure-activity relationship of the reported SDHIs, the structure of these fungicides are divided into four parts: the carboxylic acid part, the amide bond, the linker and the hydrophobic portion (*9*). An extensive survey of the commercialized fungicides and the patented products revealed that the carboxylic acid moiety may be structurally diverse aromatic substituents such as pyrazole ring, phenyl ring, pyridine ring, thiazole ring, thiophene ring and other heterocycles (*25*, *34*, *51*). The carboxylic acid moiety and the amide bond are "relatively conserved", while the amine part is structurally diverse. As a result, some empirical relationships have been established: the electron-withdrawing groups on the carboxylic acid are important to maintain the activity. On the amine side of the amide bond, the linker could be phenyl, heterocycle or alkyl (Figure 9). Meanwhile, the orientation of the amide bond is also critical, and the *cis* configuration of the amide bond might be important in SDHIs (*25*, *34*).

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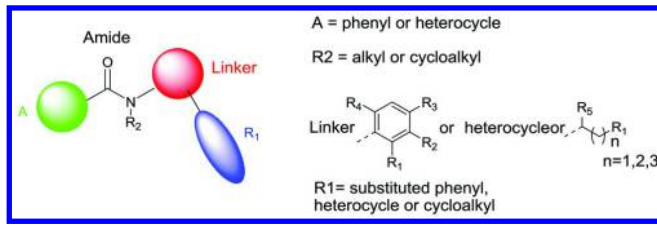

Figure 9. General chemical structures of SDHIs

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Metabolism of Benzovindiflupyr (*52*)

The metabolism of commercial SDHIs normally begins with hydroxylation of the aromatic rings (alkyl or alkoxy groups), and cleavage of the amide bond occurs at a later stage in most cases. During the study of metabolism of benzovindiflupyr in rats, at least eight types of metabolites including desmethyl, hydroxy, dihydroxy, desmethyl hydroxy, desmethyl dihydroxy, bicycle ring-opened, glucuronide conjugate and sulfate conjugate were observed with no evidence for cleavage at the pyrazole–phenyl bridge (Figure 10). The major metabolites were identified as compound **61** (26–56%), **63** (2–12%), **64** (3–8%), **65** (1–8%) and **69** (4–5%).

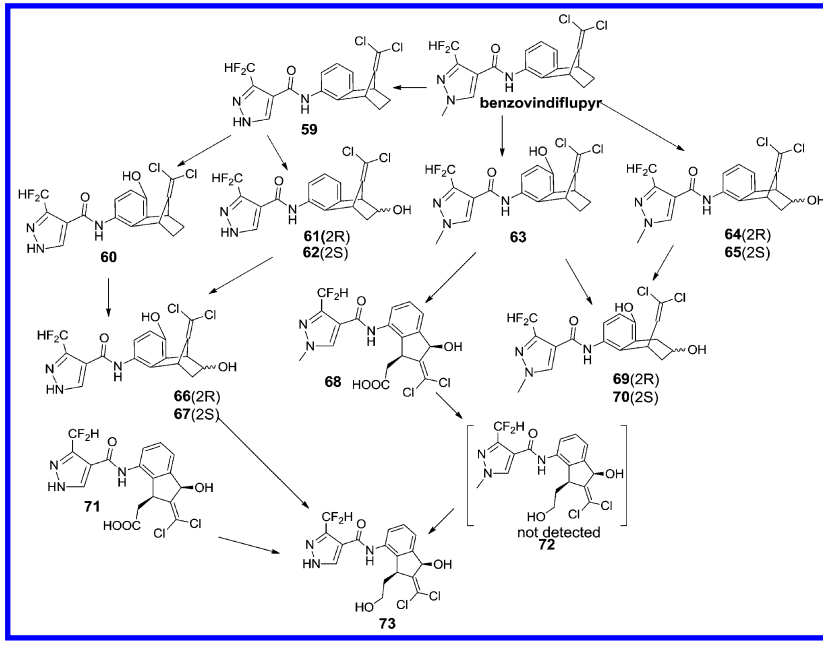

Figure 10. Metabolism of benzovindiflupyr in the rat

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ACS Symposium Series; American Chemical Society: Washington, DC, 2015.

Table 2. Resistance mutations in the SDH gene

Species name	*Origin*	*Resistance mechanism*
Aspergillus oryzae	Laboratory	B-H249Y/L/N, C-T90I, D-D124E
Alternaria alternata	Pistachio Field	B-H277Y/R, C-H134R, D-D123E, D-H133R
Botrytis cinerea	Laboratory mutants, Field	B-P225L/T/F, B-H272Y/R/L, B-N230I, DH132R
Botrytis elliptica	Field	B-H272Y/R
Corynespora cassiicola	Field	B-H287Y/R, C-S73P, D-S89P
Didymella bryoniae	Field	B-H277R/Y
Mycosphaerella graminicola	Laboratory mutants	B-H267Y/R/L, B-I269V, C-H152R, C-N86K, D-H139E
Podosphaera xanthii	Field	B-H->Y
Sclerotinia sclerotiorum	Field	D-H132R
Stemphylium botryose	Field	B-P225L, H272Y/R
Ustilago maydis	Laboratory mutants	B-H257L

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Resistance

The development of resistance is a major issue for many classes of fungicides, including SDHIs. First indications of possible mutations mediating resistance to carboxin were reported for *Ustilago maydis and* and *Aspergillus nidulans* in the 1970s, and these selected mutants were generated artificially by applying the UV irradiation on fungicide amended media (*53–55*). After that, more resistant mutants to carboxin have also found in different phytopathogenic fungus, such as *Fusarium, Botrytis and Zymoseptoria* (*56*). For instance, in the maize smut pathogen *Ustilago maydis*, carboxin resistance was observed by replacement of a highly conserved histidine residue located at subunit SDHB either by tyrosine or leucine (H257Y/L), whereas in *Coprinus cinereus*, another mutantion (N80K) has been found.

Subsequently, compared with carboxin, the following SDHI fungicide boscalid, was the first carboxamide that had truly broad-spectrum foliar activity. Mutants resistant to boscalid have been reported in fields and laboratories, expressing high levels of resistance. In *A. alternata*, between sensitive and resistant isolates, AaSDHB gene sequence showed a conserved histidine residue at position 277 in the AaSDHB protein was mutated to either tyrosine or arginine in some boscalid-resistant isolates (*57*). The amino acid substitutions in *U. maydis* and in *M. Graminicola* by artificial mutants expressed resistance to boscalid (*58*). Cross-resistance to some types of SDHIs has been shown to be connected

with boscalid resistance in some strains. In most of the boscalid-resistant strains, mutations in the Sdhb gene have been found and resulted in the following substitutions (P225F/L/T; N230I; H272L/R/Y). A survey about several crops treated with boscalid led to the characterization of *B. cinerea* strains with medium to high levels of resistance in different countries and on several crops (*59*, *60*).

With the increasing usage of SDHIs, they were classified as fungicides at medium to high risk of resistance by Fungicides Resistance Action Committee. Resistance of SDHIs has been reported in *B. cinerea, Alternaria alternate* (*61*, *62*), *Didymella brioniae*, *Corynespora cassiicola* (*63*) and *Podosphaera xanthii* (*57*). The currently known facts on target site mutations at the mitochondrial SDH genes causing resistance to SDHI were shown in Table 2 (*64*). With the wide scale use of these fungicides, which are at risk of resistance development, appropriate anti-resistance management strategies are demanded.

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Conclusion

In the past decade, SDH has been used as an ideal target for novel fungicide discovery. The SDH inhibitors display important roles in both agronomical and commercial areas, and exhibit broad spectrum fungicidal activity. In this review, we have described the structures of SDH, provided a comprehensive overview on the progress of SDH inhibitors and resistance risk to these inhibitors. In order to prevent or delay the onset of resistance, new SDH-inhibiting fungicides and practical measures are still to greatly desired.

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Chapter 14

Mollisin: A Promising Antifungal Natural Product

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Mollisin is a fungal secondary metabolite produced by several *Mollisia species,* which has been known for decades to exhibit antifungal activities. Recently, more than 60 years after its first isolation, the first total synthesis of this naphthoquinone pigment was reported enabling access to various analogues for further investigation of its biological activity. It was revealed that mollisin and its analogues show not only a good inhibition of phytopathogenic fungi, but also possess interesting pharmacological activities.

Introduction

In 1952 Gremmen cultivated several different discomycete families on maltagar in order to investigate their biological properties (*1*). The focus of these studies soon pointed towards the two species *Mollisia caesia* and *Mollisia fallens*, which attracted attention due to their fast growth and the production of a yellow substance that crystallized on the surface of the agar. Isolation and structural investigations of the pigment by van der Kerk and Overeem (*2*) revealed a chlorinated naphthoquinone motif, that was soon after named mollisin. Initial structural assignment was of mollisin was incorrect, however, the correct structure was finally elucidated by the same authors in 1964 (Figure 1) (*3*) and was later confirmed through crystallography (*4*). More than 50 years after the isolation of mollisin (**1**) two further naphthoquinone metabolites produced by the same fungus were isolated and named mollisin A (**2**) and mollisin B (**3**) (*5*).

Mollisin is not just interesting due to its unique chlorination pattern but also due to its biological activity. Gremmen already observed a strong growth

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inhibition of several fungi, such as *Heterobasidion annosum* (formerly known as *Fomes annosum*) which causes serious damage to coniferous forests (*6*). Furthermore, mollisin exhibits a strong antibiotic activity towards Gram-positive bacteria (*7*).

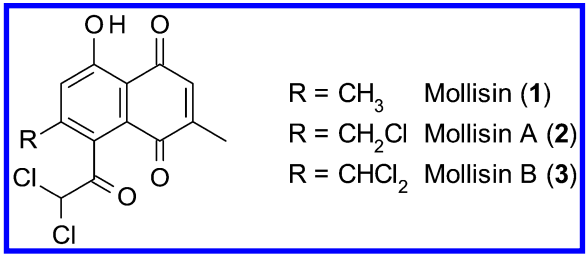

Figure 1. Structures of naphthoquinone metabolites isolated from Mollisia caesia.

Biosynthesis of Mollisin

Although the biosynthesis of mollisin by *Mollisia caesia* has been intensively investigated, the exact pathway is still not entirely elucidated (*8–11*). Feeding experiments with different sodium acetate isotopes clearly showed that mollisin is synthesized following a polyketide pathway. However, there are still two possibilities: the construction of the naphthoquinone skeleton starting from one polyketide chain (pathway 1) or out of two polyketide fragments (pathway 2) (Scheme 1) (*5*).

Pathway 1 constructs the naphthoquinone by intramolecular condensation of a C16-polyketide chain, whereas, in pathway 2 the bicycle is formed from C6- and a C10- chains. In both cases the loss of two C1-units is proposed. Feeding with chlorinated acetate did not lead to an incorporation of this building block, therefore, it is assumed that the chlorine atoms are introduced after the condensation to the naphthoquinone (*12*). This assumption is affirmed by the detection of a chloro peroxidase that is present in the fungus. Although pathway 2 cannot be excluded, several findings suggest that the biosynthesis of mollisin **1** follows the first route. First, feeding deuterated acetyl-CoA leads to high deuterium concentration at C9 only (*5*, *12*). Pathway 2 would, on the other hand, lead to incorporation of deuterium at C2′ in addition to C9‘. Furthermore, chlorination through a chloro peroxidase usually proceeds at nucleophilic substrate positions. The position C2′ in the C16-polyketide chain of route 1 is activated by two carbonyls , thus chlorination of this chain should be more likely than chlorination at monactivated C2′ of the C6-chain (route 2). The fact that only mono- or dichlorination occurs in all three naphthoquinone metabolites (also at C10 of mollisin A and B) indicates chlorination of a double-activated methylene group and not of a methyl group (as in pathway 2). Thus, it is very likely that mollisin **1** and its derivatives are produced by pathway 1 (*5*).

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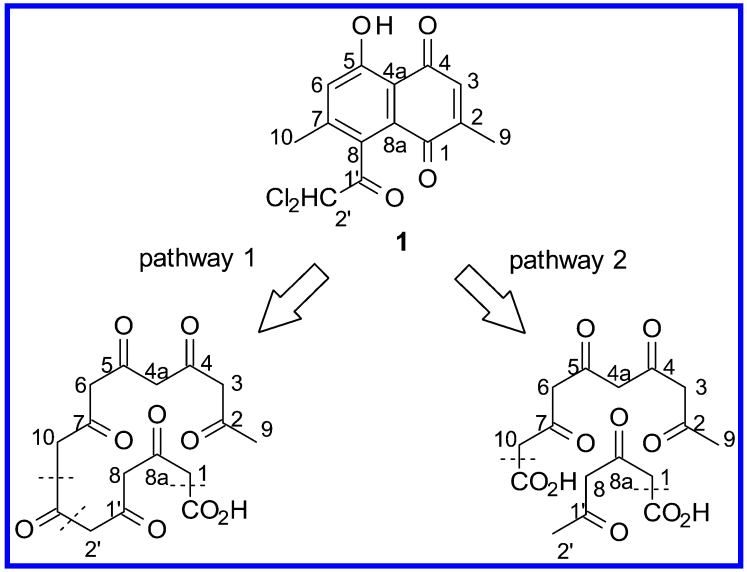

*Scheme 1. Two possible biosynthetic pathways to mollisin **1**.*

Synthesis

The first aproach to synthesize the naphtho-1,4-quinone metabolite mollisin was made in 1964 by van der Kerk and Overeem (*13*). However, they were not able to synthesize mollisin itself, but some close derivatives like dechloromollisin methyl ether (**11**) (Scheme 2).

In the first step of their synthesis two molecules of diacetylacetone (**5**) reacted in a triple Knoevenagel condensation catalyzed by piperidine to give the 2-acetyl-3,6-dimethylnaphthalene-1,8-diol (**6**). Deacetylation led to naphthol-1,8-diol **7**, which was sensitive to oxidation and, thus, immediately converted into the highly-stable dimethyl derivative **8** by methylation with dimethyl sulfate. For the synthesis of mollisin (**1**) it would have been necessary to install the dichloroacetyl group at this stage by a Friedel-Crafts reaction. However, all attempts to produce the dichloro-acetylated naphthol **9** failed. Due to this, the authors decided to prepare dechloromollisin methyl ether by treating naphthol **8** with excess acetyl chloride and subsequent oxidation to the naphthoquinone derivative **11**.

In 2013 the first successful total synthesis of mollisin was achieved by Opatz and coworkers (*14*). Based on the synthesis of dechloromollisin methyl ether by van der Kerk and Overeem naphtholether **8** was chosen as key intermediate in their synthesis (Scheme 3).

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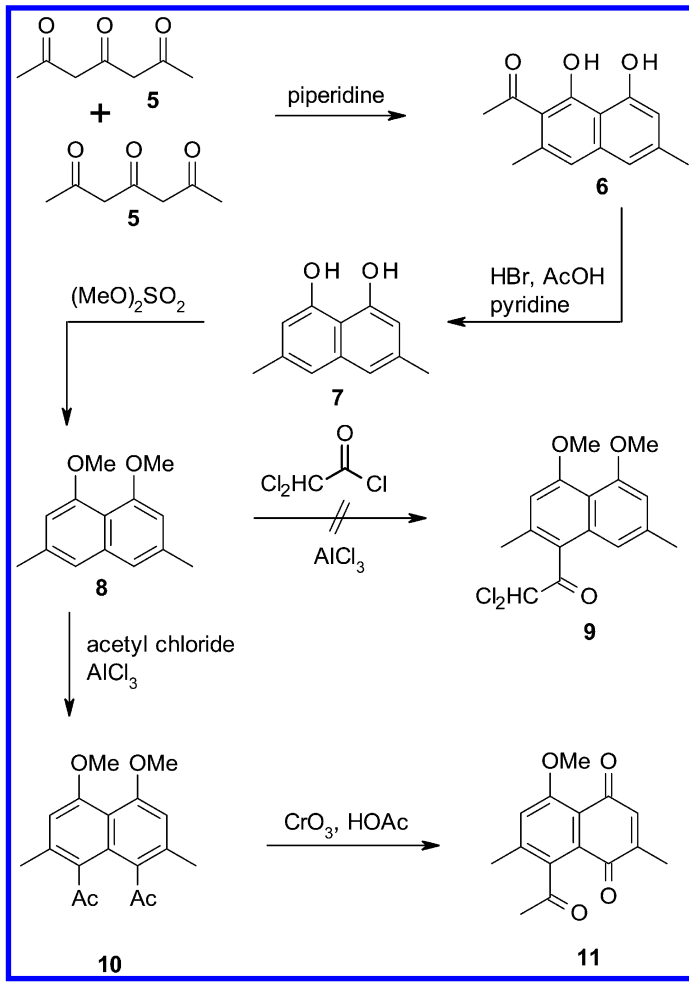
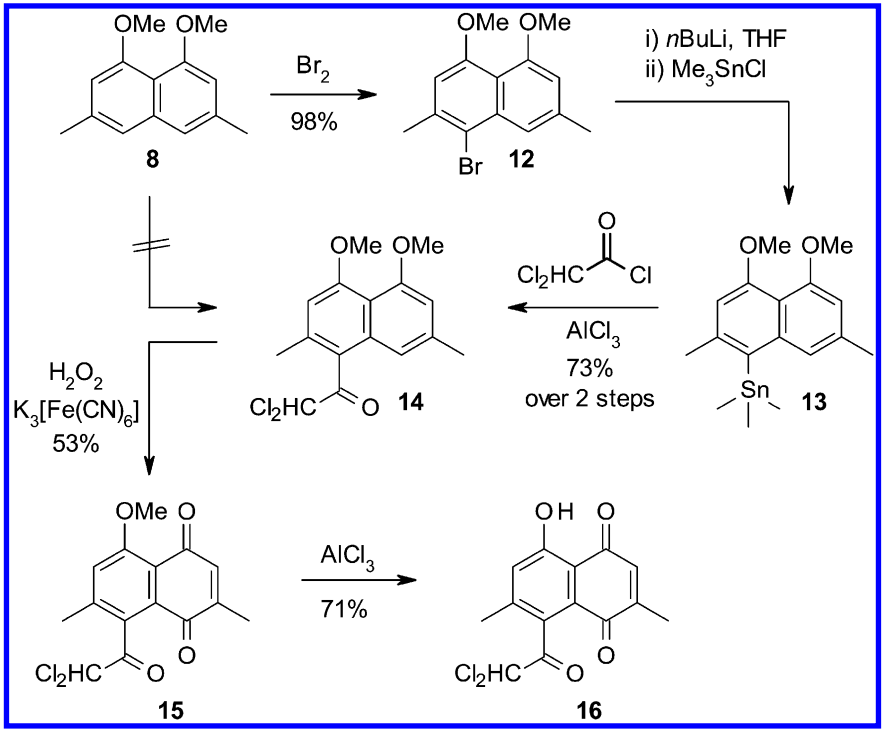


*Scheme 2. Synthesis of dechloromollisin methyl ether (**11**).*

As described by van der Kerk and Overeem naphthol ether **8** could not be acylated directly with the dichloroacetyl chloride. However, Opatz showed that the 4-position could be activated towards an electrophilic attack by bromination and subsequent conversion of the bromide **12** into stannane **13**. Subsequent *ipso*-substitution at the 4-position is favored due to the higher electrophilicity of this position and due to the weak C–Sn bond, which makes the trimethylstannyl cation a good leaving group. Hence, the dichloroacetyl group could be introduced by a Friedel–Crafts-like acylation. In the next step formation of the naphthoquinone **15** was achieved by oxidation of ether **14** and subsequent demethylation with $AlCl_3$ to provide mollisin (**1**) as yellow crystals in 27% yield over five steps.



*Scheme 3. Total synthesis of mollisin (**1**).*

Although Friedel-Crafts acylation of **8** did not work with dichloro acetyl chloride as shown in Scheme 2, reaction with acetyl chloride or even with trifluoroacetyl chloride led to the formation of monoacetylated naphthols **17** (Scheme 4). Further oxidation to the quinones **18** and demethylation led to dechloromollisin **19a** and its trifluoro analogue **19b** (*15*).

Recently a variety of synthetic analogues of mollisin have been reported by BASF (*16*). Most derivatives contain the quinone moiety typical for mollisin and differ only in the attached acyl group (Scheme 5). However, the synthesis of naphthatriol **20** was also achieved. With the synthesis route described above (see Schemes 3 and 4) not only halogenated acyl groups could be installed, but also various aliphatic and aromatic ketones, which were all tested for their antifungal properties against phytopathogenic fungi.

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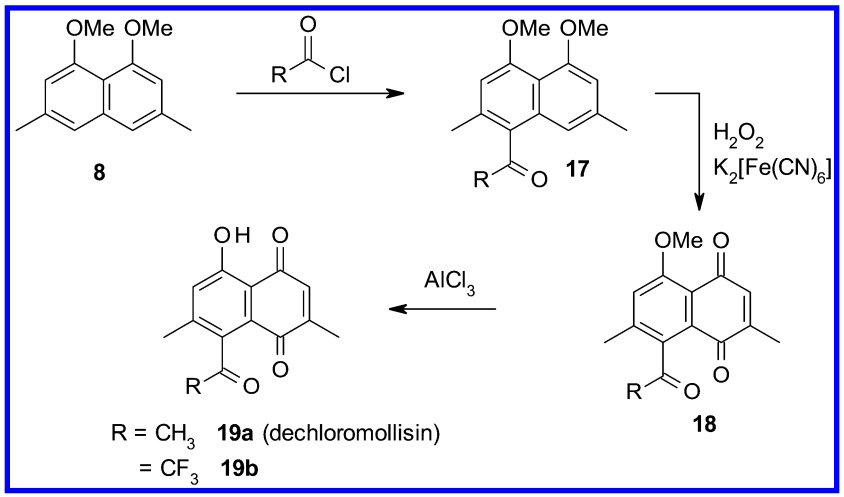
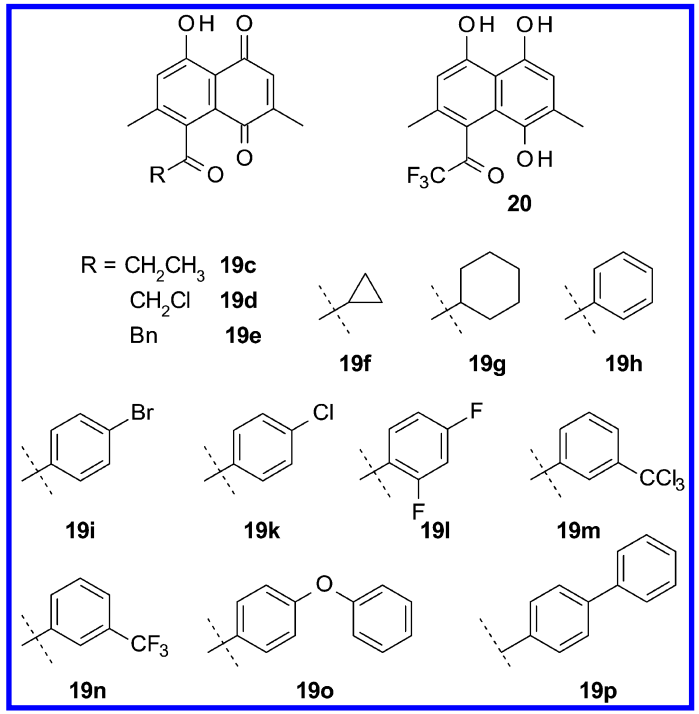


*Scheme 4. Synthesis of dechloromollisin (**19a**) and its trifluoro analogue **19b**.*



Scheme 5. Synthetic derivatives of mollisin.

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Biological Properties

Mollisin, the fungi that produce itas well as other derivatives possess various antifungal and interesting pharmacological activities. The natural product mollisin (**1**) itself, its derivatives and the deuteromycetes *Mollisia sp.* and *Mollisia fallens* have been investigated for their biological properties.

Fungicidal Activity of *Mollisia sp.*

The fungus *Mollisia sp.* was investigated for its properties against several phytopathogenic fungi occurring on trees. It showed strong antibiotic activities against the ascomycete *Pezicula cinnamomea* (DC), which causes Pezicula canker of Red Oak *(Quercus rubra* L.) (*17*), and the basidiomycete *Heterobasidion annosum*, one of the most destructive basidiomycetes in coniferous forests. In addition activity against the less-investigated ascomycetes *Amphiporthe leiphaemia* and *Diaporthe carpini* (Fr.) was found (*5*).

Fungicidal Activity of *Mollisia caesia*

The culture filtrate of *Mollisia caesia* showed a good antagonistic activity against the post-harvest pathogens *Pezicula malicorticis* and *Nectria galligena*, which can cause severe loss in stored apples (*18*).

Fungicidal Activity of Mollisin

The isolated natural product mollisin (**1**) shows various antifungal activities. For example mollisin is active against the basidiomycete fungus *Heterobasidion annosum*, an economically important forest pathogen and *Sclerophoma pityophila*, a pathogen that affects pines (*5*). It also inhibits growth of the wide-spread pests *Dothichiza populea* and *Pollaccia radiosa* (*6*).

Furthermore mollisin is also active against some economically-relevant phytopathogenic fungi. *Sclerotinia trifoliorum,* which can affect spruces (*6*), *Magnaporthe oryzae*, also known as rice blast fungus, *Phytophthora infestans*, an oomycete that causes serious potatoe disease, and *Botrytis cinerea*, a necrotrophic fungus that affects many plant species are all inhibited by mollisin (*2*, *16*).

Activity against *Penicillium notatum* has been investigated for mollisin (**1**) and its metabolites mollisin A (**2**) and B (**3**). An activity increase from the dichloro (**1**) to the trichloro (**2**) to the tetrachloro compound (**3**) has been observed (*5*).

During their studies BASF investigated the influence of several mollisin analogues on the spore germination of *Magnaporthe oryzae*, *Botrytis cinerea* and *Phytophthora infestans* (*16*). As also reported for mollisin itself, all of the derivatives show a good inhibition of *Magnaporthe oryzae* at all tested concentrations (Table 1). The spore germination inhibition of *Phytophthora infestans*, however, was not as consistent. Whereas smaller substituents (R) appeared to be favourable for the inhibition, aromatic acyl groups had a lower influence on germination. This effect is even more distinct with *Botrytis cinerea*. In this case only compounds with an acyl (**19a**) or trifluoroacyl group (**19b** and

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20) showed a significant inhibition. Notably, the naphthatriol **20** showed a very good inhibition of spore germination of all tested fungi at all concentrations (*16*).

Table 1. Test results of spore germination inhibition assay[a]

Cmp.	*Spore Germination Inhibition @ 10, 25, 50 μg/mL*								
	M. oryzae			*B. cinerea*			*P. infestans*		
	10	*25*	*50*	*10*	*25*	*50*	*10*	*25*	*50*
19a	+++	+++	+++	++	+++	+++	+++	+++	+++
19b	+++	+++	+++	-	+++	+++	+	+++	+++
19c	+++	+++	+++	-	-	-	++	+++	+++
19f	+++	+++	+++	-	-	-	+	+++	+++
19g	-	+++	+++	-	-	-	-	-	-
19h	+++	+++	+++	-	-	-	-	++	+++
19i	+++	+++	+++	-	-	-	-	-	-
19k	+++	+++	+++	-	-	-	+	+	+
19l	+++	+++	+++	-	-	-	++	+++	+++
19m	+++	+++	+++	-	-	-	-	-	-
19n	+++	+++	+++	-	-	-	-	-	-
19o	+++	+++	+++	-	-	-	-	-	-
19p	+++	+++	+++	-	-	-	-	-	-
20	+++	+++	+++	++	+++	+++	+++	+++	+++

[a] Rating: 0-25% (-), 25-50% (+), 50-75% (++), >75% (+++).

Antiproliferative Activity of Mollisin

Mollisin (**1**) and its two isolated metabolites **2** and **3** have been tested in search for antiproliferative drugs. Hereby, mollisin (**1**) and mollisin A (**2**) showed very good antiproliferative activities against two cell lines: the L-929 (murine fibroblasts) and K-562 (human leukemia). Furthermore, both compounds exhibit a weaker cytotoxic effect against the HeLa (human cervix carcinoma) cell line. Mollisin B (**3**) showed a reduced level of activity against these three cell lines (*5*).

Antiinflammatory Activity of Mollisin

A similar activity order was found with respect to phagocytosis-modulating properties. Phagocytic cells are attracted and activated by inflammatory stimuli to attack microbes by effector mechanisms. The phagocytes generate after such stimuli large amounts of reactive oxygen species (ROS).The ROS produced may be of importance in a number of inflammatory diseases, including arthritis,

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atherosclerotic lesions, and ischemic tissue injury. The degree of release of ROS can be modulated by compounds or drugs. Mollisin B (**3**) showed a strong inhibitory activity of ROS release of phagocytic cells (*5*).

In the search for new anti-inflammatory drugs, the inhibition of 3α-hydroxysteroid dehydrogenase (3α-HSD) can be a suitable measurement (*19*). The 3*a*-HSD enzyme catalyzes the reduction of 5β-dihydrocortisone under consumption of NADPH. The NADPH consumption can be determined photometrically by measuring the decrease of UV/VIS extinction at 340 nm. Indomethacin and ibuprofen were used as reference compounds. Mollisin (**1**) shows no activity, but mollisin A (**2**) and B (**3**) are good inhibitors (*5*).

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Summary

More than 60 years after its first isolation from several *Mollisia* species, mollisin and several close analogues have been investigated for their biological activity against phytopathogenic fungi and pharmacological properties. These studies were enabled by the first total synthesis of this naphthoquinone metabolite and the easy access to its derivatives. It has been shown that several fungal pathogens that can cause significant crop failures are affected by these compounds. Furthermore, interesting pharmacological activities have been found. The biological properties of mollisin make it a promising starting point for combating phytopathogens as well as for pharmaceutical studies.

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Chapter 15

Combinatorial Approach To Lead Generation and the Discovery of a Potent *Septoria tritici* Fungicide

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Developing a general protocol in which large sets of molecules are synthesized simultaneously is an efficient way to build structure-activity relationships (SARs). The preparation of several libraries around a modest screening hit led to the discovery of a novel substituted pyrimidine with excellent wheat leaf blotch (*Septoria tritici*) activity. Synthesis involved sequential displacement reactions on 2,4-dichloropyrimidines and resulted in over 400 analogs. The three dimensional array allowed rapid assessment of fungicidal trends and the identification of a particularly efficacious molecule with excellent protectant and curative activity in greenhouse tests. Combinatorial strategy, synthetic methods and biological activity are discussed.

Combinatorial chemistry first gained prominence two decades ago as a way to generate large numbers of compounds for high throughput screening (*1*). The original concept, however, which relied heavily on the testing of crude mixtures, proved to be impractical as hit rates for quality starting points in lead generation did not substantially increase despite the development of innovative technologies and significant investment across the industry. The appeal of simultaneously generating multiple analogs, however, did not diminish and so the approach continued to evolve. It has since been reshaped into a simpler yet

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more sophisticated paradigm: the preparation of smaller focused libraries where compounds are purified and individually tested.

A much touted advantage of the combinatorial approach is the possibility of discovering unexpected beneficial combinations of substituents. This is because multi-dimensional arrays address chemical space more comprehensively than linear strategies do. While finding such molecules certainly does happen (as it did in the work described in this chapter) it is a rare occurrence, and should not be relied upon. The main motivation for using combinatorial strategies should be the efficient generation of high quality structure-activity relationships (SARs) by rapidly preparing larger sets of compounds.

While combinatorial methods are applicable at any level of discovery research, they fit particularly well into lead generation. It is important to remember, however, that they are not suitable in every situation. Molecules where a late stage disconnection can be addressed with robust chemistry – that is, with a selective high yielding reaction – are the best candidates for a combinatorial approach.

At Dow AgroSciences, there is no separate high throughput chemistry group and all lead generation and optimization chemists are encouraged to use combinatorial methods as needed. Experienced chemists mentor novices and only straightforward standard equipment is utilized. This integrated and pragmatic arrangement has led to several significant discoveries. One of these is discussed in detail in this chapter.

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Background

Original Screening Hit

A molecule showing activity against wheat leaf rust (*Puccinia recondita,* PUCCRT) was found during a review of historical crop disease screening hits. Additional testing revealed that compound **1** shown in Figure 1 also had activity on wheat leaf blotch (*Septoria tritici*, SEPTTR) in a one day protectant (1DP) test. The economic importance of this disease today and the need to control it, especially in the European cereals market, cannot be overstated (*2*). A thorough investigation of this compound was therefore undertaken.

The tetrahydroquinazoline **1** was an attractive molecule to explore because it was novel from a fungicidal perspective and conceptually straightforward to modify. It seemed particularly well suited to a combinatorial approach because sequential functionalization at the 4- and 2-positions of a pyrimidine is well established in the synthesis literature (*3*–*7*).

Although the novelty of the tetrahydroquinazoline ring system was appealing, the desire to simplify the structure and explore broadly around the pyrimidine core was also important. The hypothesis that the fused cyclohexyl ring was not necessary for activity ultimately drove the decision to begin with simpler substituents in the 5- and 6-positions of the pyrimidine ring. The overall effort was envisioned as a series of 8 x 11 combinatorial plates in which substitution

at the 5- and 6-position would be held constant for each plate, but varied across plates. Some initial experiments, however, were needed to validate the targets and to help guide input and route selection.

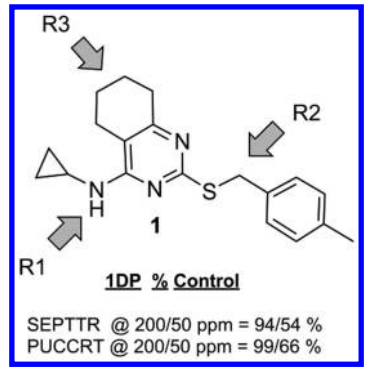
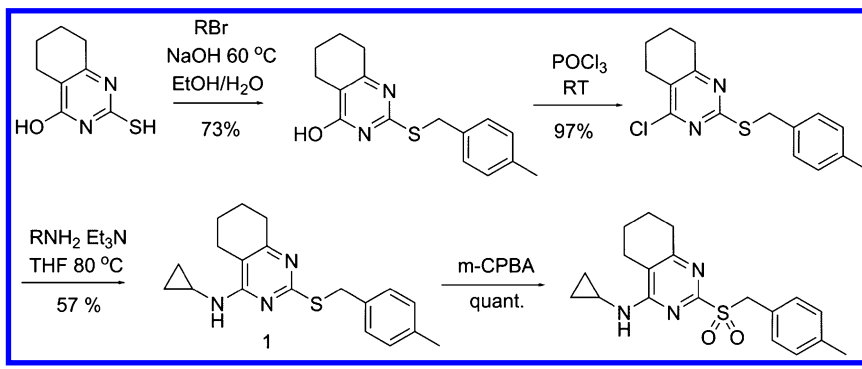


Figure 1. Original screening hit.

Initial Investigations

The first question to consider was whether the sulfur atom was oxidized or not *in vivo* and if the resulting sulfone was actually the active molecule. Compound **1** was prepared as shown in Scheme 1 from commercially available 2-mercapto-5,6,7,8-tetrahydro-quinazolin-4-ol by selective alkylation of the thiol followed by conversion of the hydroxyl to chlorine and displacement with cyclopropylamine. Treatment with *m*-chloroperoxybenzoic acid yielded the sulfone. This compound turned out to be inactive, and so efforts were refocused on the sulfide.



Scheme 1. Synthesis of 4-alkylamino-2-alkylthiopyrimidines from 4-hydroxy-2-mercaptopyrimidines by alkylation and displacement

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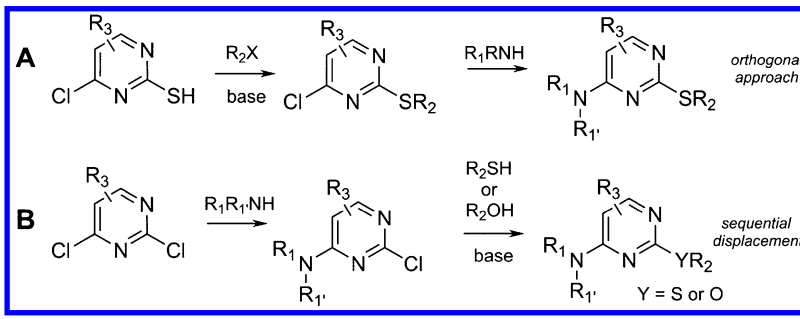
Protocol Development

Route Selection

The synthetic route described in Scheme 1 appears to be well suited for developing into a combinatorial protocol because the two reactions used to functionalize the pyrimidine core are orthogonal. The same type of product, however, could also be obtained by sequential displacement on a 2,4-dihalopyrimidine core. Both options in generalized form are shown in Scheme 2 for comparison. The R_3 substituent is held constant – R_1 and R_2 are variable. Note that in both cases, it is the last step that is combinatorial. In option A, the 4-chloro-2-mercaptopyrimidine starting materials needed for preparing R_2 inputs must be synthesized. Several functionalized dichloropyrimidines, on the other hand, are commercially available for option B. Furthermore, the displacement reaction of the second step could potentially provide access to both sulfur and oxygen analogs. This was particularly appealing because of our intention to explore broadly around screening hit **1**. Option B was therefore selected for protocol development.



Scheme 2. Comparison of possible synthesis routes

R_3 Substituents and Regiochemical Selectivity

The selection of 5- and 6-position substituents was based on the hypothesis that the fused cyclohexyl ring was not critical for activity. The following three options were chosen for consideration: 5,6-unsubstituted, 5-fluoro and 6-methyl. They were viewed as simple and diverse replacements of the fused cyclohexyl ring. Although nucleophilic displacement occurs preferentially at the 4-position of 2,4-dichloropyrimidines, the selectivity of the reaction does vary substantially depending on both the nature of the nucleophile and substitution on the ring.

In two of the substrates illustrated in Scheme 3, the natural propensity of the pyrimidine to favor alkylamine displacement at the 4-position over the 2-position was only about a ratio of 2:1. Fortunately, these regioisomers were easily prepared and separated. The 5-fluoro substituted substrate, on the other hand, was completely selective at the 4-position and so was chosen to produce the first set of plates.

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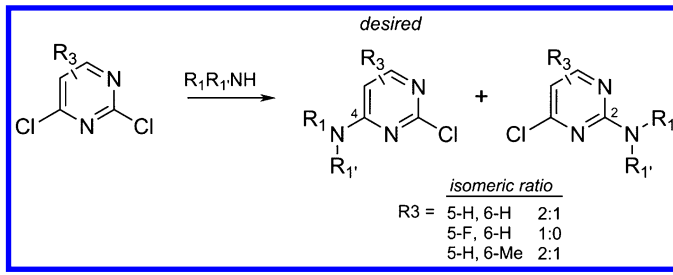

Scheme 3. Selectivity of nucleophilic displacement on 2,4-dichloropyrimidines

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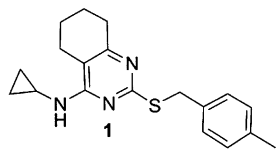
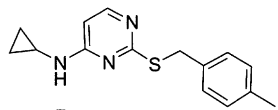
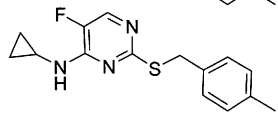
Physical properties measurements supported our choices for substituents in the 5- and 6-position. The analogs tested in Table 1 indicated that partitioning as predicted from log K_{ow} measurements would likely be similar to the original screening hit **1** but uptake might be improved due to increased water solubility.

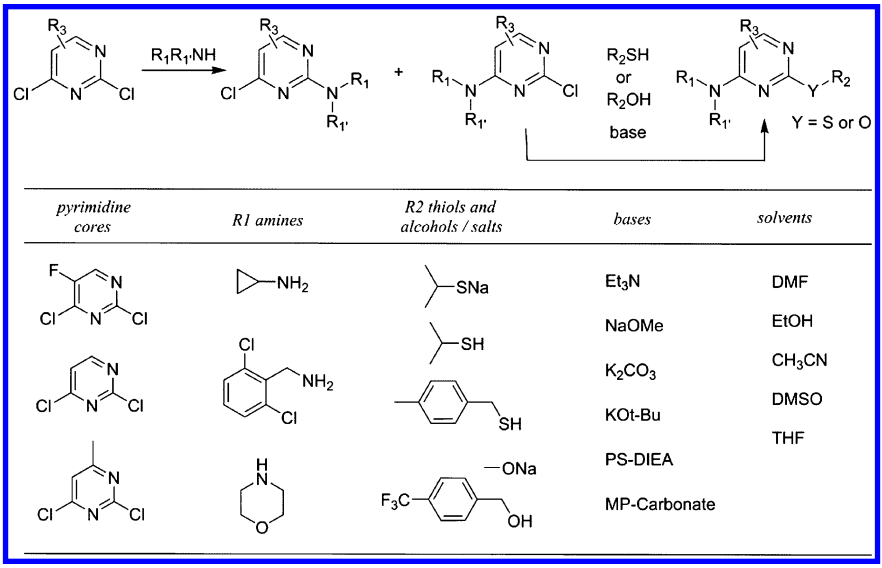
Table 1. Physical Properties Comparison for R_3 Substituents

Structure	*Solubility +/- 10% ppm*	*log K_{ow} +/-0.35*	*pK_a +/- 0.20 (predicted)*
 **1**	<1	4.28	5.42
	10	3.88	5.17
	8	4.09	4.12

Reaction Conditions

Arguably, the most important part of combinatorial library synthesis is protocol development. Starting materials must be completely consumed and byproduct formation needs to be minimal. Several inputs and a variety of reagents and conditions were investigated as shown in Table 2.

Table 2. Components Investigated During Protocol Development



Much effort was spent finding the right base and solvent for the combinatorial second step. Though the reaction occurred in all the solvents tried, the cleanest results were obtained in tetrahydrofuran. It was important to have the reaction be as complete as possible with one equivalent of thiol or alcohol because excess alkylthiolate or alkanoate invariably led to displacement of the 5-fluoro substituent as well. Resin bound bases did not work at all, whereas sodium methoxide competed with the alkylthiolates during the displacement and gave mixtures. Potassium *t*-butoxide turned out to give the best results, allowing the use of as little as 1.1 equivalents of thiol or alcohol. It was also particularly convenient to handle, due to its availability as a 1.0 M solution in *t*-butanol. Modest heating aided in driving the reactions to completion.

The first step was much less problematic, and the solvent chosen was simply the one that worked best in the second step. A base was utilized here as well, so that the alkylamine input could be used stoichiometrically. Triethylamine was chosen for this purpose as it is volatile and so can be used in excess.

The reaction conditions described above worked remarkably well, making purification by column chromatography unnecessary. Excess reagents and their byproducts still had to be removed, however, and so the scavenger resin MP-Isocyanate was employed to eliminate unreacted nucleophiles and MP-Carbonate was used to manage the triethylamine hydrochloride generated.

Input Selection

Library inputs were selected so that the synthesized targets would be as diverse as possible, yet still meet criteria considered optimal for agricultural

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compounds. At Dow AgroSciences, such parameters are referred to as AgLike (*8*) and were determined empirically using very large sets of internal biological data. A wide selection of substituted alkyl, benzyl and heteroarylmethyl moieties were incorporated into the amine, thiol and alcohol inputs as illustrated in Figure 2.

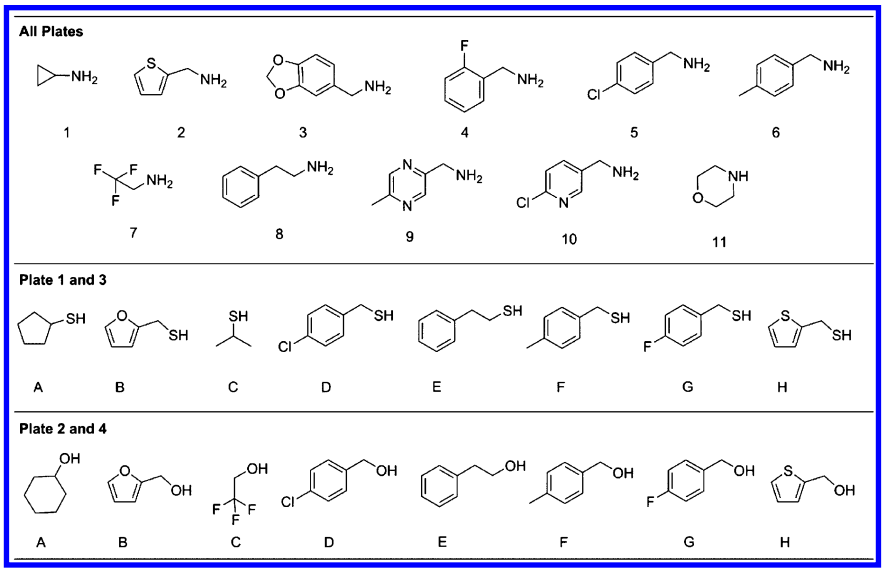

Figure 2. Library inputs selected.

Library Production

Four combinatorial plates were prepared according to the protocol shown in Scheme 4. Each reaction of the library was run in a simple 8 mL vial using a multi-pipettor to add stock solutions of components in dry tetrahydrofuran and an oscillating 96 sample block heater to stir and heat. The chloride (1.0 equivalent) was added first, followed by the thiol or alcohol (1.5 equivalents) and finally the 1.0M potassium *t*-butoxide solution in t-butanol (1.5 equivalents). The vials were agitated for 18 hours at >50 °C. Upon completion of the reaction, MP-NCO scavenger resin (4.0 equivalents) was added with a calibrated scoopula. The solids were removed by filtration after 18 hours at room temperature by using individual membrane cartridges attached to syringes. The solvents were then evaporated and the remaining solids and syrups analyzed. The isolated materials were sufficiently pure at this point for biological testing.

The production rates (targets obtained/reactions attempted x 100) for the 4 plates were 100% for the 5-F-2-RS- plate, 97% for the 5-F-2-RO- plate, 99% for the 6-Me-2-RS- plate and 86% for the 6-Me-2-RO- plate. The yields were generally >75%. The purity as determined by LC/MS was typically >90%. The LCMS method was considered a reliable estimate of the true purity based on ^{1}H NMR spectra obtained for selected compounds.

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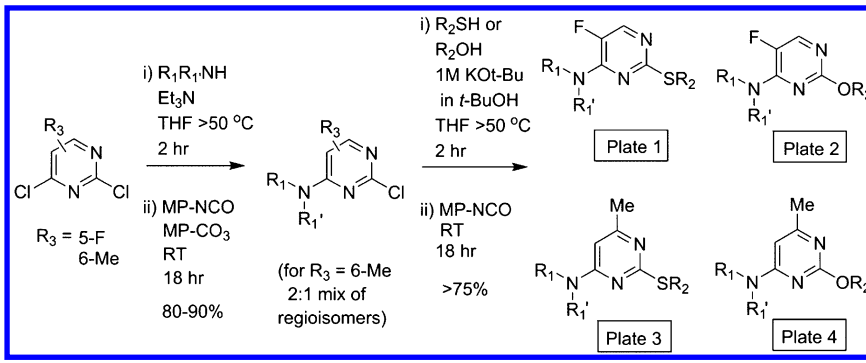
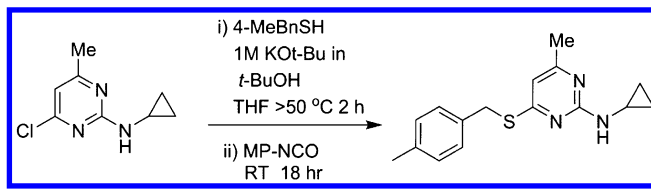


Scheme 4. Final library protocol and plates

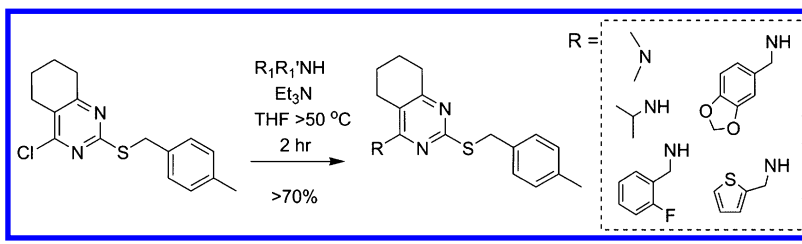
Additional Analogs

Only the 4-alkylamino-2-chloro-6-methylpyrimidine regioisomers isolated from the alkylamine displacement reactions were used in the combinatorial format to generate targets. The possibility of making additional plates using the other regioisomer was considered, but a probe molecule synthesized to explore this idea shown in Scheme 5 proved to be inactive.



Scheme 5. Synthesis of a regioisomeric analog

In an attempt to specifically capitalize on the novelty of the tetrahydroquinazoline core structure, several more analogs of **1** were prepared as shown in Scheme 6. These were also tested for fungicidal activity.



Scheme 6. Synthesis of additional tetrahydroquinazoline analogs

Plates based on the 5,6-unsubstituted pyrimidine were not prepared as they were thought to be too similar to the 6-Me targets. The 6-Cl substitution, however, was a logical next step in expanding the SAR. As a consequence, a 3 x 4 array of

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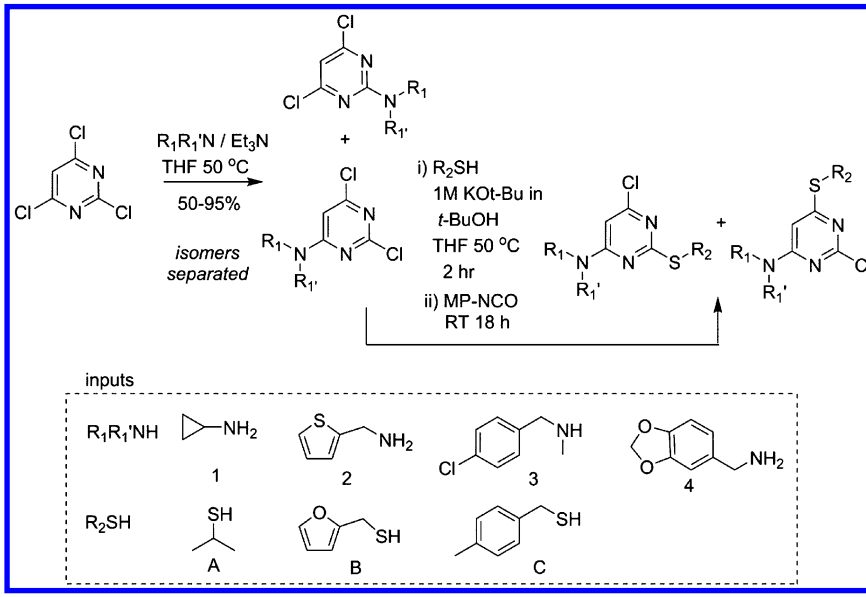
6-chloro-4-alkylamino-2-alkylthiopyrimidine targets were prepared as shown in Scheme 7.



Scheme 7. Synthesis of 6-chloropyrimidine analogs

A complicating factor with the 6-position chlorine was that it was quite susceptible to displacement by the thiolate so that regioisomeric 1:1 mixtures were obtained. These were difficult to separate and so most were tested as mixtures. Only the 4-cyclopropylamino regioisomers were separable.

Biological Testing

The synthesized target molecules can be grouped into six sets based on the substitution of the pyrimidine ring (four combinatorial plates and two smaller arrays) as shown in Table 3.

Compounds were initially assessed for in vitro growth inhibition of *Septoria nodorum*, *Phytophthora infestans*, *Pyricularia oryzae*, *Saccharomyces cerevisiae*, *Septoria tritici* and *Ustilago maydis* in microtiter plate fungitoxicity assays.

Compounds providing >70% growth inhibition on any of the 6 species *in vitro* at 25 ppm were promoted to one day protectant evaluations at 200 ppm in the greenhouse. Greenhouse evaluations were made for control of spot blotch of barley caused by *Helminthosporium sativum*, cucumber anthracnose caused by *Colletotrichum lagenarium*, wheat glume blotch caused by *Phaeosphaeria nodorum*, cucurbit downy mildew caused by *Pseudoperonospora cubensis*, wheat brown rust caused by *Puccinia recondita* and rice blast caused by P*yricularia oryzae*.

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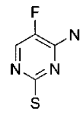
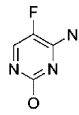
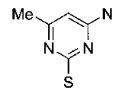
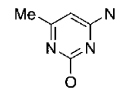
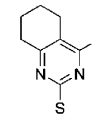
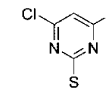
Table 3. Overall Fungicidal Activity of Substituted Pyrimidine Analogs

	Plate 1	Plate 2	Plate 3	Plate 4		
Number of Compounds:						
synthesized	88	85	87	76	6	12
active in vitro[a]	30	42	53	16	3	7
active in greenhouse[b]	0	4	19	9	3	2

[a] >70% growth inhibition at 25 ppm against at least one organism

[b] >85% control at 200 ppm against at least one disease

The level of *in vitro* activity obtained across all four combinatorial plates was encouraging. It appeared that the third plate (6-Me-2-RS-) was the most successful in terms of number of compounds with activity and this advantage was maintained in greenhouse testing. Results from the limited number of tertrahydroquinazoline molecules also looked promising, whereas the 6-chloro materials were clearly less compelling.

Further testing on PUCCRT and SEPTTR in the greenhouse revealed some interesting trends, as can be seen in Table 4. For example, the activity of the original hit on SEPTTR (entry 1) could be improved by varying nitrogen substitution (entry 2 and 3), but at the expense of control on PUCCRT.

Replacing the fused cyclohexyl moiety with a 6-methyl substituent (entry 1 and 4) actually improved activity on both PUCCRT and SEPTTR.

Compounds from the second plate (5-F-2-RO-), however, turned out to be the most interesting, despite the low overall initial activity observed in the greenhouse. The most obvious trend to note, was the complete lack of potency on PUCCRT (entry 5-10). Testing on SEPTTR, however, revealed that some of these compounds had exceptional activitiy in both the one day protectant and two day curative tests, the best of which ran down well below 6.25 ppm (entry 10).

The ability of a combinatorial strategy to uncover unexpected activity is also highlighted by this set of analogs. The point is often made, that SAR trends are not necessarily additive and seams of activity can be missed by a linear strategy. Comparing the 2-(thiophen-2-ylmethoxy)- analogs (entry 5 and 6) in isolation may lead one to conclude that the benzo[d][1,3]dioxol-5-ylmethanamine moiety in the 4-position is not effective. Pairing it with a 4-fluorobenzyloxy substituent in the 2-position (entry 7), however, clearly demonstrates that such a conclusion would be wrong. The fact that the remaining permutation (entry 8) was not very active further underscores this point. Finding the best pair of inputs is not typically predictable (entry 8, 9 and 10), and so increasing the probability of success by preparing and testing a greater number of combinations makes good sense. In this case, it led to the discovery of a novel class of agricultural fungicides.

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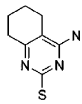
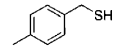
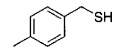
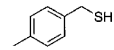
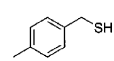
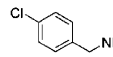
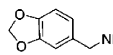
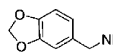
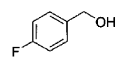
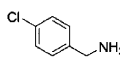
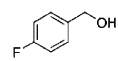
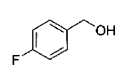
Table 4. Fungicidal Activity of Selected Pyrimidine Analogs

pyrimidine core	*entry*	*amine input*	*alcohol or thiol input*	*% 1 DP[a] @ 50 ppm PUCCRT*	*% 1 DP @ 50 ppm SEPTTR*	*% 1 DP @ 6.25 ppm SEPTTR*	*% 2 DC[a] @ 6.25 ppm SEPTTR*
	1			69	47	9	0
	2			50	79		
	3			27	99	19	35
	4			88	84	12	20
	5			0	97	71	74
	6			0	39		
	7			6	98	88	
	8			0	69		
	9			0	25		
	10			0	97	97	99

[a] 1DP = 1day protectant; 2DC = 2 day curative

Due to its significant protectant and curative activity on SEPTTR and its novelty, compound **2** shown in Figure 3 became a lead molecule in an extensive optimization program.

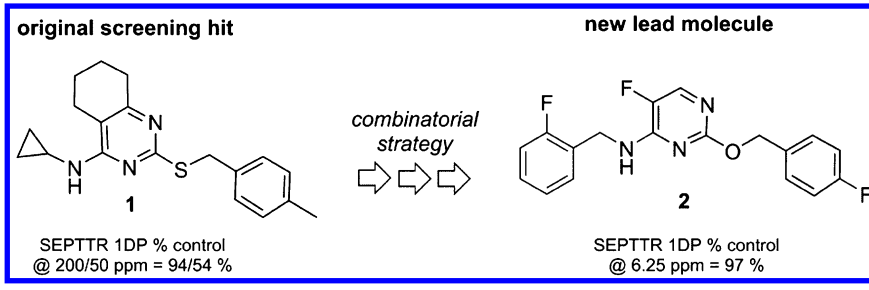


Figure 3. Screening hit to lead molecule comparison.

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ACS Symposium Series; American Chemical Society: Washington, DC, 2015.

The mode of action is currently under investigation and it is anticipated that the data obtained will form the basis of a future publication.

Conclusions

A combinatorial approach was used to explore a fungicidal screening hit and find analogs with significantly improved efficacy. Compounds were prepared by a sequential substitution protocol based on a central pyrimidine scaffold. Production rates were excellent and the yields were high. Scavenger resins were employed for the isolation of targets and as a result additional purification was not required before biological testing.

A potent new chemistry with excellent activity against wheat leaf blotch *Septoria tritici* has been discovered. This area represents a potentially new mode of action for crop disease control which would be of great benefit due to the ever increasing need to combat resistance and emerging new diseases. This is especially true in the cereal market where resistance to the strobilurins and azoles has created a pressing need for new control methods. In particular, sustaining robust control of wheat leaf blotch, *Septoria tritici,* in cereals is always a challenge. This class of compounds could therefore provide a valuable new tool for crop protection.

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Chapter 16

HPPD Herbicide-Safener Combinations as Resistance Breaking Solutions for 21st Century Agriculture

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Modern agricultural practices are facing a major challenge to their sustainability due to the worldwide spread of herbicide resistant weeds. To combat this problem, it is important that existing herbicides are preserved and that new herbicidal solutions are developed. Herbicide-safener combinations, as exemplified by the HPPD inhibitor pyrasulfotole, which is sold as a mixture with the safener mefenpyr-diethyl for broadleaf weed control in cereals, offer a new way to manage resistance issues. The safener ensures good crop selectivity, which had previously been problematic with this mode of action (MoA) and had prevented the use of HPPD chemistry in cereals. This represents the first new MoA introduced into cereal cultivation for over 20 years and provides cereal farmers with a valuable tool for the management of resistant weed populations. The usage has been extended to enable the control of herbicide resistant Amaranths in grain sorghum.

The Competition between crops and weeds has been a problem for farmers ever since agriculture first became established in the Neolithic Period. If weeds are not controlled, then substantial reductions in crop yield of around 25 to 50% are to be expected, depending upon the crop and level and type of weed

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infestation (*1*). Traditionally weeds have been controlled by a combination of tillage, hand weeding and crop rotation. However, in the twentieth century these methods were increasingly replaced by the usage of synthetic herbicides. The first major herbicide for selective in-crop use was 2,4-D which was commercialized in 1946 for the control of broadleaf weeds in cereals (*2*). More recently, the introduction of transgenic glyphosate-resistant crops in 1996 allowed the use of the non-selective herbicide glyphosate for the broad-spectrum control of weeds in crops (*3*). The introduction of synthetic herbicides has revolutionized modern agriculture leading to increased crop yields, reduced soil erosion and increased production efficiency. However, these very significant and important gains are being increasingly threatened by the development of herbicide resistance amongst weed populations throughout the world (*4*).

Herbicide resistance cannot truly be considered as surprising or unexpected. In 1859 the English scientist Charles Darwin wrote, "As many more individuals of each species are born than can possibly survive; and as, consequently, there is a frequently recurring struggle for existence, it follows that any being, if it vary however slightly in any manner profitable to itself, under the complex and sometimes varying conditions of life, will have a better chance of surviving and thus be naturally selected" (*5*). The widespread application of herbicides can be considered as a modern variation of the conditions of life for weed species and it was and is to be expected that they would adapt and become resistant. For example, just eleven years after its introduction, the first report of weeds resistant to 2,4-D was published (*6*). Although some scientists considered that the development of agronomically relevant levels of resistance to glyphosate was unlikely to occur (*7*), here too resistance has developed, the first case being found following fifteen years of usage in Australian orchards (*8*). Currently the resistance of certain weeds to glyphosate has developed to become a serious problem in some areas of the world that rely heavily upon glyphosate for weed control, and is threatening the sustainable future usage of this very important herbicide (*9*).

The problem of weed resistance to herbicides is compounded by the relatively small number of commercial herbicide modes of action (MoAs). The twelve most important herbicide MoAs in terms of market value are shown in Table 1 (*10*). Taken together they make up 93% of the herbicide market. However, the six top MoAs alone make up 75% of the market. This means that these six MoAs are under a tremendous selection pressure and not surprisingly significant resistance problems have developed against five of them, namely EPSPS, ALS, PSII, ACCase and synthetic auxins (*11*). With the exception of the long established PSI herbicides, the bottom six MoAs, which together make up about 18% of the market, generally suffer less from resistance problems, consistent with their lower levels of usage. Of particular note, are the five MoAs where resistance problems are currently at low levels, namely VLCFA, HPPD, PPO, GS and carotenoid biosynthesis inhibitors (See Table 1 for key to herbicide MoA abbreviations).

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Table 1. Important herbicide modes of action and occurrence of resistance

Mode of Herbicide Action[a]	*Herbicide World Market Share (10)*	*No. of Resistant Weeds Globally (11)*	*No. of Resistant Weeds North America (11)*
EPSPS Inhibitor	21%	29	14
ALS Inhibitor	17%	144	54
VLCFA Inhibitor	11%	4	1
PSII Inhibitor	10%	72	29
ACCase Inhibitor	8%	46	15
Synthetic Auxin	8%	31	12
HPPD Inhibitor	5%	2	2
PSI Electron Diverter	4%	31	5
PPO Inhibitor	3%	6	2
Microtubule Inhibitor	3%	12	7
GS Inhibitor	2%	2	1
Carotenoid Biosynth. Inhibitor	1%	3	1

[a] Abbreviations: EPSPS = 5-enolpyruvylshikimate 3-phosphate synthase; ALS = acetolactate synthase; VLCFA = very-long-chain fatty acid biosynthesis; PSII = photosystem II; ACCase = acetyl-coenzyme A carboxylase; HPPD = 4-hydroxy-phenylpyruvate dioxygenase; PSI = photosystem I; PPO = protoporphyrinogen oxidase; GS = glutamine synthetase.

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The continuing spread of resistant weeds means there is a real need to implement additional measures to preserve the utility of existing herbicides and to discover new herbicides able to control resistant weed species. Ideally, these new herbicides should be compounds with a completely new commercial mode of action. However, despite the combined efforts of all agrichemical companies, working at times together with researchers from academia, no major new mode of action has been introduced into the market in the past thirty years (*10*). In the absence of new modes of herbicide action companies have concentrated their search for new herbicides upon those existing modes of action where resistance problems are less prevalent. In this regard, two strategies have proven to be especially successful:

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1) The development of new chemical classes where the chemical structure varies significantly from existing herbicidal classes acting at a specific herbicide target. This strategy has the potential to overcome both target site and metabolic resistance. In the case of target site resistance, the herbicide binding site on the target enzyme has undergone changes such that existing herbicides no longer bind effectively, meaning that a new herbicide must be sufficiently different to enable it to overcome these changes and bind once again. In the case of metabolic resistance, the new herbicide must be sufficiently different that it is no longer a substrate for the metabolizing enzymes present in the resistant weeds.

2) The introduction of an existing herbicide class into a completely new crop where no similar chemistry has been previously utilized. In as much as different weed populations are associated with different crops, this means that even if resistance is present in some existing crop usages, it may be absent in the new crop usage. A good example of such a product is the Bayer herbicide pyrasulfotole which was introduced for the post-emergence control of broadeaf weeds in cereals in 2008 (*12*). Its introduction represented the first new mode of action for broadleaf weed control in cereals for over 20 years and the product enables effective control of ALS-, ACCase- and glyphosate-resistant weeds. The usage in cereals was enabled by the addition of a proprietary safener.

Pyrasulfotole exhibits its herbicidal activity through inhibition of the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD) in plants (*13*). This mode of action was first commercialized for use in rice in 1979 with the introduction of pyrazolynate by Sankyo (*14*). In the 29 years between then and the introduction of pyrasulfotole, numerous other herbicides with this mode of action were introduced and some of these are depicted in Figure 1. These herbicides are used in numerous crops including rice, corn and soybean but crop selectivity problems prevented their use in cereals prior to the introduction of pyrasulfotole in 2008 (*14*).

Pyrasulfotole was discovered by combining the 1,3-dimethylpyrazolone moiety of pyrazolynate with the 2-methansulfonyl-4-trifluoromethyl benzoic acid moiety present in isoxaflutole (Figure 2) (*12*). This “mix and match” approach is a quite commonly used discovery strategy within the agrisciences. An early laboratory synthesis of pyrasulfotole is outlined in Figure 3 (*15*). It starts from commercially available 2-bromo-5-trifluoromethylaniline and yields pyrasulfotole in six steps, beginning with the introduction of the methyl mercapto group *via* a diazotization reaction in the presence of dimethyl disulfide. In the next step the carboxylic acid group is introduced by a metal-halogen exchange reaction followed by quenching over solid carbon dioxide. The methyl mercapto group is then oxidized to a sulfone, the acid is converted to an acid chloride which is condensed with 1,3-dimethyl-5-hydroxypyrazole to give the kinetic *O*-benzoyl adduct. The final step involves an isomerization to give the thermodynamically more stable *C*-benzoyl product pyrasulfotole.

The herbicide pyrasulfotole exhibited excellent post-emergence control of broadleaf weeds in cereals, including resistant biotypes. However, although it showed good selectivity in cereals, crop tolerance was not assured under all conditions. Consequently it was highly desirable to find a way to guarantee the required crop safety.

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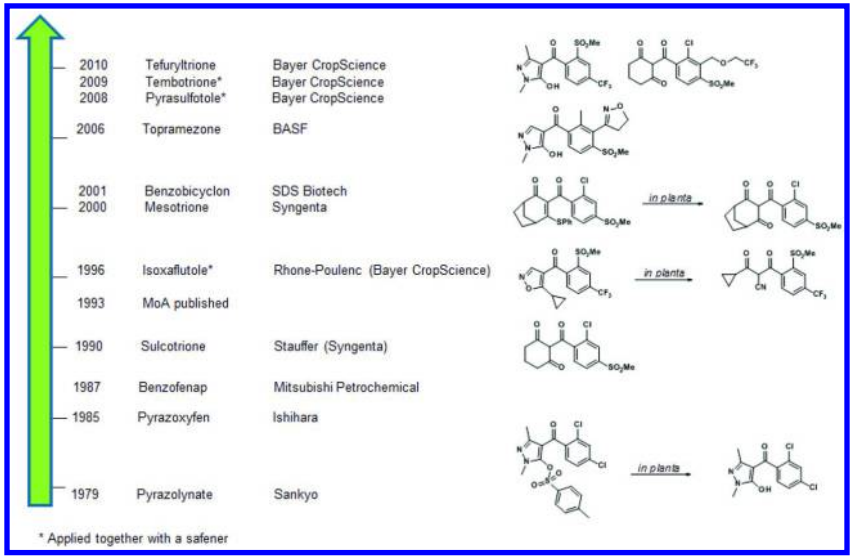

Figure 1. Selected milestones for herbicides inhibiting the HPPD enzyme

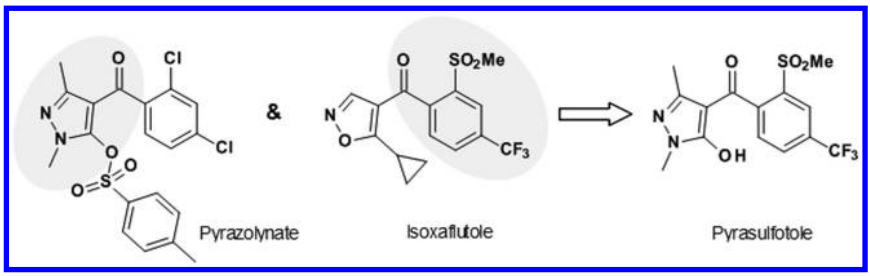

Figure 2. The discovery of pyrasulfotole

In modern agriculture there are two ways of achieving crop safety when using herbicides that are not inherently selective in their own right. One is the safener approach, and the other involves the development of a herbicide tolerant (HT) crop. These two possibilities are compared in Table 2. Both start from a non- or only partially selective herbicide. In the safener approach, a compound (the safener) is co-applied with the herbicide to the crop and selectively protects it from herbicide injury without protecting the weeds. In the HT crop approach, herbicide resistance is achieved either by genetic modification or induced mutation and breeding. This means that whereas the HT approach has a high dependence upon crop varieties, the safener approach is independent of the crop variety. At Bayer CropScience, the two approaches are considered complimentary and both are actively pursued.

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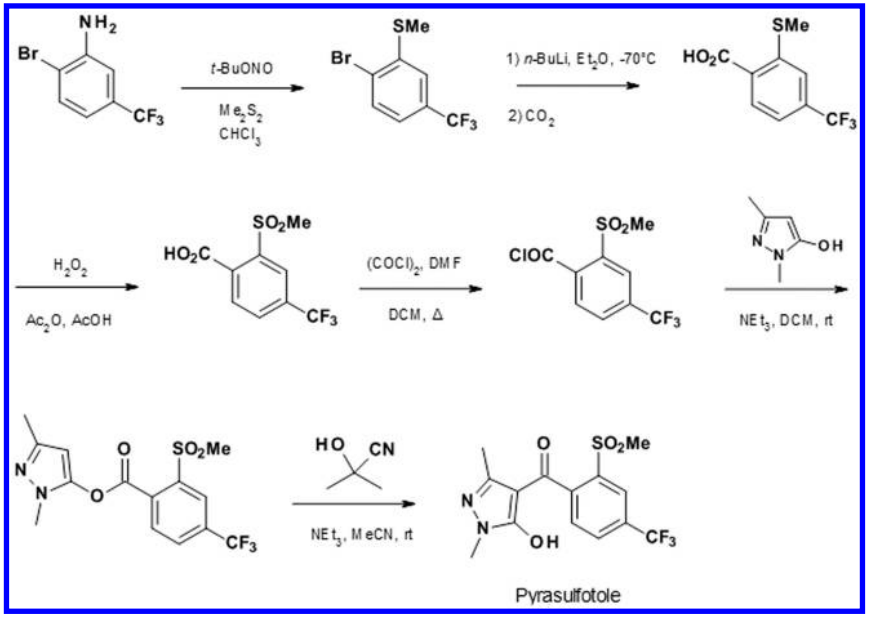

Figure 3. Synthesis of pyrasulfotole

Table 2. Achieving crop safety in modern agriculture

The Safener Approach	*The Herbicide Tolerant Crop Approach*
• Non- or only partially selective herbicide • Safety through addition of a herbicide safener • Independent of crop variety	• Non-selective herbicide • Safety through genetic modification (GM) or induced mutation (non-GM) • Dependent on crop variety

In the case of pyrasulfotole, the safener approach was used to ensure crop selectivity. Following an extensive program of biological screening, mefenpyr-diethyl was identified as the best safener for combination with pyrasulfotole in cereals. Mefenpyr-diethyl was already in the Bayer portfolio as a safener for use in cereals, but in combination with the herbicides fenoxaprop-ethyl or iodosulfuron, which are inhibitors of the enzymes ACCase and ALS, respectively. It was discovered by systematically varying the heterocyclic core of the older safener, fenchlorazole-ethyl (*14*). This led initially to experimental safeners containing a pyrazole ring and then following further modification, to the pyrazolines and mefenpyr-diethyl (Figure 4). The compound can by synthesized as shown in Figure 5, starting from 2,4-dichloroaniline (*16*). The key step involves a Japp-Klingemann reaction between the diazonium salt derived from the aniline and ethyl 2-chloroaceloacetate to give a chlorohydrazone intermediate.

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Under basic conditions this generates a 1,3-dipole which undergoes a [2+3] cycloaddition with ethyl methacrylate to yield mefenpyr-diethyl.

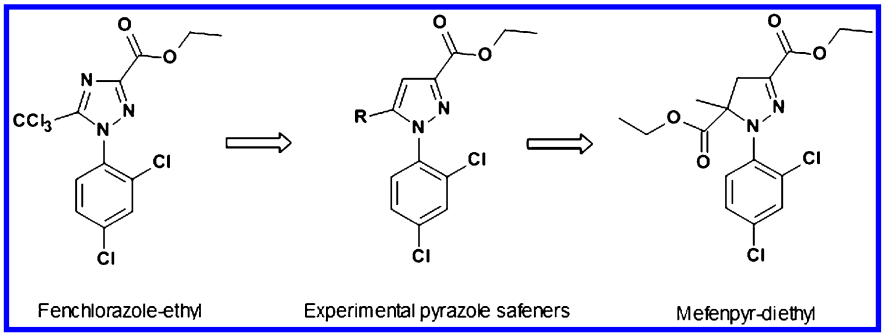

Figure 4. The discovery of mefenpyr-diethyl

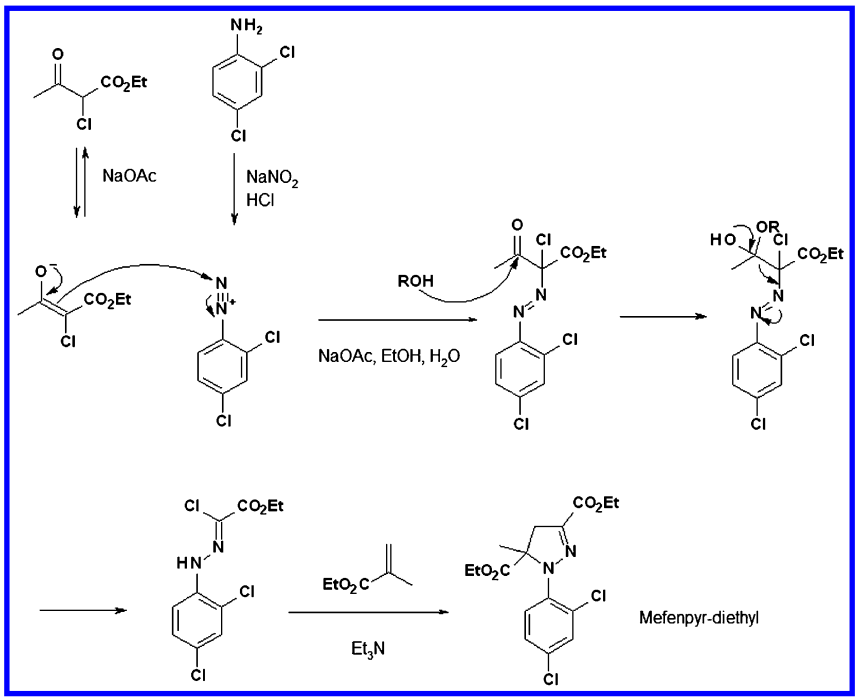

Figure 5. Synthesis of mefenpyr-diethyl via a Japp-Klingemann reaction

Mefenpyr-diethyl, like most other safeners, works by increasing the rate of herbicide metabolism in crops but not in weeds (*17*). This involves an increased expression of genes encoding enzymes responsible for xenobiotic degradation (eg. cytochrome P450s, glutathione S-transferases and ATP-binding casette transporters). This increased rate of metabolism has been nicely demonstrated in plants fed ^{14}C-labelled pyrasulfotole *via* their transpiration stream (Figure 6)

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(*18*). One day after treatment, radio-HPLC analysis of shoot extracts showed the extent of herbicide metabolism. Selected results are shown in Figure 6 where the addition of the safener mefenpyr-diethyl can clearly be seen to enhance pyrasulfotole metabolism in wheat, whereas no effect is seen in black bindweed (*Polygonum convolvulus*).

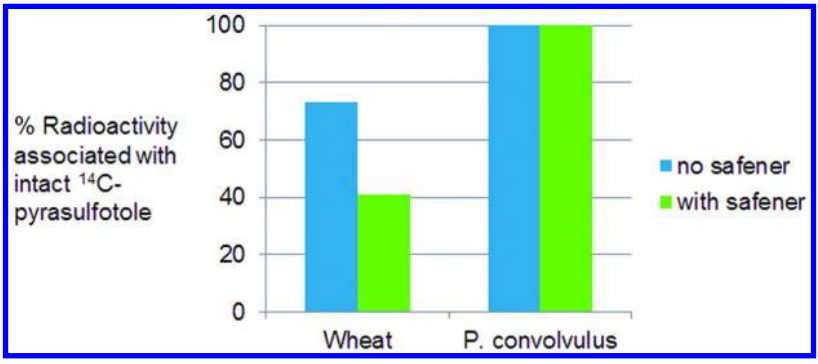

Figure 6. Effect of mefenpyr-diethyl on pyrasulfotole metabolism

Pyrasulfotole was launched in 2008 under the trade name Huskie® in the U.S.A. and as Infinity® in Canada. Complete crop tolerance is achieved by combining the herbicide with the safener mefenpyr-diethyl in a ratio of 4:1 by weight. In addition, the herbicide bromoxynil is added to the mixture in order to improve the weed control spectrum and for two other important reasons. Firstly, bromoxynil has a different mode of action to pyrasulfotole and using herbicidal mixtures with different modes of action is an effective way of decreasing the selection pressure for herbicide resistance and controlling its spread in the field. Secondly, mixtures of the two herbicides show significant synergistic effects which can be understood by examining their modes of action in more detail (*13*, *19*). Pyrasulfotole inhibits the HPPD enzyme which results in the inhibition of carotenoid and plastoquinone biosynthesis. Bromoxynil, on the other hand, directly competes with plastoquinone for binding to the photosystem II (PSII) complex. In other words, the concentration of plastoquinone in the plant will be lower following treatment with pyrasulfotole, which should in turn increase the efficacy of bromoxynil. This synergy is nicely illustrated in Figure 7 which shows the effects of herbicide applications to chickweed (*Stellaria media*). Whereas 18.8 g/ha of pyrasulfotole gives only 50% control and 240 g/ha of bromoxynil no control at all, a mixture containing 18.8 g/ha of pyrasulfotole and 105 g/ha of bromoxynil achieves close to 100% control (*19*). Similar synergistic effects between HPPD and PSII herbicides have been previously reported for other herbicides (*20*).

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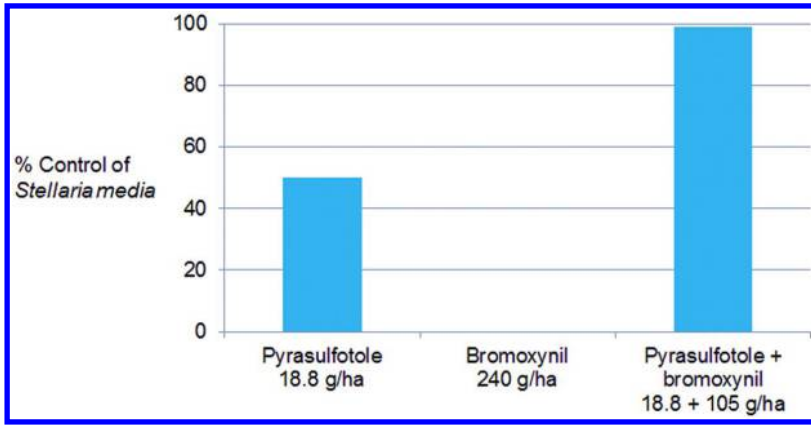

Figure 7. Synergic effects between pyrasulfotole and bromoxynil

As previously mentioned, pyrasulfotole exhibits excellent post-emergence control of resistant broadleaf weeds in cereals, including ACCase-, ALS- and glyphosate-resistant weeds. In particular, ALS inhibitors have been used for many years to control dicotyledonous weeds in cereals and substantial resistance has now developed in certain weed species. North American ALS-resistant populations of, for example, chickweed (*Stellaria media*), Kochia (*Kochia scoparia*), spiny sow thistle (*Sonchus asper*) and cleavers (*Galium spurium*) can all be effectively controlled using Infinity® (*19*), a mixture of pyrasulfotole (37 g/L) and bromoxynil (210 g/L; present as mixed octanoate and heptanoate esters). Figure 8 shows field trial results from North Dakota in which ALS resistant Kochia in wheat was effectively controlled using Infinity® (205 g/ha) (*21*).

Figure 8. Control of ALS resistant Kochia in N. Dakota. Left: Untreated; Right: Treated in strips with Infinity® (205 g/ha). Note the heavy weed pressure under dry conditions with light crop competition

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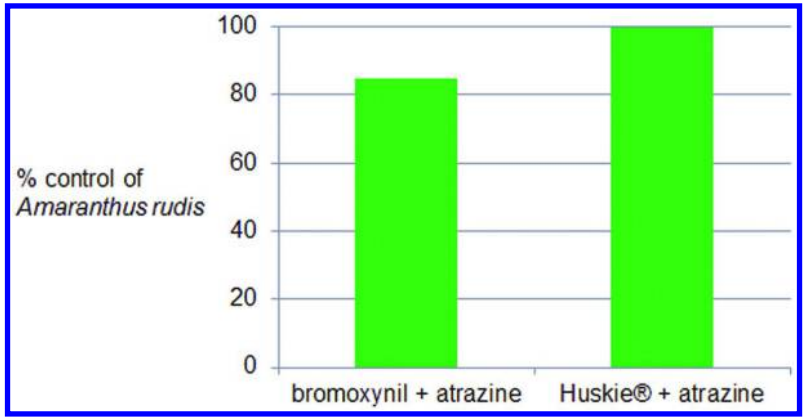

Figure 9. Control of ALS resistant common waterhemp (Amaranthus rudis) in Kansas. Application rates: Huskie® (235g/ha); bromoxynil (280 g/ha); atrazine (560 g/ha)

Figure 10. Control of ALS resistant Palmer amaranth in Kansas. Left: Untreated; Right: Two weeks after treatment with Huskie® (425 g/ha) and atrazine

No discussion of resistant weed problems in North America can avoid the tremendous challenges posed by the two Amaranths, common waterhemp (*Amaranthus rudis*) and Palmer amaranth (*Amaranthus palmeri*). The prolonged germination period, rapid growth and extremely high fecundity of these species mean that their complete control in crops is essential in order to stop fields being completely overgrown by these weeds. However, resistant biotypes of these two species are spreading throughout North America and threatening the sustainability of modern agricultural practices (*9*). For example, ALS and PSII resistant common waterhemp infests many grain sorghum fields throughout Kansas and neighboring states. Figure 9 shows that ALS resistant waterhemp can be completely controlled in grain sorghum by applying mixtures of Huskie® (235g/ha; mixture of pyrasulfotole and bromoxynil mixed octanoate and heptanoate esters) and atrazine (560 g/ha) (*22*). The 85% control shown by the bromoxynil plus atrazine combination may seem relatively good, but the huge numbers of seeds produced by even a few survivors means that as close to 100%

control as possible is essential in order to prevent the rapid establishment of resistant populations. Figure 10 shows field trial results from Kansas in which ALS resistant Palmer amaranth in grain sorghum was very effectively controlled using a mixture of Huskie® (425 g/ha) and atrazine (*23*).

Weed resistance to herbicides is increasing globally, with ever more weeds showing resistance to multiple modes of action (*11*, *24*). While the development of resistance cannot be completely prevented, it can be substantially delayed, provided that current agricultural practices are modified. It is important that herbicides are used at their full recommended use rates and that herbicide application is not delayed until weeds become too large for effective control. The use of herbicide mixtures with multiple modes of action and the rotation of herbicides with different modes of action both help reduce the selection pressure for resistance. There is a need to move away from an over-reliance on herbicides and to introduce more diversity into weed control practices. For example, crop rotation, the use of cover crops, mechanical weed control and post-harvest weed-seed destruction are all important resistance management measures which should be considered in order to ensure the sustainability of modern agriculture.

In summary, safener technology offers an alternative and complementary strategy to the herbicide tolerant (HT) crop approach for achieving herbicide crop selectivity. Pyrasulfotole is an inhibitor of the HPPD enzyme introduced by Bayer CropScience for post-emergence control of broad leaved weeds in cereals. It is sold as a mixture with the PSII inhibitor bromoxynil and the safener mefenpyr-diethyl under the trade names Huskie® and Infinity.® This introduction was the first time that HPPD chemistry could be used in cereals and represented the first new mode of action for broadleaf weed control in cereals for over 20 years. Subsequently the usage of pyrasulfotole has been extended to include grain sorghum. The product, particularly when applied in mixtures, enables the effective control of ALS-, ACCase- and glyphosate-resistant weeds, including Amaranths, providing farmers with a valuable new option for weed resistance management.

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Chapter 17

Indaziflam: An Innovative Broad Spectrum Herbicide

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Indaziflam is the innovative active ingredient in the herbicides Specticle™ and Alion™ (first registrations in 2010/2011), which were followed by other brands. This new compound from Bayer CropScience belongs to the alkylazine chemical class and inhibits cellulose biosynthesis in plants. It is effective against a very wide range of weeds and offers excellent long-term results at very low dose rates. The discovery process and the optimization of the alkylazine class which led to *indaziflam*, including biological structure activity relationship (SAR) profiles, are presented in this chapter.

Historical Background

At the end of the nineties Idemitsu Kosan launched *triaziflam* (Figure 1), which was the first alkylazine herbicide (*1*). It is used for weed control in turf and shows a high level of herbicidal activity, with particularly efficient broadleaf weed control.

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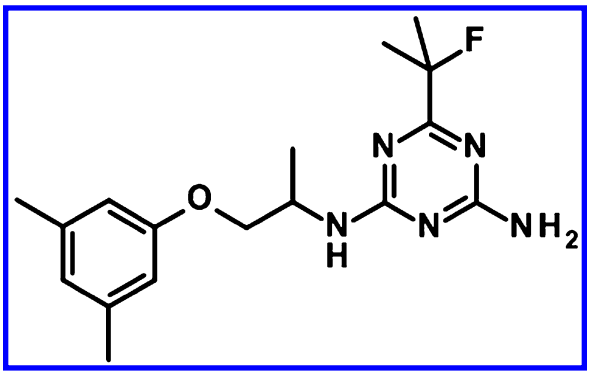

Figure 1. Triaziflam

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Interestingly the mode of action involves not only the inhibition of the photosystem II electron transport, but also the inhibition of cellulose biosynthesis. This second novel mode of action, combined with the high level of weed control, raised the question as to whether it might be possible to further improve the herbicidal profile, based solely upon inhibition of cellulose biosynthesis. The introduction of a novel mode of action is a more and more pressing need in order to control and eradicate resistant weeds in agricultural plantations.

First Optimization of Herbicidal Activity

In order to try and optimize the herbicidal activity, several sites in the *triaziflam* molecule were structurally varied (e. g. molecules with different chain lengths between the aryl and the triazine moieties; substitution of a carbon atom in this chain with a heteroatom; introduction of substituents into the aryl ring and/or on the aliphatic side). Unfortunately biological results from greenhouse tests were not encouraging as the level of herbicidal activity was at best equal and often lower than that of *triaziflam*. However, the introduction of bicyclic systems boosted the herbicidal activity significantly and with the indanyl ring system a new peak of weed control was achieved (Figure 2).

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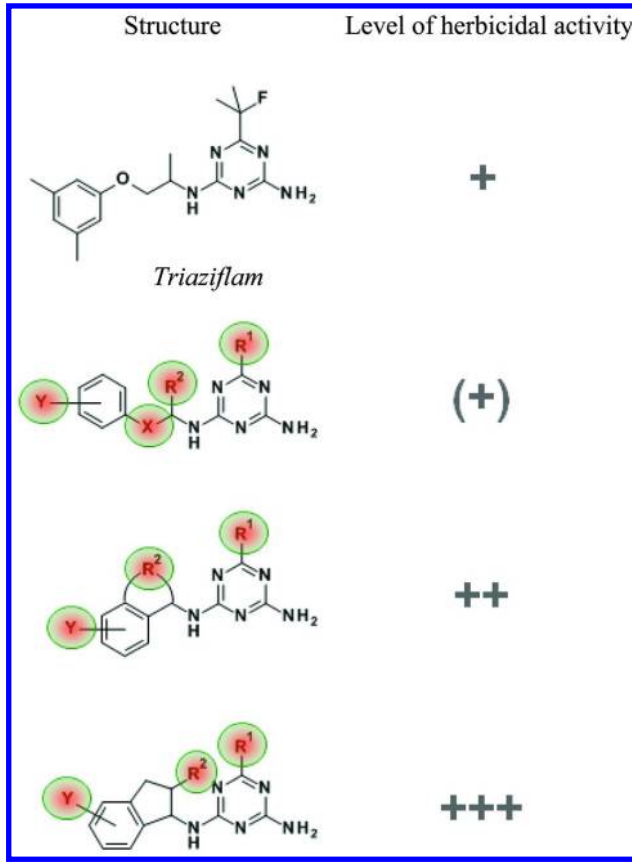

Figure 2. Structure variations at positions R^1, R^2, X and Y

Chemical Synthesis I

The general synthetic access to indanylamino triazines comprised a reductive amination of the indanone followed by conversion of the indanylamine to the target product (Figure 3).

Figure 3. General synthetic access to indanylamino triazines

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The key question for the design of synthesis programs was how to most efficiently access highly diverse indanones with different aromatic substitution patterns (Figure 4).

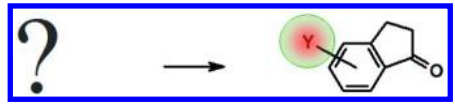

Figure 4. Key question for the design of synthesis programs

A one-step approach, based on well known Friedel Crafts chemistry, afforded a broad range of indanones based on readily available starting materials (Figure 5).

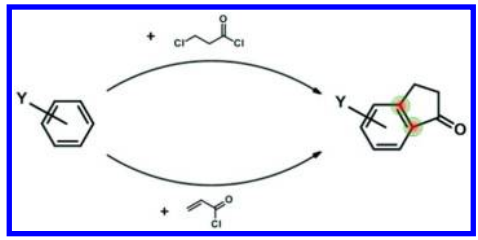

Figure 5. One-step approach to indanones

This route provided a very efficient access to screening compounds and enabled detailed structure activity relationships to be established. Whereas the formation of regioisomers contributed towards obtaining a comprehensive SAR picture, their separation was often difficult. Thus, this synthesis strategy was less appropriate for larger amounts.

Depending on the targeted substitution pattern, alternative approaches not involving the formation and laborious separation of regioisomers were investigated. For example the 1,2- and 1,4-addition of aryl nucleophiles to α,β-unsaturated systems, followed by acid-catalyzed ring closure, proved to be an efficient approach to indanones with substitution in the 4- or 7-position (Figure 6).

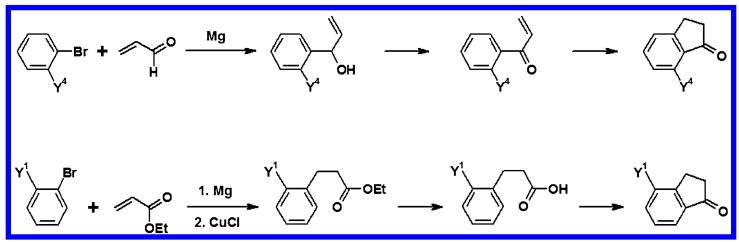

Figure 6. Alternative approaches to indanones

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For the second step, i. e. the reductive amination, several methods were tested. The cyanoborohydride based approach proved to be very sluggish but, after prolonged reaction times, it afforded pure product. The reductive cleavage of oximes with sodium in isopropanol was only appropriate if halogens were absent. An alternative approach was the iron-mediated reduction of oximes followed by formylation of the corresponding enamine. Subsequent hydrogenation, followed by acidic deformylation, yielded the desired indanylamine (Figure 7).

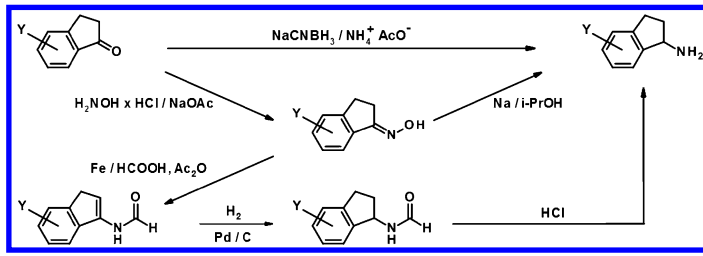

Figure 7. Reductive amination

The final step was the installation of the triazine ring. Basically there were two routes. One method was the reaction of the indanylamine with a chlorotriazine. The other, was a two-step approach involving a reaction of the indanylamine with cyanoguanidine and subsequent ring closure of the biguanidine to the triazine (Figure 8).

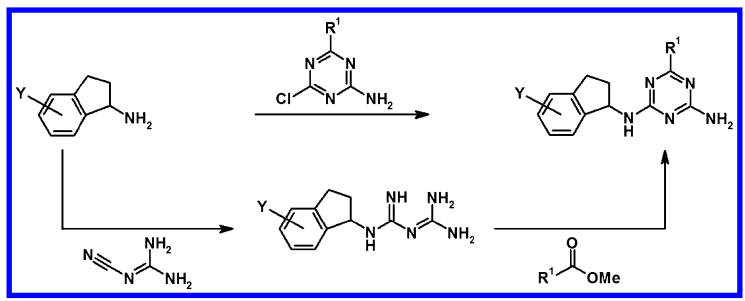

Figure 8. Installation of the triazine ring

As an example Figure 9 shows the synthesis of a 5-fluoro-6-methylindanylaminotriazine. The sequence started with a Friedel Crafts type alkylation. In this case a second step is necessary to achieve ring closure and this delivers two isomers. After their separation, the 5-fluoro-6-methylindanone is treated with sodium cyano borohydride and ammonium acetate in methanol to achieve a reductive amination. Following condensation with a chloro triazine the desired product is isolated.

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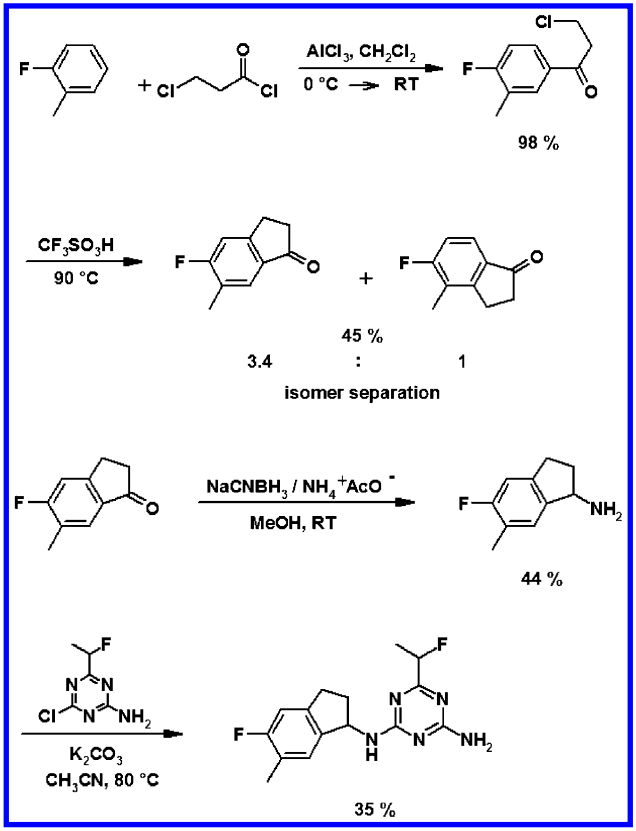

Figure 9. Preparation of 5-fluoro-6-methylindanylaminotriazine

Due to their strong herbicidal activity in the greenhouse, the 2-methyl indanyl derivatives were also synthesized (Figure 10). This indanone was prepared in a one-step manner by conversion of ortho-fluoro toluene with methacrylic acid chloride. Again, two isomers were isolated. Following isomer separation, reductive amination and reaction with the chlorotriazine, the 5-fluoro-2,6-dimethylindanylaminotriazine could be isolated.

As these compounds were only prepared for screening tests the yields in Figures 9 and 10 are not optimized.

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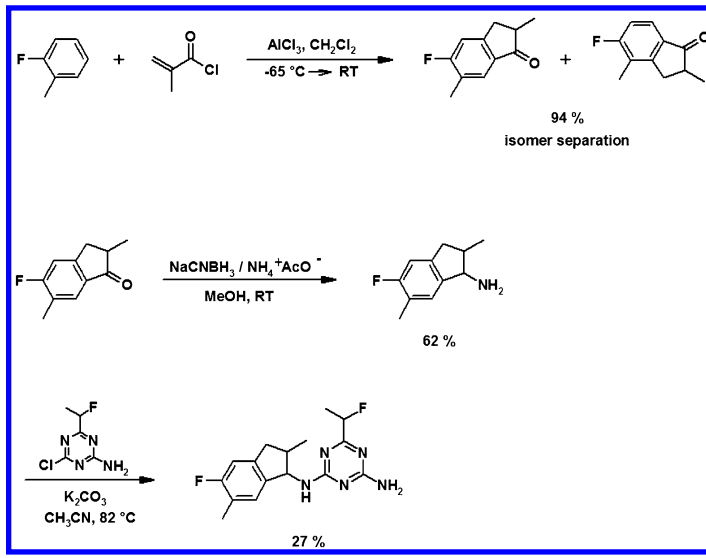

Figure 10. Preparation of 5-fluoro-2,6-dimethylindanylaminotriazine

Structure Activity Relationships

Using this "toolbox" of synthetic methods a broad range of indanylaminotriazines were prepared and tested for herbicidal activity, establishing a detailed picture of structure activity relationships (Figure 11).

On the triazine ring, a free amino group and a haloalkyl group, notably fluoro alkyl groups, proved to be the most appropriate substituents. The strongest bicyclic substituent was the indanyl system. Within this group the introduction of a methyl group into the 2-position often further increased the herbicidal potency. Numerous aromatic substitution patterns lead to attractive compounds, particularly those with a methyl group or a halogen atom, almost independent of position and number of substituents. Eventually, fine tuning led to the 2,6-dimethylindanylamino backbone. Combination with an aminotriazine bearing a 1-fluoroethyl group afforded the compound with the highest level of weed control (Figure 12).

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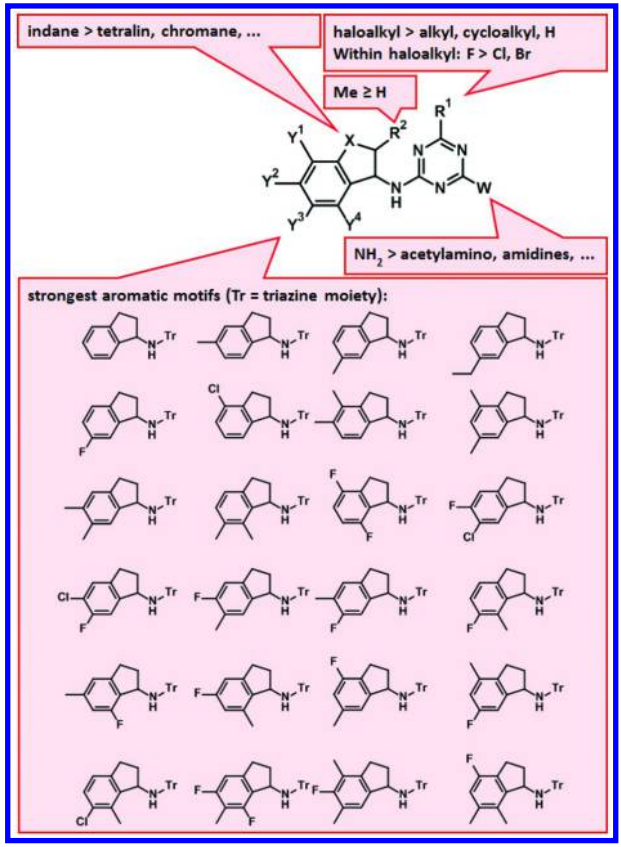

Figure 11. Structure activity relationships

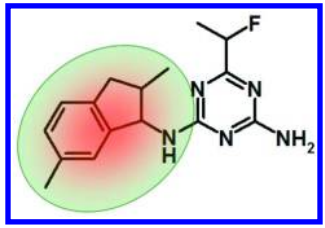

Figure 12. 2,6-Dimethylindanylamino backbone

However, this compound still showed two modes of action, i. e. inhibition of cellulose biosynthesis and inhibition of the photosystem II electron transport. As the compound contains three chiral centers and thus consists of a mixture of eight stereoisomers, this raised the question as to what influence the configuration of the chiral centers might have upon the mode of action.

Investigation of the Influence of the Chiral Centers

To clarify the influence of chirality on activity, all eight stereoisomers (Figure 13) were separated and tested for both their herbicidal activity and their mode of action.

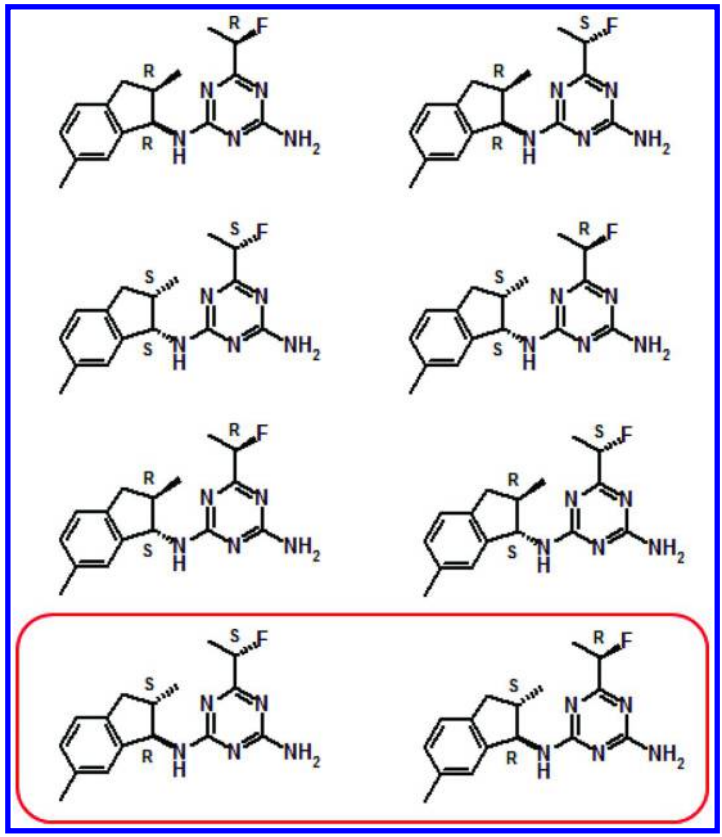

Figure 13. The eight stereoisomers

The result was that the configuration of the indanyl backbone is decisive for herbicidal activity and for the mode of action. The (*1R*,*2S*)-indanyl backbone proved to be biologically the most potent, combined with a clear shift in the mode of action away from the inhibition of the photosystem II electron transport towards the inhibition of cellulose biosynthesis. The corresponding two stereoisomers showed the highest level of weed control and are highly effective inhibitors of cellulose biosynthesis – they are the constituents of *indaziflam* (Figure 13, box).

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The 2,6-dimethylindanone is easily accessible via a sequence consisting of benzylation of diethyl methylmalonate, followed by ester saponification with decarboxylation and finally acid catalysed ring closure (Figure 14) (*2*).

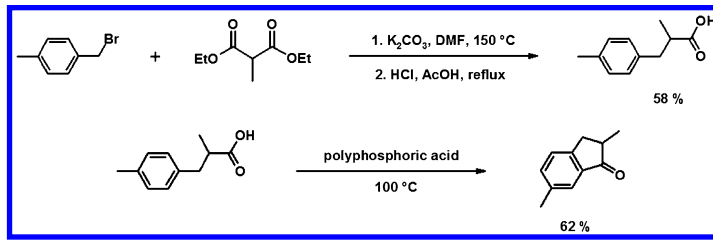

Figure 14. Synthesis of 2,6-dimethylindanone

The enantiopure indanylamine can be prepared via a reductive amination, followed by separation of stereoisomers (Figure 15) (*3*).

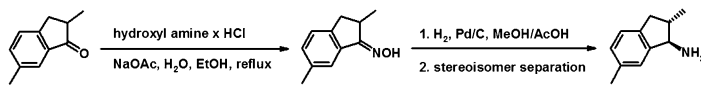

Figure 15. Preparation of the (1R,2S)-indanylamine

This method is suitable for comparative testing of stereoisomers but is not economically viable due to the loss of unwanted stereoisomers. Thus there was a strong need for an enantioselective access to the (*1R*,*2S*)-indanylamine. One appropriate method was established in a cooperation with J. M. Lassaletta, and involves a novel dynamic kinetic resolution followed by a nucleophilic substitution (Figure 16) (*4*).

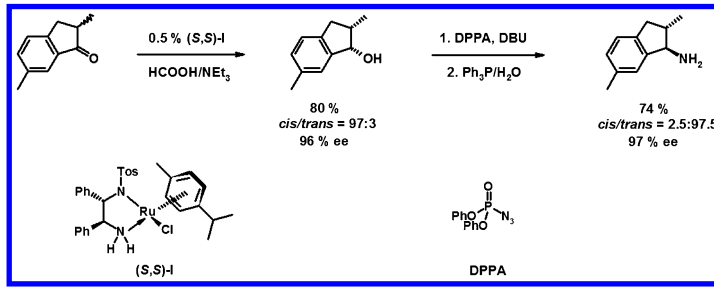

Figure 16. Dynamic kinetic resolution, followed by installation of the amino group

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In the first step the indanone is hydrogenated to the corresponding indanol, mediated by the ruthenium based Noyori/Ikariya type catalyst. The approach of the reductant occurs from the sterically less hindered side, i. e. opposite to the methyl group, leading to the cis-configured indanol. With the chiral induction of the ruthenium catalyst only the (*2S*)-indanone is reduced, leaving the (*2R*)-indanone. However, the (*2R*)-isomer undergoes equilibration under the reaction conditions to give (*2R/S*)-indanone. Therefore, the racemic indanone is transformed completely into the (*1S,2S*)-indanol with a high diastereo- and enantioselectivity. In a second step the hydroxy group is replaced by an azide group (via conversion with diphenylphosphorylazide) with strict inversion of configuration. This is followed by a Staudinger-type reduction with triphenyl phosphine to yield the desired (*1R,2S*)-indanylamine.

For completion of the *indaziflam* synthesis, the (*1R,2S*)-indanylamine was converted with cyano guanidine to the corresponding biguanidine. However, the biguanidine synthesis based on literature precedent had several problems and was technically unacceptable. High temperatures of at least 140 °C were needed, which caused several side reactions. The addition of aluminium alkoxide simplified the biguanidine synthesis significantly (Figure 17) (*5*).

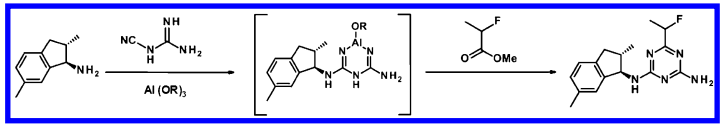

Figure 17. Biguanidine synthesis and conversion to indaziflam

The coordination of this Lewis acid with the cyanoguanidine activates the nitrile group towards nucleophilic attack by the indanylamine. The resulting biguanidine is stabilised via an aluminium chelate. Consequently, a much lower reaction temperature is needed and the product is formed with a good yield and in high purity. The aluminium atom is released smoothly via ring closure of the biguanidine with 2-fluoro propionic acid ester. The fluorinated ester is generated from lactic acid in an enantiomeric ratio of approx. 95 : 5, in favor of the (*2R*)-fluoro propionic acid ester (*6*). As this stereoconfiguration is stable under the reaction conditions, this ratio is reflected in the ratio of the two stereoisomers of *indaziflam*.

Herbicidal Profile and Application

In numerous field trials *indaziflam* has shown broad weed control of both grasses and broadleaf weeds, in most cases achieved with low application rates of about 50 – 75 g/ha (Figure 18).

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Figure 18. Pome fruit, untreated versus application of 75 g/ha indaziflam

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With the novel mode of action, i. e. the inhibition of cellulose biosynthesis, *indaziflam* is an effective tool to manage weed populations that are resistant to other herbicidal modes of action. So far there is no evidence for any cross-resistance. The residual pre-emergence weed control lasts for several months and consequently the number of applications can be reduced, thereby saving time, fuel and labor costs.

Indaziflam is used for weed control in established permanent crops such as citrus, grapes, fruit trees, tree nuts, industrial plantations, and for use in perennial sugarcane, lawns, golf course, turf farms, recreational turf, ornamentals, non-crop areas, Christmas tree farms and forested areas.

Summary

Indaziflam proved to be the strongest alkylazine herbicide and was discovered following an extensive program of optimisation. The optimisation of the herbicidal profile also resulted in a highly effective inhibitor of cellulose biosynthesis. An efficient technical synthesis was realised via a novel biguanidine intermediate. *Indaziflam* from Bayer CropScience represents a breakthrough for broad spectrum residual weed control. It is an innovative tool for effective resistance management. *Indaziflam* is the active ingredient in brands like Specticle®, Alion®, Esplanade® and DuraZone®.

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Chapter 18

Discovery and SAR of Halauxifen Methyl: A Novel Auxin Herbicide

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The most recent discovery in the area of auxin herbicides was the identification of novel 6-aryl-picolinate herbicides that exhibit post-emergent control of key dicot weeds at low application rates. Research of the SAR associated with this compound class led to the identification and field testing of a highly active analog. While the herbicidal activity of this analog was compelling, the soil half-life was too long to support advancement toward registration. To address this long soil half-life, additional research focused on the incorporation of a methoxy group on the 6-phenyl tail. A novel 6-aryl-picolinate analog was subsequently discovered that exhibited potent dicot weed control and a soil half-life between 10-25 days. This compound advanced into the development process and received the common name of halauxifen methyl. This report describes the discovery process that led from the first picolinate herbicide picloram to halauxifen methyl.

Introduction

The first organic compounds used to selectively control dicot weeds in grass crops were auxin herbicides. Auxin herbicides derive their name and classification from the fact that they cause effects similar to those caused by

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indole-3-acetic acid, a natural plant hormone of the auxin class (*1*). Auxin herbicides cause physiological changes in plants that result in phenotypic effects such as tissue swelling, root growth inhibition, epinasty and plant death. The first commercial auxin herbicides were 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) (Figure 1) which were both discovered in the early 1940s (*2*). These aryloxyacetate herbicides are inexpensive and are highly effective at use rates between 125-4800 g ha^{-1} (*3*). These factors result in large yearly sales volumes for 2,4-D and MCPA (84,085 and 16,500 MT respectively for the year 2012) (*4*).

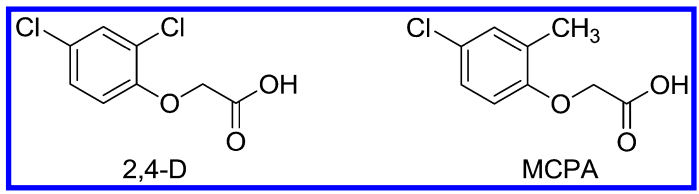

Figure 1. First commercial auxin herbicides.

In the late 1950s, a new auxin herbicide class was unexpectedly discovered at The Dow Chemical Company (Dow) by scientists researching 2-trichloromethylpyridine nitrification inhibitors exemplified by nitrapyrin (Figure 2). Nitrification inhibitors reduce nitrogen loss in the soil by inhibiting the microbe-catalyzed production of nitrate ions from ammonium ions. Field studies assessing various 2-trichloromethylpyridine analogs showed unexpected crop injury.

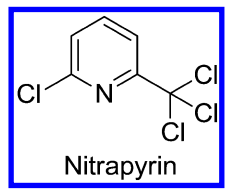

Figure 2. Commercial nitrification inhibitor nitrapyrin.

Analysis of the soil samples from the field trials showed that some of the nitrapyrin analogs tested as nitrification inhibitors were being converted in the soil to new compounds. Unique picolinic acid molecules were identified in the soil samples where the experimental nitrification inhibitors were applied. 4-Amino-3,5,6-trichloropicolinic acid was one of the picolinic acid molecules identified in the soil samples. Researchers at The Dow Chemical Company synthesized this molecule and assessed it for herbicidal utility. It was later named picloram (Figure 3) and first introduced as a commercial herbicide in 1963 (*5*). The commercialization of picloram was followed by clopyralid in the 1970s and aminopyralid in 2006 (Figure 3).

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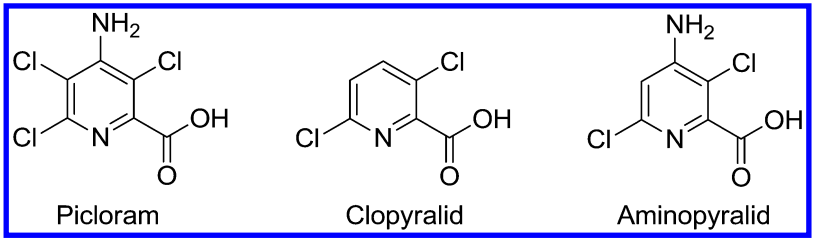

Figure 3. Commercial picolinic acid herbicides.

In the years following the discovery of aminopyralid, picolinate analogs stemming from aminopyralid were synthesized and found to exhibit even more potent herbicidal activity toward a large number of key dicot weed species. One of the most active series of picolinate herbicides produced to date is the 6-aryl-picolinates (6-AP) shown in Figure 4. This report provides a short description of the innovative steps that led to the discovery of the 6-AP herbicides and ultimately to halauxifen methyl (Figure 4).

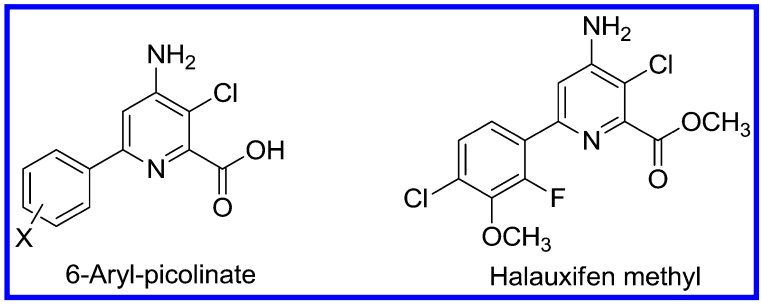

Figure 4. Synthetic manipulation following the discovery of aminopyralid led to the 6-aryl-picolinate herbicides including halauxifen methyl.

History of Picolinate Herbicide Discovery

Field studies were conducted with experimental nitrification inhibitors including nitrapyrin and **1A** (Figure 5). Nitrapyrin eventually became a commercial nitrification inhibitor that is an important agricultural chemical tool to this day; however, a high degree of phytotoxicity was observed in test plots sprayed with **1A**. It was subsequently determined that **1A** reacted with ammonia applied as fertilizer to produce **1B** (Figure 5), and that the trichloromethyl group of compound **1B** was converted to a carboxylic acid by soil microbes to generate a picolinate-based herbicide that was ultimately named picloram (*6*). Furthermore, the herbicidal symptoms were consistent with an auxinic mode of action. Eventually, this serendipitous discovery resulted in the commercial launch of a novel auxin herbicide in 1963. Picloram is applied at rates between 125 and 1120 g ha^{-1} (*3*). In 2012, the picloram sales volume was 2,737 MT (*4*).

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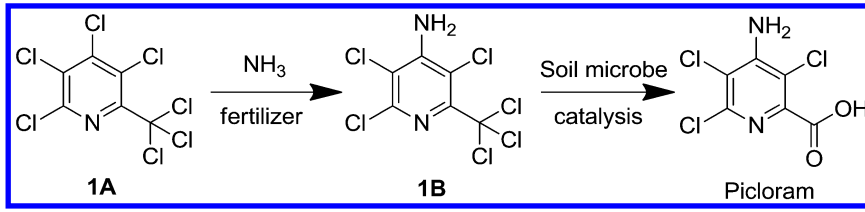

*Figure 5. Production of picloram from 2,3,4,6-tetrachloro-6-(trichloromethyl)pyridine **1A** via 2,3,5-tetrachloro-6-(trichloromethyl)pyridin-4-amine **1B** in soil.*

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Clopyralid (Figure 3) is another picolinate auxin herbicide closely related to picloram. While it was discovered in the same timeframe (the early 1960s) as picloram, clopyralid was not launched as a commercial herbicide until 1975. The narrow herbicidal spectrum of weed control provided by clopyralid was probably a contributing factor in this delayed launch, but the relatively low cost of manufacture and superior control of a small number of key dicot weed species (Canada thistle for example) eventually resulted in the commercial launch of this herbicide. Clopyralid is typically applied at rates between 105-500 g ha^{-1} (*3*). In 2012, the clopyralid sales volume was 1,811 MT (*4*). Currently, the world's supply of clopyralid is generated by four different commercial synthesis routes. One of these synthesis routes includes an electrochemical reduction as the final step of the synthesis route to remove two chlorine atoms attached to the pyridine ring of 3,4,5,6-tetrachloropicolinic acid **1C** (Figure 6).

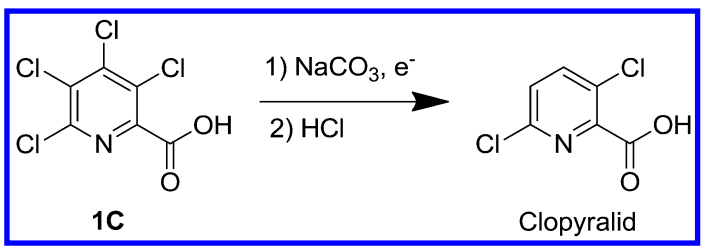

*Figure 6. Electrochemical production of clopyralid from 3,4,5,6-tetrachloropicolinic acid **1C**.*

In 1998, chemists and chemical engineers at Dow were developing the large scale electrochemical production of clopyralid from 3,4,5,6-tetrachloropicolinic acid **1C** (Figure 6). As a part of this process, they envisioned a hypothetical scenario in which picloram might be subjected to the electrolysis reaction instead of the intended tetrachloropicolinic acid **1C**. In small-scale electrochemical experiments designed to probe this hypothetical scenario, a high percentage of 4-amino-3,6-dichloropicolinic acid (later named aminopyralid) was produced from picloram (Figure 7) (*7*).

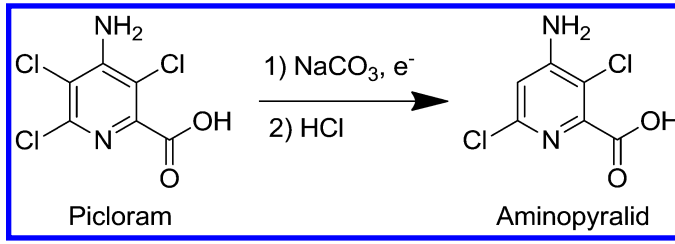

Figure 7. Electrochemical production of aminopyralid from picloram.

Aminopyralid was found to exhibit significantly greater herbicidal activity toward many key dicot weeds than picloram. Greenhouse trials demonstrated that aminopyralid controlled many dicot weed species at rates 2-10 times lower than the rates of picloram required to obtain the same level of control (*8*). Aminopyralid was also found to be 4 times more active than clopyralid on Canada thistle, one of the few key weed species completely controlled by commercial formulations of clopyralid (*9*). The end result of this second serendipitous synthesis of a picolinate-based herbicide was the commercial launch of aminopyralid in 2006. Aminopyralid is typically applied at rates between 5 and 120 gha^{-1} (*3*). In 2012, the aminopyralid sales volume was 250 MT (*4*).

Herbicidal Utility of 6-Aryl-picolinates

Expanding the Picolinate SAR

Synthesis to determine structure activity relationship (SAR) patterns of diverse analogs of aminopyralid led to the discovery of a number of 6-AP analogs through the general synthetic routes depicted in Scheme 1.

Reaction of intermediate **2B** (see US patent 6,297,197 B1 for preparation of this key intermediate) (*10*) with various boronic acids **2A** (purchased or synthesized by routes reported in the chemical literature) yielded N-acyl-6-AP methyl esters **2C**. Alternatively, reaction of **2E** (see US patent 6,784,137 for preparation of this key intermediate) (*11*) with commercially available bromobenzenes **2D** produced N-acyl-6-AP methyl esters **2C**. Hydrolysis of the **2C** compounds in acidic methanol provided the final 6-AP methyl esters **2F**. Finally, other 6-AP methyl esters were synthesized directly by the reaction of aminopyralid (see US patent 6,297,197 B1 for preparation of this key intermediate) (*9*) with various boronic acids **2A** (purchased or synthesized by routes in the chemical literature).

The replacement of the chlorine at the 6-position of aminopyralid with a phenyl ring provides many options for new, diverse picolinate analogs. The number of substituents and substituent combinations is so large that only a systematic analysis of the herbicidal activity associated with these combinations allowed for synthetic prioritization. Initial synthesis focused on various mono- and di-substituted phenyl analogs.

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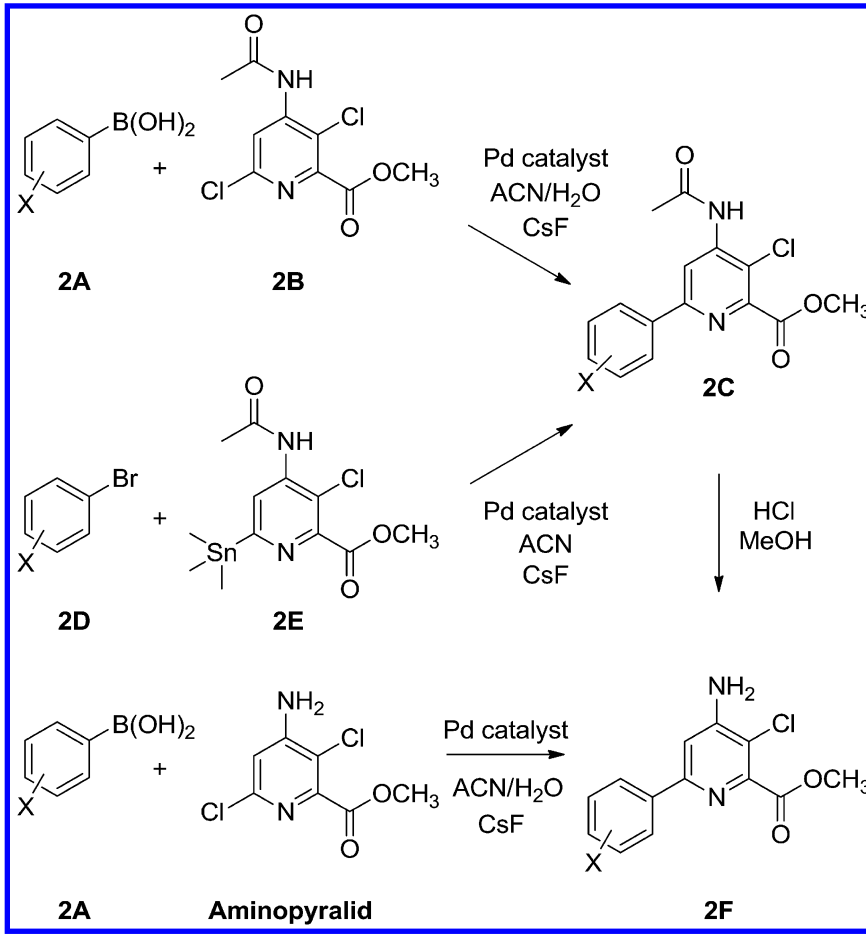

Scheme 1. General synthetic routes used to produce 6-Aryl-picolinate herbicides.

Herbicidal Assessment of Key 6-AP Analogs

Herbicidal activity was assessed in foliar applications on nine dicot weed species (Table 1) representing diverse species encountered in various crops and global cropping systems. Plants were incubated in a greenhouse and evaluated for percent visual injury 14 days after application.

The quantitative data comparison of analogs that differ in herbicidal potency and species specificity can pose a challenge. For ease of comparison, the cumulative visual herbicidal injury of the nine dicot weed species at an application rate of 17.5 grams per hectare (g ha^{-1}) were tabulated and compared graphically. For these graphical comparisons, the highest score possible score is 900 and requires 100% control of all nine dicot weed species shown in Table 1.

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Table 1. Dicot weed species evaluated for herbicidal activity in foliar applications and data was used to provide direction in SAR development.

Scientific Name	*Common Name*	*Bayer Code*
Chenpodium album	Lambsquarter	CHEAL
Ipomea hederacea	Ivyleaf Morningglory	IPOHE
Amaranthus retroflexus	Redroot Pigweed	AMARE
Abutilon theophrasti	Velvetleaf	ABUTH
Viola tricolor	Viola	VIOTR
Polygonum convolvulus	Wild Buckwheat	POLCO
Euphorbia heterophylla	Wild Poinsettia	EPHHL
Cirsium arvense	Canada Thistle	CIRAR
Helianthus annuus	Sunflower	HELAN

The unsubstituted phenyl 6-AP analog **3** and the mono-chlorophenyl analogs **4**, **5**, and **6**, shown in Figure 8, were synthesized and tested in the early stages of the 6-AP SAR development. These analogs were targeted to probe the steric and electronic influence of the addition of a single chlorine atom on the overall herbicidal activity.

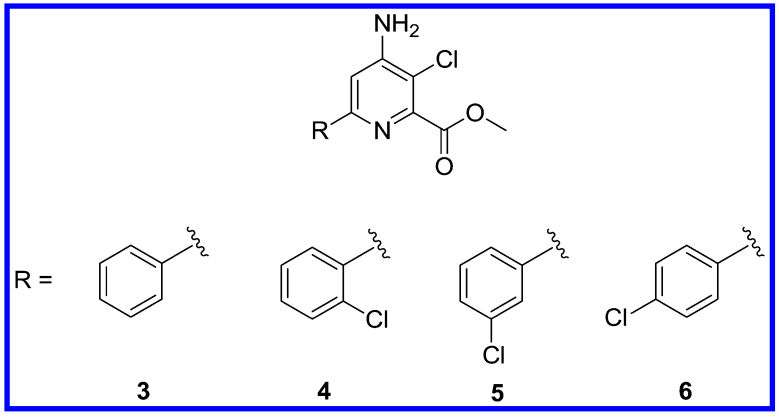

Figure 8. Initial research focused on understanding the herbicidal activity of key mono-substituted 6-AP analogs.

The bar graph in Figure 9 shows that all three of the mono-chlorophenyl 6-AP analogs provided greater cumulative herbicidal activity than the unsubstituted phenyl analog **3**. The 2′-chlorophenyl **4** and 3′-chlorophenyl **5** analogs provided only incrementally higher herbicidal activity than the unsubstituted phenyl analog. However, the 4′-chlorophenyl analog **6** provided considerably greater herbicidal activity than the unsubstituted phenyl analog and the other two monochlorophenyl analogs. The sum of the % herbicidal activity of **6** on the nine dicot weed species reached 700 out of a possible 900 at the application rate of 17.5 g ha^{-1} (Figure 9).

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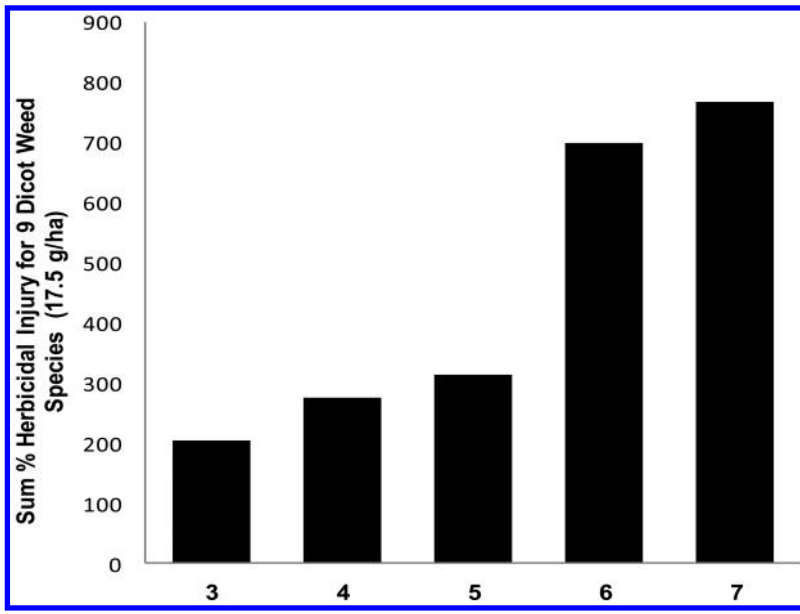

*Figure 9. Comparison of the cumulative herbicidal activity of the unsubstituted phenyl analog (**3**), the monochlorophenyl analogs (**4**, **5**, **6**) and the 2′-fluoro-4′-chlorophenyl analog (**7**) on nine dicot weed species (Maximum score = 900).*

The herbicidal activity of **6** prompted a synthetic effort to understand the SAR of 4′-chlorophenyl analogs. A number of di-substituted phenyl analogs were synthesized and evaluated. The addition of a fluorine in the 2′-position of the 4′-chlorophenyl ring led to **7** (Figure 10). Compound **7** provided consistently higher herbicidal activity than **6** on the nine dicot weeds at an application rate of 17.5 g ha^{-1} (Figure 9). The sum of the % herbicidal activity of **7** on the nine dicot weed species reached nearly 800 out of a possible 900.

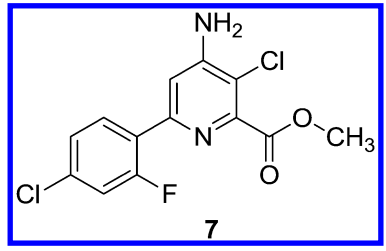

*Figure 10. The SAR development associated with 6 led to the identification of methyl 4-amino-3-chloro-6-(4′-chloro-2′-fluorophenyl)picolinate (**7**).*

The herbicidal activity of **7** observed in greenhouse trials led to evaluations of this compound in the field. In parallel with the field trials, early stage environmental degradation studies were completed. While field trials showed potential utility of **7** to control a broad spectrum of dicot weeds, the aerobic soil studies of **7** identified slow degradation (soil half-life for **7** was greater than 180 days) as a significant problem. The longevity of **7** in soil limited the potential commercial utility of this compound in certain herbicide markets and development on this analog was therefore suspended.

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Discovery of Halauxifen Methyl

The compelling herbicidal activity of **7** provided the impetus for a more direct approach of identifying an analog that retained the herbicidal activity of **7** with potential for more rapid degradation in aerobic soil. While SAR development around analog **7** may have eventually led to the discovery of a new compound with similar herbicidal activity and lower stability in the soil, this process could have involved the synthesis of thousands of compounds over multiple years. The more direct approach that was pursued comprised the introduction of chemical functionality to 6-AP analogs that may allow for microbial metabolism leading to more rapid soil degradation.

The introduction of chemical functionality into herbicidal compounds that increase soil degradation has occurred either through SAR synthesis or specific design. Aichele and Penner (*12*) showed that at neutral pH, the herbicide imazamox degraded in soil significantly faster than the related herbicides imazethapyr and imazaquin. Although minimal analysis of degradates was presented, the methoxymethyl substitution on imazamox was a probable key structural feature affecting soil degradation. It is important to note that when the soil degradation of the herbicides imazapyr and imazapic were compared only minor differences were observed (*13*). This suggests that the methoxy substitution on the methyl group of the pyridine ring of imazamox allows for more rapid soil degradation than occurs with the methyl substitution on imazapic. The data in Table 2 shows that the addition of the methoxy substitution added to the methylpyridine ring of imazapic to form imazamox lowers the soil half-life by 4 – 6 fold. The soil half-life for imazethapyr with an ethyl group in the same position was only 1.5 – 2 fold lower than imazapic suggesting that the soil degradation differences are not due to spatial or steric properties of the pyridine ring systems.

Application of this approach to the 6-AP herbicide class led to the design and synthesis of some specific compounds with methoxy substituent on the phenyl ring. The goal was to incorporate a methoxy group on the phenyl ring to provide the potential for increased environmental dissipation without reducing the high level of herbicidal activity associated with **6** and **7**.

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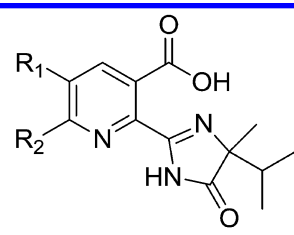
Table 2. Soil half-life values of Imidazolinone herbicides.



Herbicide	R_1	R_2	Soil Half-life[3] (days)
Imazapyr	H	H	25-142
Imazapic	CH_3	H	120
Imazethapyr	CH_3CH_2	H	60-90
Imazaquin	-CH=CH-CH=CH-		60
Imazamox	CH_3OCH_2	H	20-30

Accordingly, compound **8** (Figure 11) was synthesized to assess the effect of adding a methoxy substitution to the 3′-position of the phenyl ring independent of other phenyl substitution. The herbicidal activity of **8** on the nine dicot weeds at 17.5 g ha^{-1} was moderate (Figure 12) but surprisingly higher than the activity noted with the 3-chlorophenyl analog **5** (Figure 8). The higher activity of **8** relative to **5** is an indication that the methoxy group at the 3′-position of the phenyl ring may be involved in a specific molecular recognition within susceptible plants. This specific hypothesis led to the synthesis of the 4′-chloro-3′-methoxyphenyl analog **9** and the 4′-chloro-2′-fluoro-3′-methoxyphenyl analog **10** (Figure 11).

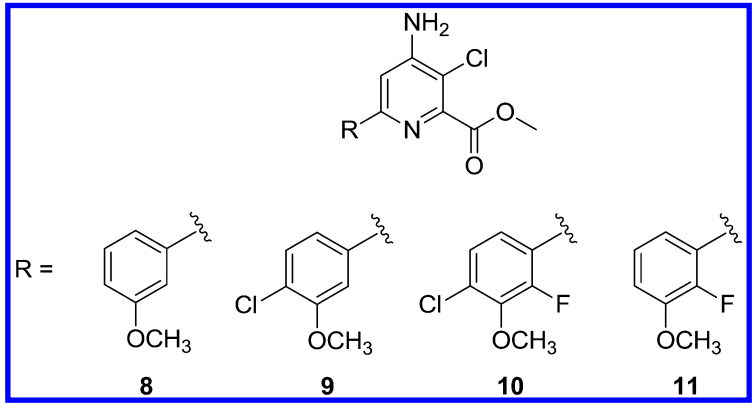


Figure 11. A methoxy group was incorporated into multiple key 6-AP phenyl tail analogs.

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The cumulative herbicidal activity of **9** was found to be considerably higher than the herbicidal activity identified for **8** (Figure 12). In fact, the herbicidal activity of **9** was similar to that of the 4′-chlorophenyl analog **6** (Figure 8). The addition of the fluorine at the 2′-position of **9** to produce the 4′-chloro-2′-fluoro-3′-methoxyphenyl analog **10** provided another increase in herbicidal activity (Figure 12). The difference in herbicidal activity between **9** and **10** was similar to the difference in herbicidal activity between the corresponding *des*-methoxy analogs **6** and **7** (Figure 11). The 2′-fluoro-3′-methoxy phenyl analog **11** (Figure 11) was synthesized and evaluated to assess the importance of combining all three substituents on the phenyl ring. The herbicidal activity associated with **11** (Figure 12) was slightly lower than the herbicidal activity of the unsubstitued phenyl analog **3** (Figure 8). The low herbicidal activity of **11** demonstrated the necessity of combinig all three substituents on the phenyl ring of **10** to achieve a high level of herbicidal activity.

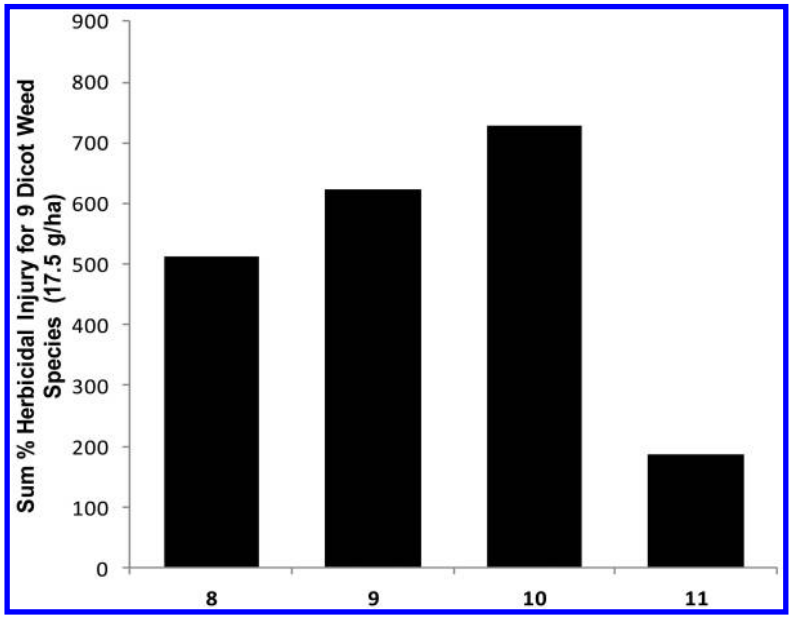

*Figure 12. Comparison of the cumulative herbicidal activity of the 3′-methoxyphenyl analog (**8**), the 4′-chloro-3′-methoxyphenyl analog (**9**), the 4′-chloro-2′-fluoro-3′-methoxyphenyl analog (**10**) and the 2′-fluoro-3′-methoxyphenyl analog (**11**) on nine dicot weed species (Maximum score = 900).*

The weed control associated with **10** led to additional research to analyze the soil stability of **10** in multiple soil types. Aerobic soil degradation studies showed that **10** was degraded fairly rapidly with a soil half-life range of 10 – 25 days (depending on soil type). Analytical analysis demonstrated that compound **10** was initially de-esterified and then de-methylated to the hydroxy acid analog

12. Further degradation in the soil leads to carbon dioxide and non-extractable residues (Figure 13) (*14*).

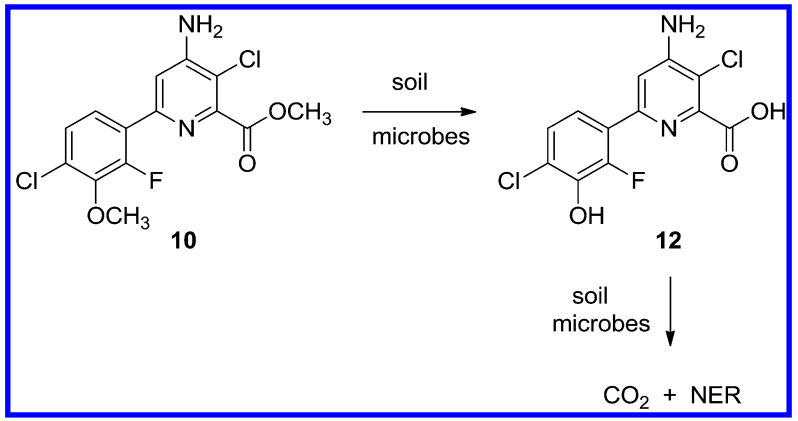

*Figure 13. Compound **10** is metabolized under aerobic conditions by soil microbes to the hydroxy acid **12** and then further to CO_2 and non-extractable soil residues (NER).*

The broadspectrum herbicidal activity of **10** in greenhouse trials coupled with a favorable soil degradation profile catalyzed field research to define the commercial utility in the global herbicide market. Multiple years of field trials in many countries demonstrated the ability of **10** to control economically important dicot weed species in a variety of wheat and barley cropping systems. The common name of halauxifen methyl has been approved for **10** by the ISO (International Organization of Standards). Commercialization of halauxifen methyl will occur in multiple herbicide formulations under the Dow trademarked name Arylex.

Conclusions

The discovery of the commercial herbicidal aminopyralid in 1998 catalyzed synthesis efforts to identify novel picolinate herbicides and led to the discovery of the 6-aryl-picolinate (6-AP) herbicide class. Initial SAR development of the 6-AP herbicide class identified methyl 4-amino-3-chloro-6-(4′-chloro-2′-fluorophenyl)picolinate **7** as a highly active herbicide. When aerobic soil degradation studies with this 6-AP analog indicated a high potential for extended environmental persistence, a directed synthesis effort focused on the introduction of a metabolic handle to a 6-AP analog was conducted. Ultimately, the addition of a methoxy group at the 3′-position of methyl 4-amino-3-chloro-6-(4′-chloro-2′-fluorophenyl)picolinate **7** provided a compound that exhibited a high level of herbicidal control of key dicot weeds and rapid degradation in aerobic soils. Further evaluation of 4-amino-3-chloro-6-(4′-chloro-2′-fluoro-3′-methoxyphenyl)picolinate **10** in

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global field trials identified utility for dicot weed control in the global wheat and barley markets. Compound **10** has received the ISO approved common name of halauxifen methyl.

Acknowledgments

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Chapter 19

3-Sulfonylisoxazoline Derivatives as Novel Herbicides

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A series of novel 3-sulfonylisoxazoline derivertives show good herbicidal activity against annual weeds. 3-sulfonylisoxazoline derivatives, consisting of isoxazoline and benzene rings, have unique physicochemical properties that enable them to provide stable efficacy under flooded rice culture systems and to prevent the risk of leaching into groundwater. Optimization of these compounds as a new herbicide for use in rice culture has led to the discovery of Fenoxasulfone. In this chapter, the structure–activity relationship, influence of physicochemical properties and biological activities of Fenoxasulfone are reported.

Introduction

A series of novel 3-sulfonylisoxazoline derivatives (Figure 1) were found to show good herbicidal activity against annual weeds, especially grasses, when applied pre-emergence (*1*). Structure modifications of the skeletal structure of 3-sulfonylisoxazolines were made at the substituents R^1–R^4 and the aromatic ring Ar, including heterocyclic groups. As a result, the substituents and aromatic moiety were optimized, and some compounds showed enhanced herbicidal activity with improved crop safety. Ultimately, Pyroxasulfone (Figure 1) was selected as a promising compound for development as a pre-emergence herbicide in corn (*2*, *3*).

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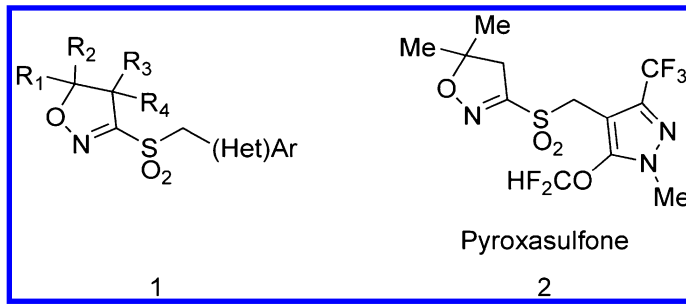

Figure 1. 3-sulfonylisoxazoline derivatives

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On the other hand, compounds that had a benzene ring showed unique physicochemical properties (low solubility in water and strong adsorption on soil), enabling them to provide stable efficacy under flooded rice culture systems and to prevent the risk of runoff from a rice paddy field.

We therefore focused on substituents on the benzene ring and evaluated the herbicidal performance of the resulting compounds. (Figure 2) In this chapter, we describe the process of optimizing the benzene ring to obtain Fenoxasulfone, as well as the herbicidal activity of this compound.

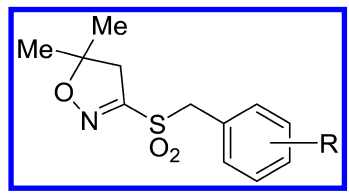

Figure 2. Compounds that had a benzene ring

Discovery of Fenoxasulfone (*4*)

Effects of Substituents on the Benzene Ring – Mono-Substituted Ring

We understood that compounds with a benzene ring had unique physical properties, which would not be suitable for use as a corn herbicide. However, we considered that their low solubility in water and strong adsorption on soil would be suitable for use in paddy field rice cultivation. Therefore, we started to optimize the benzene ring from another point of view.

Initially, substituent positions on the benzene ring were replaced with an Methyl group (Figure 3).

Table 1 shows the effects of Methyl substituents on every position. The ortho-substituent derivative expressed stronger herbicidal activity as compared with the others.

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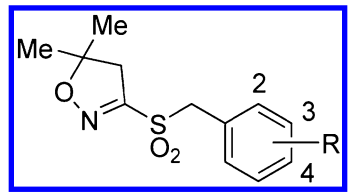

Figure 3. Substituent positions on the benzene ring

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Table 1. Effect of substituent position (g a.i./ha)[a]

	ED_{20}	ED_{90}		
R	*ORYSA*	*ECHOR*	*MOOVA*	*SCPJO*
2-Me	63	32	250	250
3-Me	250	63	1,000	250
4-me	250	63	1,000	250

[a] Abbreviations: ORYSA, *Oryza sativa* (Transplanted rice; cv. Kinmaze, 2-leaf stage); ECHOR, *Echinochloa oryzoides*; MOOVA**,** *Monochoria vaginalis*; SCPJP, *Scripus juncoides*. Treatment: A drop of diluted solution was applied directly into paddy water. Evaluation: 30 days after application (herbicidal activity and crop injury were visually evaluated on the basis of percentage of the growth relative to that of untreated control). ED20: the dosage of 20% crop inhibition by visual assessment. ED90: the dosage of 90% weed control by visual assessment.

Next, substituent groups other than Methyl were examined and the results showed the same tendency. These findings indicated that introduction of a functional group at the ortho position was necessary to express stronger herbicidal activity.

The effect of the substituent group at the ortho position was then examined. The size, electronic properties and stability were evaluated, and the results are shown in Table 2.

Ethoxy and chlorine substituents showed good efficacy against weeds and some safety toward rice. However, these mono-substituted derivatives did not exhibit sufficient efficacy for use as a herbicide in paddy field rice cultivation. Under the supposition that ethoxy and chlorine were effective substituents for this structure, we further investigated the effect of substituents on the benzene ring by introducing a second substituent.

Table 2. Effect of different ortho substituents (g a.i./ha)[a]

Orth position	*ED_{20}*	*ED_{90}*		
	ORYSA	*ECHOR*	*MOOVA*	*SCPJO*
OMe	250	63	1,000	250
OEt	250	16	250	63
OCF_2H	32	16	125	32
Cl	250	16	500	500
CF_3	63	16	250	125
CN	63	63	500	125
COOMe	>1,000	250	>1,000	>1,000
$MeSO_2$	250	250	>1,000	250

[a] See Table 1 for abbreviations and conditions.

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Effects of Substituents on the Benzene Ring – Di-Substituted Ring

Ethoxy and chlorine seemed to be favorable substituents of this structure for use in rice cultivation. Therefore, di-substituted benzene rings (a combination of ethoxy and chlorine) were investigated next. The results are shown in Table 3.

Table 3. Effect of di-substituent positions (g a.i./10a)[a]

R	*ED_{20}*	*ED_{90}*		
	ORYSA	*ECHOR*	*MOOVA*	*SCPJO*
2-OEt-3-Cl	250	32	250	63
2-OEt-4-Cl	250	16	250	250
2-OEt-5-Cl	63	4	63	63
2-OEt-6-Cl	250	16	250	63
4-OEt-2-Cl	500	16	125	63
5-OEt-2-Cl	250	63	500	125

[a] See Table 1 for abbreviations and conditions.

The 2-OEt-5-Cl derivative (Figure 4) showed excellent efficacy against weeds and had some safety toward rice. The 4-OEt-2-Cl derivative also showed good efficacy and better selectivity as compared with the others.

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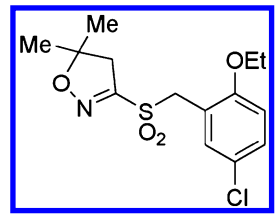


Figure 4. Lead Compound

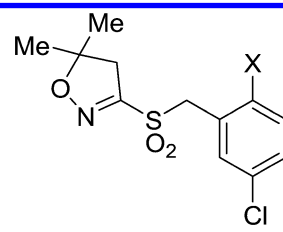
Unlike when used as a pre-emergence herbicide for corn, 2,5-"di-substituted" derivatives exhibited stronger efficacy as a rice herbicide than the commercial standards.

On the basis of these results, the 2-EtO-5-Cl derivative (Figure 4) was selected as a lead compound.

Next, to improve crop safety, the effect of functional groups other than Ethoxy and chlorine at the 2- and 5-positions was investigated.

As shown in Tables 4 and 5, the introduction of other functional groups only reduced herbicidal activity, and an improvement in selectivity was not observed.

Table 4. Effect of substituent at the 2-position (g a.i./ha)



R	ED_{20}	ED_{90}		
	ORYSA	ECHOR	MOOVA	SCPJO
OMe	32	63	250	125
OEt	63	4	63	63
OCF_2H	63	32	63	125
OCH_2CF_3	63	32	63	125
CF_3	63	4	500	63
Cl	63	16	250	63
Me	63	16	125	125

See Table 1 for abbreviations and conditions.

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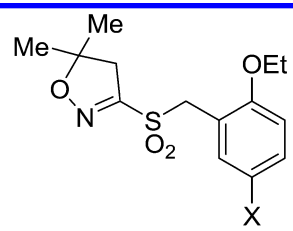
Table 5. Effect of substituent at the 5-position (g a.i./ha)



R	ED_{20}	ED_{90}		
	ORYSA	ECHOR	MOOVA	SCPJO
OMe	250	63	500	63
OCF_2H	250	32	250	250
CF_3	250	32	63	125
F	63	32	500	125
Cl	63	4	63	63
Me	63	16	125	125

See Table 1 for abbreviations and conditions.

We therefore compared the physical properties (logP, Soil Adsorption) of the 2-OEt-5-Cl derivative with those of commercial products to decide how to proceed with this project (Figure 5).

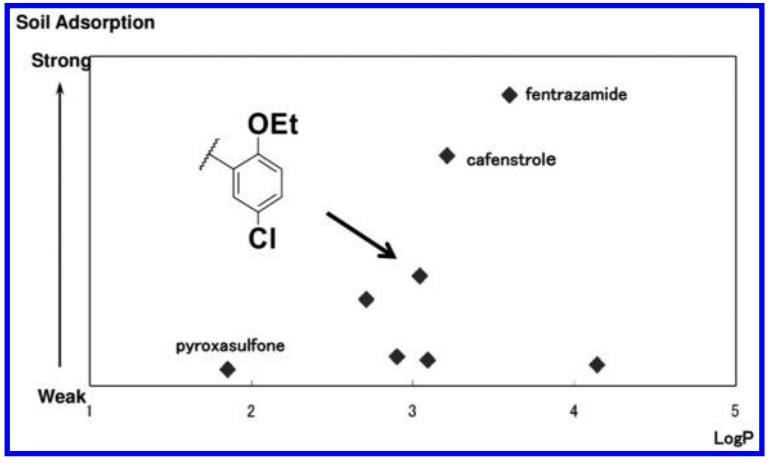


Figure 5. Correlation diagram of Soil adsorption and logP

The logP of the lead compound was lower and the soil adsorption was lower than that of commercial products such as Fentrazamide and Cafenstrole. It was assumed that weaker soil adsorption leads to less residual activity. It was clear that an improvement of physicochemical properties was necessary for further

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development of a rice herbicide. To improve crop safety and physicochemical properties, we therefore aimed to introduce further functional groups onto the benzene ring.

Effects of Substituents on the Benzene Ring – Multi-Substituted Ring

On the basis of the results obtained so far, multi-substituted compounds with EtO and Cl were synthesized and examined. The results are shown in Table 6.

Table 6. Effect of tri-substituents (g a.i./ha)[a]

	ED_{20}	ED_{90}		
R	*ORYSA*	*ECHOR*	*MOOVA*	*SCPJO*
2-OEt-3,4-Cl_2	500	16	500	500
2-OEt-3,5-Cl_2	1,000	16	125	125
2-OEt-3,6-Cl_2	63	63	63	250
2-OEt-4,5-Cl_2	1,000	16	250	250
2-OEt-4,6-Cl_2	63	63	125	125
2-OEt-5,6-Cl_2	1,000	16	125	125
4-OEt-2,3-Cl_2	250	16	63	250
4-OEt-2,5-Cl_2	>1,000	16	63	63
4-OEt-2,6-Cl_2	16	16	32	63

[a] See Table 1 for abbreviations and conditions.

The compound with the 4-ethoxy-2,5-dichloro benzene ring (Fig. 6), termed Fenoxasulfone, exhibited excellent herbicidal activity and was found to be very safe.

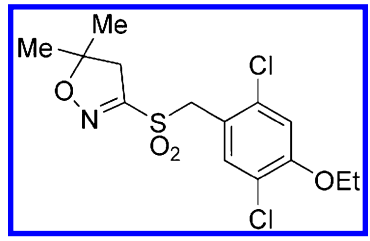

Figure 6. Structure of Fenoxasulfone

The performance of multi-substituted derivatives with other functional groups was not sufficient (data not shown).

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The physicochemical properties of Fenoxasulfone (Figure 7) were also measured and compared with those of commercial products.

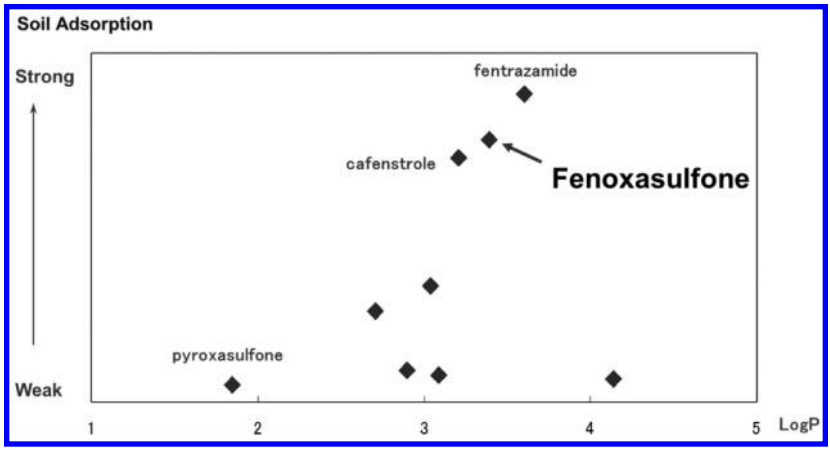

Figure 7. Correlation diagram of Soil adsorption and logP

The physicochemical properties of Fenoxasulfone were improved as expected with appropriate values for use in paddy field rice cultivation. For example, Fenoxasulfone exhibited excellent herbicidal activity and sufficient selectivity. In addition, Fenoxasulfone had appropriate physicochemical properties for use in paddy fields. Therefore, Fenoxasulfone was selected as an agrochemical candidate with excellent efficacy and high safety.

The physical properties of Fenoxasulfone are summarized below.

- Water solubility 0.17 mg/L
- LogP 3.30 (25°C
- Adsorption/desorption in soil K_{oc} 436-3295
- Vapor pressure 2.9×10^{-7}Pa (25°C
- Hydrolysis Stable (pH=5,7,9; 25°C 30d)

Synthesis

The isoxazoline and Fenoxasulfone were synthesized in the laboratory as illustrated in Figure 8.

The isoxazoline moiety was prepared in five steps and the benzyl moiety was prepared in three steps. Next, the isoxazoline and benzyl moieties were reacted and oxidized, giving Fenoxasulfone as a white solid. When the hydroxymethylation of 2,5-dichlorophenol was carried out in this reaction, 2,5-dichloro-4-hydroxymethlphenol alone was obtained selectively. This is a huge advantage of Fenoxasulfone synthesis.

In the following section, we present the biological data of Fenoxasulfone.

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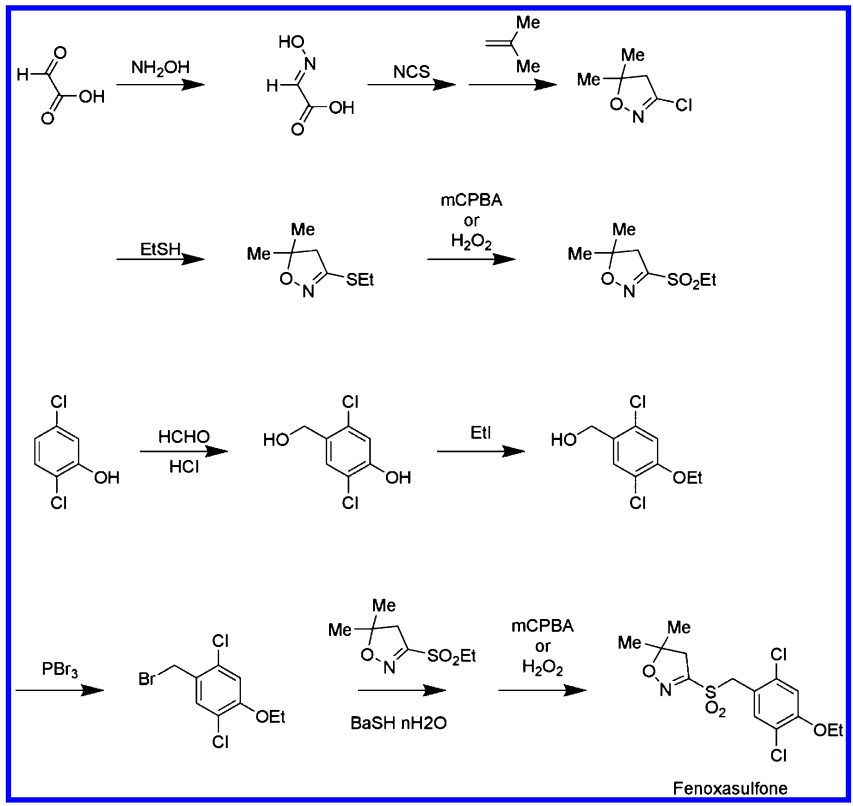

Figure 8. Synthetic route

Biological Aspects of Fenoxasulfone (*5*)

Greenhouse tests and a field trial were conducted to evaluate the herbicidal efficacy of Fenoxasulfone. The compound exhibited excellent herbicidal efficacy at a lower application rate (200g a.i./ha) as compared with standards and also showed sufficient residual activity.

Effects on Weeds

Fenoxasulfone is absorbed mainly by the shoots and roots of germinating weeds. In the case of *Echinochloa oryzoides*, symptoms of wilting and growth inhibition symptoms begin to appear within a week of application and death occurs in approximately 2 weeks (Figure 9).

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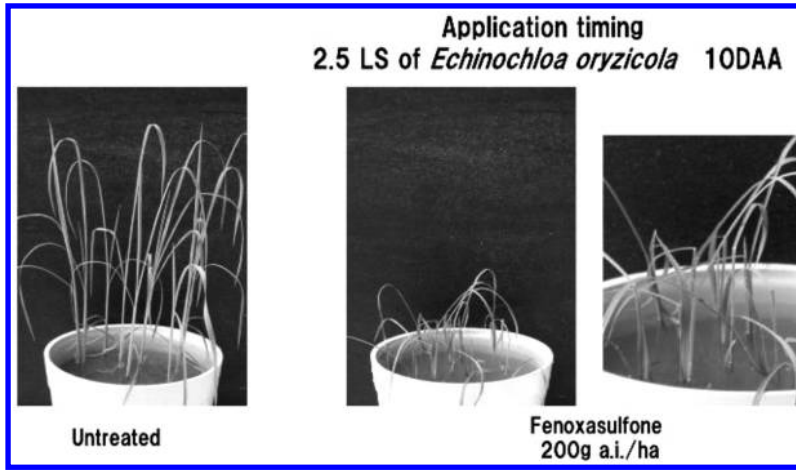

Figure 9. Symptoms of Fenoxasulfone

Efficacy

Greenhouse experiments were conducted at the Kumiai Chemical Life Science Research Institute in Shizuoka, Japan, to evaluate the herbicidal efficacy of Fenoxasulfone against *E. oryzicola* and *Echinochloa crus-galli*.

As shown in Figure 10, Fenoxasulfone exhibited excellent herbicidal activity against *Echinochloa oryzicola* and *Echinochloa crus-galli* at 100g a.i./ha.

The spectrum of Fenoxasulfone activity was also evaluated by greenhouse experiments. The results are shown in Figure 11.

Fenoxasulfone provided more than 90% control of weed species at 200g a.i./ha when applied pre-emergence until the 2.5 leaf stage (LS) of *Echinochloa* spp.

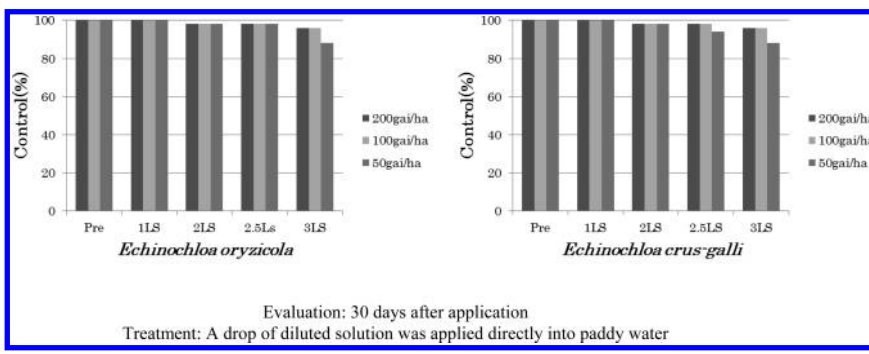

Figure 10. Herbicidal efficacy of Fenoxasulfone

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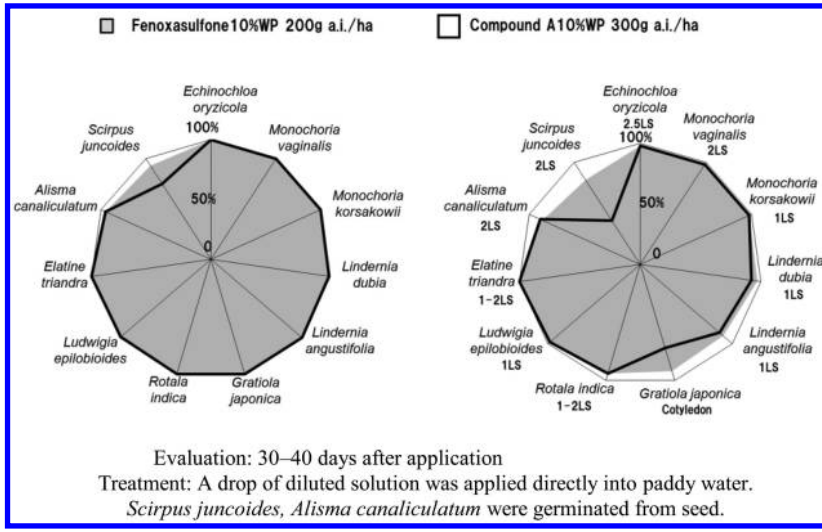

Figure 11. Spectrum of Fenoxasulfone

Residual Activity of Fenoxasulfone

The residual activity is one of the key factors required in a herbicide for paddy field rice cultivation. As shown above, the physicochemical properties of Fenoxasulfone were favorable for use in paddy fields. To confirm the residual activity, a greenhouse experience was conducted at the Kumiai Chemical Life Science Research Institute in Shizuoka, Japan.

Fenoxasulfone applied at 200ga.i./ha provided excellent residual activity against *E. oryzoides* and *Monochoria vaginalis*. The length of activity was superior to both compound A at 300 ga.i/ha and compound B at 300 ga.i./ha (Figure 12).

During the normal period of rice cultivation in Japan, there is frequent heavy rainfall. An effective herbicide will be required to exhibit enough activity even under such circumstances. Therefore, under an assumption of heavy rain, the residual activity of Fenoxasulfone in overflow conditions was examined.

In general, Overflow conditions did reduce the residual activity somewhat; however, the reduction in residual activity was not as significant for Fenoxasulfone as it was for the standards (Figure 13). This is due to the strong soil adsorption and low water solubility of Fenoxasulfone, which was adsorbed into the soil quickly and was not moved much in a vertical or horizontal direction by the movement of water. Thus, Fenoxasulfone exhibited stable herbicidal activity even in conditions of overflow.

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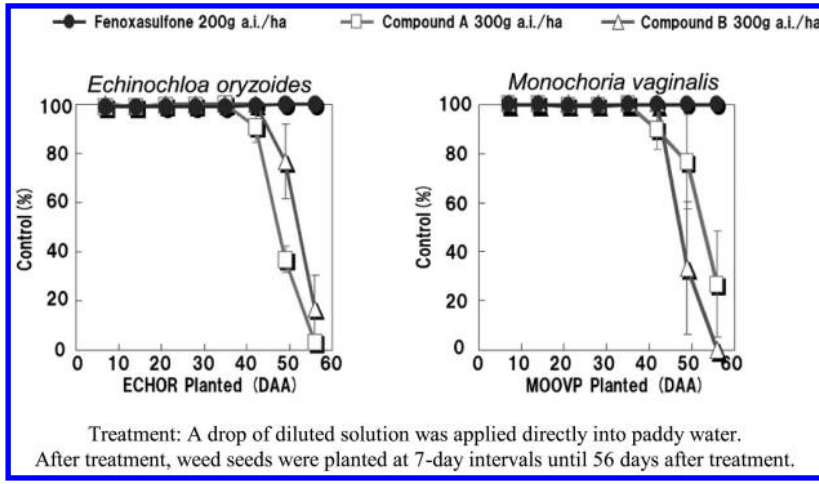

Figure 12. Residual activity of Fenoxasulfone

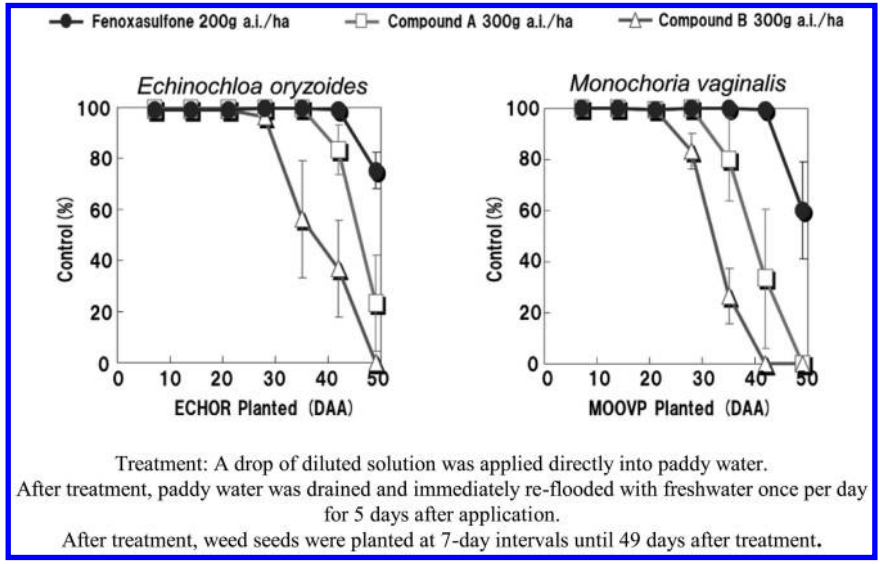

Figure 13. Residual activity of Fenoxasulfone in overflow conditions

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Crop Safety

The phytotoxicity of Fenoxasulfone was compared to that of commercial standards which have been used for rice cultivation. Greenhouse experiments were conducted at the Kumiai Chemical Life Science Research Institute in Shizuoka, Japan.

Transplanted rice shows good tolerance to Fenoxasulfone when applied 0~10 days after transplanting and a planting depth of 2 cm or over. Shallow planting depth (less than 2 cm) may cause injury such as rice growth reduction (Figure 14).

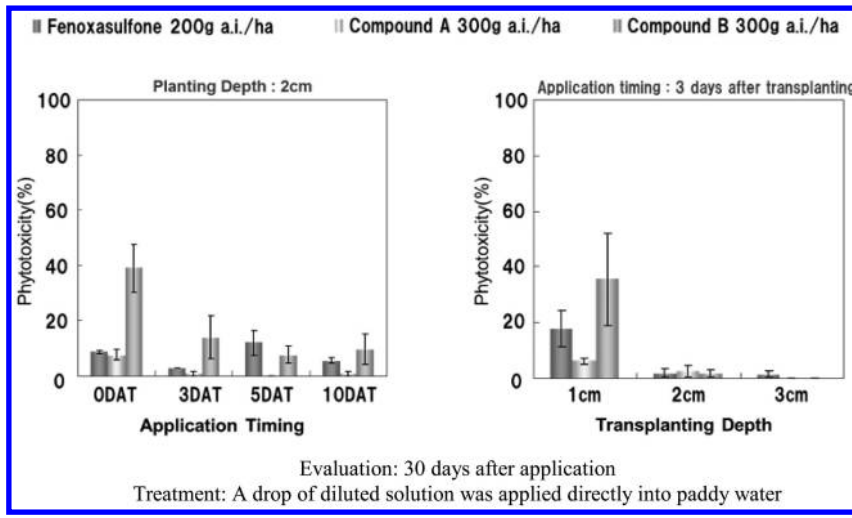

Figure 14. Phytotoxicity of Fenoxasulfone

Field Trials

Next, a large number of trials were conducted on transplanted rice grown under flood culture in Japan. The application rate of Fenoxasulfone was 200 g a.i./ha.

Fenoxasulfone exhibited excellent control of *Echinochloa* spp., *Monochoria* spp., and *Lindernia* spp at the application range and provided better control of *Scirpus* spp. as compared with commercial standards. It also exhibited a wide range of application timings (Figure 15). Thus, the applicability of Fenoxasulfone for use in paddy fields was proven in the field trials.

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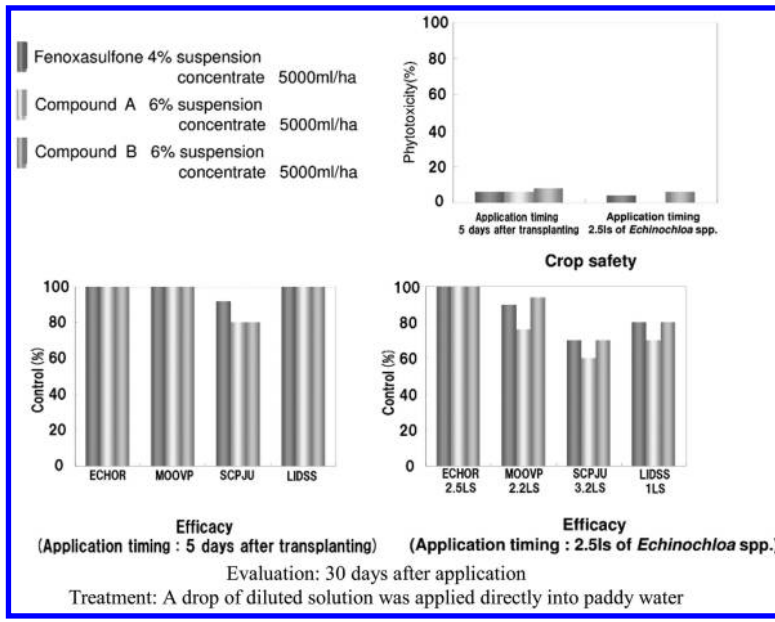

Figure 15. Results of field trials

Mode of Action (*6*)

The mechanism of Fenoxasulfone activity was studied by examining the inhibitory effects of this herbicide on the biosynthesis of very-long-chain fatty acids (VLCFAs) (Figure 16).

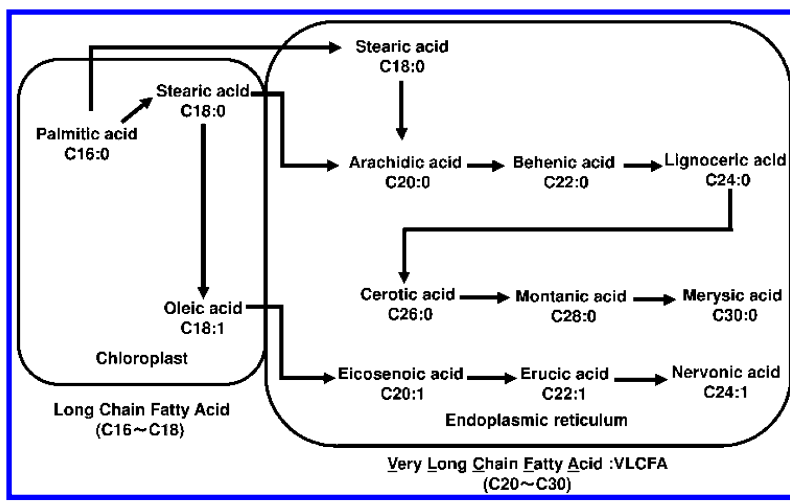

Figure 16. Biosynthesis of very-long-chain fatty acids (VLCFAs)

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Fenoxasulfone treatment decreased the proportion of VLCFAs, such as C20:0, C20:1, C22:0, C24:0, C24:1 and C26:0 fatty acids, in barnyard millet cultured cells, and increased that of long-chain-fatty acids and medium-chain-fatty acids, such as C18:0 and C15:0, which are precursors of VLCFAs. Fenoxasulfone potently inhibited the activity of VLCFA elongase (VLCFAE) in the microsomal fraction of etiolated barnyard millet seedlings, which catalyzes the elongation steps from C22:0 to C24:0 and C24:0 to C26:0, respectively. These results strongly suggest that fenoxasulfone is a potent inhibitor of plant VLCFAEs and should be categorized within the K3 group of the Herbicide Resistance Action Committee.

The VLCFAE activity of recombinant Fatty acid elongation 1 (FAE1) of *Arabidopsis*, which catalyzes the elongation step from C18:1 to C20:1, was inhibited by Fenoxasulfone in a time-dependent manner, a feature that has been observed in the inhibition of VLCFAEs by other well-known VLCFAE-inhibiting herbicides. In addition, the VLCFAE activity of the microsomal fraction of etiolated barnyard millet seedlings, which catalyzes the elongation step from C24:0 to C26:0, was inhibited by Fenoxasulfone in a time-independent manner. This time-independent inhibition indicates a new inhibitory mechanism of VLCFAE by Fenoxasulfone, possibly similar to that of Pyroxasulfone, which is classified in the same chemical class as Fenoxasulfone.

Conclusion

This chapter has described the discovery and biological aspects of Fenoxasulfone (Figure 17). Fenoxasulfone exhibits excellent efficacy against grass weeds in paddy field rice cultivation and broad-spectrum weed control. In addition, Fenoxaslfone shows outstanding residual activity on *Echinochloa* spp. and *Monochoria* spp. Fenoxasulfone also has favorable toxicological, environmental, and ecotoxicological properties.

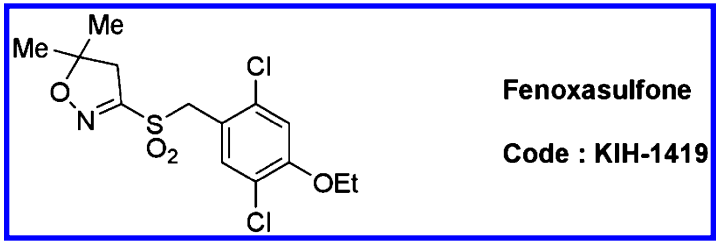

Figure 17. Fenoxasulfone

In Japan, Fenoxasulfone was registered for use on turf on May 16th, 2014, and for use on rice on October 3rd, 2014.

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Chapter 20

Broad-Spectrum PPO-Inhibiting *N*-Phenoxyphenyluracil Acetal Ester Herbicides

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Substituted N-aryluracils were first discovered in the late eighties at Hoffman-La Roche as an extremely active subclass of herbicidal protoporphyrinogen-IX oxidase inhibitors. Interest by Uniroyal followed but it was not until 2001 that uracil-based butafenacil was commercially introduced by Syngenta and saflufenacil by BASF in 2009. In the mid-nineties, unique 3-ring *N*-phenoxy-phenyl-triazolinones with an oxyproprionate side-chain on the diphenylether part of the molecule were reported by FMC to be very active protox-inhibiting herbicides. Structurally-related *N*-aryloxy-phenyl-uracils having an oxyacetate group on the diaryl ether were subsequently patented by Sumitomo. Here, we report on the synthesis and herbicidal activity of a series of acetal ester-substituted *N*-phenoxy-phenyl-uracils where we had interest as potential short-residual herbicides for postemergent-applied weed control (pre-plant) in row crops, especially for control of glyphosate and ALS resistant weeds.

Protoporphyrinogen-IX oxidase (PPO, protox), catalyzes the last common enzymatic step in the biosynthesis of chlorophyll and heme where protoporphyrinogen-IX is oxidized to protoporphyrin IX. Compounds that inhibit this step give quick burn symptoms on plants when applied postemergnece,

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generally with limited crop safety and plant systemic properties due to their fast-acting nature that results from singlet oxygen generation and ultimately membrane disruption. Generally higher levels of crop selectivity are obtained preemergence. Traditional PPO-inhibiting herbicides tend to be more effective for control of broadleaf than grass weeds at low rates of application.

Although discovered decades ago and commercially used for many years, sales of herbicidal PPO inhibiting products have grown more recently due in part to the on-set of weed resistance to both glyphosate and acetolactate synthase (ALS) inhibitors. Use of both diphenyl ether inhibitors, i.e. fomesafen, and aryl heterocycles (also referred to as "cyclic imides"), i.e. flumioxacin, have increased, especially for control of glyphosate and ALS resistant amaranthus weed species such as palmer amaranth and common waterhemp.

Substituted N-aryluracils were first discovered in the late eighties at Hoffman-La Roche as an extremely active subclass of "cyclic imide" PPO-inhibiting herbicides and Uniroyal soon developed commercial interest in flupropacil (*1*). Uracil-based PPO inhibitors were typically found to be more active than other similarly substituted heterocyclic chemotypes (*2–4*). However, it was not until 2001 when Syngenta introduced the uracil-containing butafenacil into the marketplace, followed by BASF's commercialization of saflufenacil in 2009 (Figure 1).

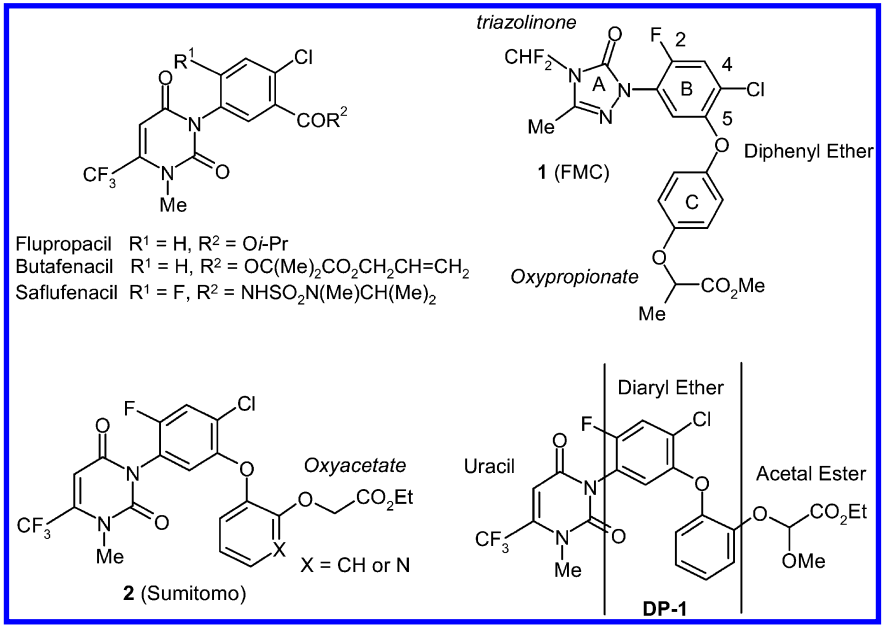

Figure 1. N-Aryluracil Herbicidal Inhibitors of Protoporphyrinogen-IX Oxidase

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In the mid-nineties, George Theodoridis at FMC reported that *N*-phenoxyphenyltriazolinones, where the diphenylether fragment attached to the ring nitrogen is substituted with a *para*-oxyproprionate side-chain, i.e. **1**, were very active as PPO-inhibiting herbicides (*5*). As substrate inhibitors, these large molecular weight 3-ring containing compounds were described as potential mimics of the tetrapyrrole, protoporphyringen-IX (*5–7*).

Sumitomo later patented related *N*-(hetero)aryloxyphenyluracils, preferably with an *ortho*-oxyacetate side-chain on the diaryl ether group, i.e. **2**, as broad-spectrum herbicides with broadleaf and grass activity (*8*, *9*). Here, we report on the synthesis and herbicidal activity of structurally-related acetal ester-substituted *N*-phenoxyphenyluracils, i.e. **DP-1** (*10*). We had special interest in this chemistry for postemergent applications to glyphosate and ALS resistant weeds (i.e. pre-plant "burndown" in row crops) where short-residual properties might stem from the hydrolytically sensitive acetal ester group.

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Chemistry

The general synthetic route for making *N*-methyl-4-trifluoromethyluracil N-diphenyl ether acetal esters is outlined in Figure 2. Cyclization of ethyl 3-amino-3-trifluoromethylpropenoate (**3**) and benzyl isocyanate in the presence of sodium hydride in DMF gave N-benzyl uracil **4**. Following methylation and de-benzylation, uracil 5 was reacted with 2,4,5-trifluoronitrobenzene in DMF with potassium carbonate to afford N-aryluracil **6** as the major poduct. Some isomeric product (< 20%), which resulted from displacement of the fluorine *ortho* versus *para* to the nitro group by the uracil ring nitrogen, required separation. Reaction of **6** with (un)substituted 2-methoxyphenols, again in DMF with potassium carbonate, give N-diphenyl ether substituted uracils **7** where displacement of fluorine ortho to the nitro group on the phenyl occurred. In the preparation of the chorodiphenyl ether containing uracils **8**, introduction of chlorine was achieved via reduction of the nitro group with iron in acetic acid followed by diazotization of the produced aniline with isoamyl nitrite in the presence of copper chloride salts in acetonitrile. De-protection of the methoxy group on the diphenyl ether with boron tribromide gave the free uracil phenols **9**, which on alkylation with alkyl 2-bromoalkoxyacetates with sodium hydride in THF or potassium carbonate in DMF afforded final products **10**, trifluoromethyl uracils N-substituted by a diphenyl ether with an acetal ester group. In most cases, the alkylating agents were made by bromination of the corresponding alkoxyacetates with NBS.

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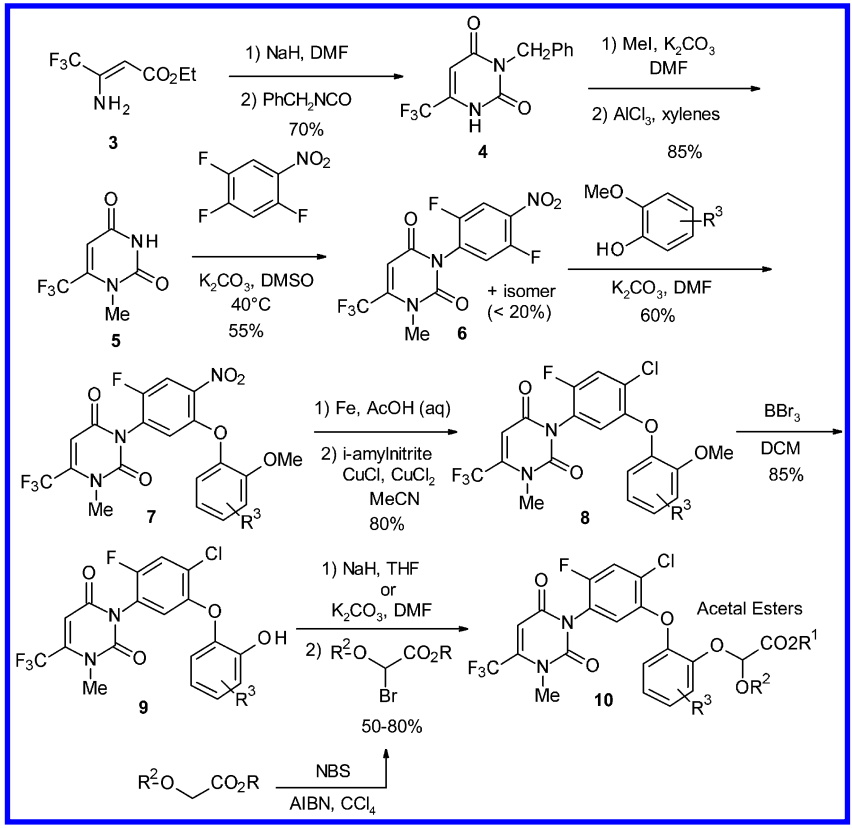

Figure 2. Synthetic route to N-phenoxyphenyluracil acetal esters

We were interested in the free acids of uracil acetal esters **10** for conversion to other ester and amide derivatives (Figure 3). However, attempted hydrolysis of uracils **10** in aqueous base resulted in mainly decomposition due in part to the hydrolytic instability of the acetal ester functionality. However, we found that transesterification of the ethyl methoxyacetate **DP-1** with excess allyl alcohol and a catalytic amount of indium trichloride produced allyl methoxyacetate **10a** in good yield. Treatment of **10a** with palladium tetrakis-triphenylphosphine in the presence of sodium *para*-toluenesulfonate in methanolic THF gave reasonable yields of the free acid **11** that could be further purified by reverse phase chromatography. Alternatively, the benzyl acetal ester **10b**, which was made by the chemistry outlined in Figure 1, underwent catalytic hydrogenolysis to also provide the free acid **11**.

Coupling of acid **11** and amines with BOP reagent and diisopropylethylamine in tetrahydrofuran gave amides **12** in reasonable yield. Esterification of **11** with alcohols (generally used in excess) to provide a range of esters (**10c**) was typically done with 2-chloro-1-methylpyridium iodide and diisopropylethylamine in tetrahydrofuran.

The preparation of substituted uracil diphenylether thioacetal esters **13** is shown in Figure 4. Made by the chemistry route in Figure 1, uracil diphenylether phenol **9a** was alkylated with 2-bromoalkylthioacetates using sodium hydride in tetrahydrofuran or potassium carbonate in DMF.

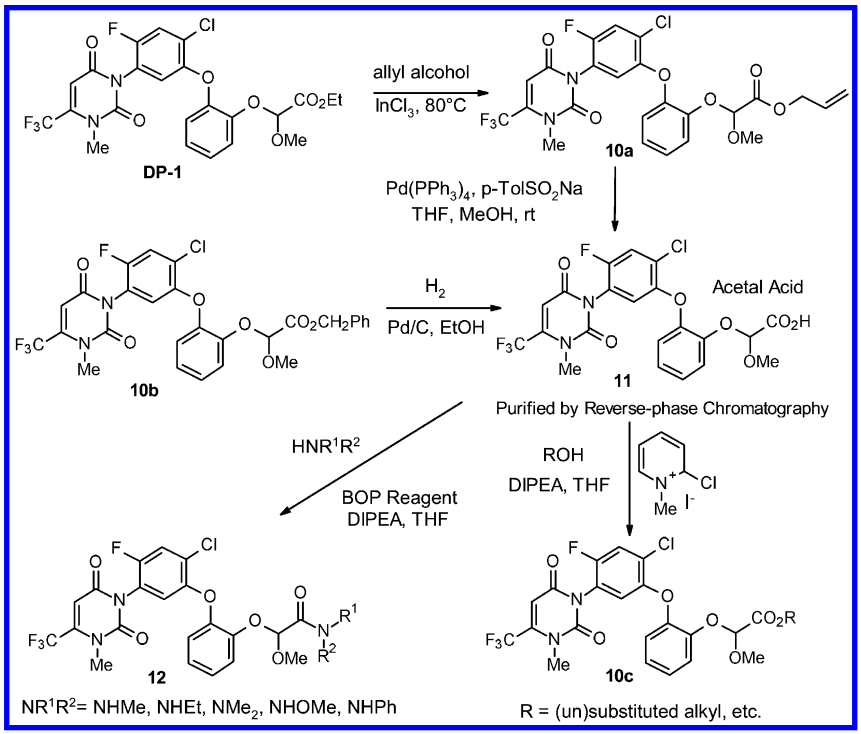

Figure 3. Converison of a N-phenoxyphenyluracil acetal ester to the free acid, other esters and amides

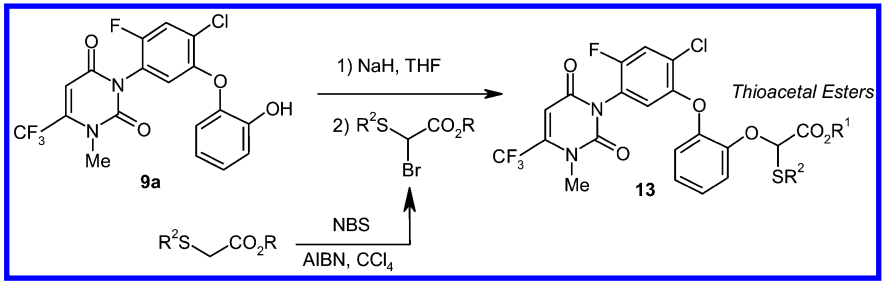

Figure 4. Synthesis of N-phenoxyphenyluracil thioacetal esters

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In Figure 5, the synthesis of a uracil N-phenoxypyridyl acetal methyl ester is outlined. Coupling of *N*-2,5-difluoro-4-nitrophenyluracil **6** with 3-hydroxy-2-methoxypyridine by heating in DMF with potassium carbonate gave the uracil *N*-phenoxypyridyluracil **14**. Reduction of the nitro group on **14** with iron in acetic acid gave the aniline which on diazotization in the presence of copper chloride salts gave the chlorine-containing phenoxypyridyluracil **15**. Treatment of **15** with boron tribromide resulted in de-protection of the pyridyl methoxy groug to generate the free pyridinol **16**. Although **16** may exist in part as the pyridone tautomer, re-alkylation with methyl 2-bromo methoxyacetate in acetonitrile with potassium carbonate occurred predominantly on oxygen versus nitrogen to give uracil N-phenoxypyridyl acetal ester **17** in good yield.

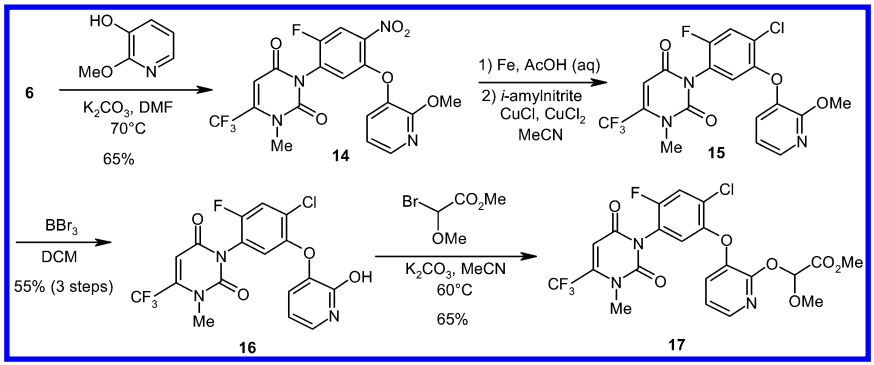

Figure 5. Synthesis of a N-phenoxypyridyluracil acetal ester

Following chemistry similar to that outlined in Figure 1, regioisomers of **DP-1** were also made from a common intermediate, *N*-2,5-difluoro-4-nitrophenyluracil **6** (Figure 6). The two ether linkages on the terminal phenyl ring of the diphenylether are *meta* on **18** and *para* on **19** versus *ortho* on **DP-1**.

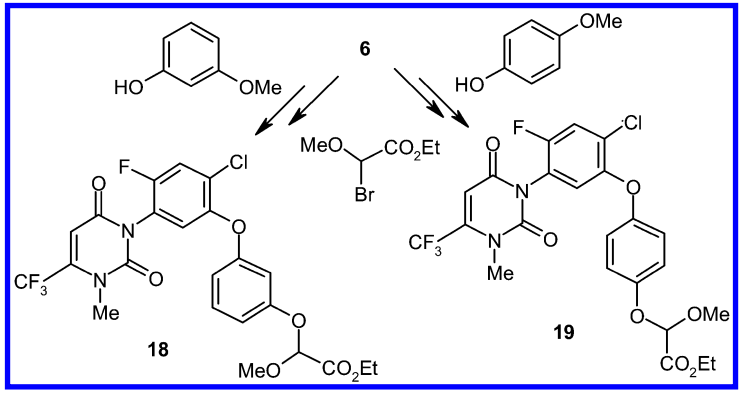

Figure 6. Synthesis of N-phenoxyphenyluracil acetal ester regioisomers

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Herbicidal Activity

Substituted *N*-phenoxyphenyluracil acetal esters and derivatives made by the described chemistry methods were evaluated postemergence (POST) and preemergence (PRE) against a range of broadleaf and grass weeds with crop selectivity assessed. Table 1 summarizes POST activity for a series of esters and free acids at an application rate of 4 g/Ha against 7 broadleaf weeds, 5 warm season grass weeds and 3 crops. Weed activity is reported as averaged percent control and crop damage as percent injury.

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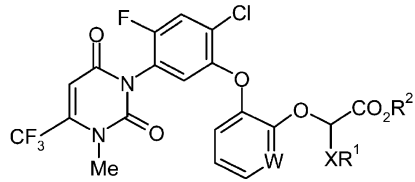
Table 1. Postemergent Weed Control and Crop Injury by *N*-Phenoxyphenyluracil Acetal Esters and the Free Acids at 4 g/Ha



Entry	*W*	*XR*[1]	*R*[2]	*% BLW Control*[1]	*% GW Control*[1]	*% Crop Injury*[2]
DP-1	CH	OMe	Et	100	95	90
DP-2	CH	OMe	Me	97	80	88
DP-3	CH	OMe	*n*-Pr	100	70	97
DP-4	CH	OMe	*i*-Pr	100	68	90
DP-5	CH	OMe	*n*-pentyl	100	71	73
DP-6	CH	OMe	CH_2CF_3	100	85	90
DP-7	CH	OMe	CH_2CH_2OMe	100	90	85
DP-8	CH	OMe	CH_2CH_2OEt	100	95	95
DP-9	CH	OMe	CH_2OEt	98	76	88
DP-10	CH	OMe	CH_2Ph	100	69	78
DP-11	CH	OMe	$(CH_2)_3F$	100	95	98
DP-12	CH	OMe	H	95	61	82
DP-13	CH	OEt	Et	98	75	78
DP-14	CH	OEt	Me	96	65	80
DP-15	CH	OEt	H	99	58	90
DP-16	CH	SMe	Et	99	54	62
DP-17	CH	SEt	Me	99	80	53
DP-18	N	OMe	Et	80	45	39

[1]Averaged % postemergent control of 7 broadleaf weeds (BLW) and 5 warm season grass weeds (GW). [2]Averaged % injury of three crops (maize, soybean and winter wheat).

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Broadleaf weeds tested included: Amaranthus *retroflexus* (redroot pigweed), Chenopodium *album* (lambsquarter), Stellaria *media* (chickweed), Ipomoea *hederacea* (ivy morninglory), Ambrosia *artemisiifolia* (ragweed), Abutilon *theophrasti (*velvetleaf) and Kochia *scoparia* (kochia). Warm Season grass weeds included: Digitaria *sanguinalis* (large crabgrass), Setaria *faberii* (giant foxtail), Sorghum *halepense (*johnsongrass), Cynodon *dactylon* (bermudagrass) and Eleusine *indica* (goosegrass). Crop injury was averaged for maize, soybean and wheat.

Like traditional PPO inhibitors, all of analogs in Table 1 showed high POST broadleaf weed activity with limited crop safety due to plant necrosis (burn) resulting from quick contact potency. However, our focus was to find analogs with a combination of excellent broadleaf activity and enhanced levels of grass control. Where W is CH and XR^1 is methoxy, the highest levels of cross-spectrum weed control were obtained for acetal esters where R^2 is ethyl (**DP-1**), trifluoroethyl (**DP-6**), methoxyethyl (**DP-7**), ethoxyethyl (**DP-8**) and 3-fluoropropyl (**DP-11**). All of these acetal esters performed similarly in advanced greenhouse testing as non-selective POST contact herbicides. The ethyl acetals **DP-14** and **DP-15** (where XR^1 is ethoxy) and the alkyl thioacetals **DP-16** (where XR^1 is methylthio) and **DP-17** (where XR^1 is ethylthio), were less active than their methyl acetal ester counterparts. Free acids **DP-12** and **DP-15** also had diminished activity versus the esters with the most noticeable activity drop on grasses.

Finally, unlike oxyacetate-substituted pyridyloxyphenyluracils previously patented by Sumitomo, significantly lower activity was unexpectedly observed for **DP-18**, the last entry in Table 1 where the terminal phenyl ring of **DP-1** was replaced by pyridine (W is N). The cause for this activity loss was unclear.

Table 2 summarizes preemergent activity for a series of acetal esters and a free acid in a sandy loam soil at 16 g/Ha against the same weeds (not including Stellaria *media*) and crops listed for Table 1.

Overall PRE efficacy was less than that of POST applications with the highest activity levels expressed against broadleaf weeds. PRE versus POST crop safety was dramatically improved, but generally with some chlorotic crop effects, still observed. Wheat tended to have better PRE tolerance to this chemistry than maize or soybean. Optimum levels of PRE activity were obtained with acetal esters **DP-1**, **DP-8** and **DP-10**, revealing that the best POST compounds tended to be the best PRE. Interestingly, the free acid **DP-14** was not only less efficacious than the esters PRE on weeds but more damaging to crops. In heavier soils, these compounds usually showed some improvement of PRE crop tolerance but accompanied with some reduction of overall weed control. Although not shown in Tables 1 and 2, most cool season grasses were less susceptible than warm season grasses to this class of chemistry PRE or POST. The reason for this was unclear.

Tables 3-5 summarizes POST activity for other analogs averaged against 3 broadleaf weeds (Amaranthus *retroflexus*, Ipomoea *hederacea* and Abutilon *theophrasti* Medik) and 3 warm-season grasses (Digitaria *sanguinalis*, Setaria *pumila* and Echinochloa *crus-galli*). In Table 3, herbicidal activity of several acetal amides is reported at 8 g/Ha. The secondary alkyl amide **DP-19** (NR^2R^3 = NHEt) was more active than the anilide **DP-21** (NR^2R^3 = NPh) which in turn was superior to the tertiary amide **DP-20** ($NR^2R^3 = NMe_2$). However, the Weinreb

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amide **DP-22** [NR^2R^3 = N(Me)OMe)] was most active with a level of activity approaching that of the best acetal esters. The significant activity difference between **DP-20** and **DP-22** might be due to the ability of **DP-22** to hydrolyze more readily to the free acid.

Activity of uracils with substituents on the terminal phenyl ring containing the acetal ester side-chain is given in Table 4 at rates of 8 and 31 g/Ha. Fluorine substitution at the 3- and 5-positions gave analogs **DP-23** and **DP-24** with roughly comparable activity to that of the parent **DP-1**. A small drop in overall activity occurred with going to 4-methyl and 4-chloro substitution (**DP-25** and **DP-26**) followed by another activity drop for 6-methoxy (**DP-27**) and 4,5-benzo-ring fusion to give naphthalene-containing **DP-28**.

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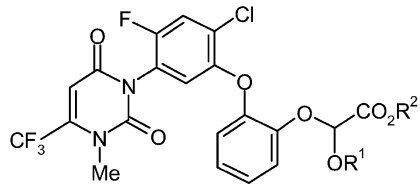
Table 2. Preemergent Weed Control and Crop Injury by *N*-Phenoxyphenyluracil Acetal Esters and the Free Acids at 16 g/Ha



Entry	R^1	R^2	*% BLW Control*[1]	*% GW Control*[1]	*% Crop Injury*[2]
DP-1	Me	Et	97	80	25
DP-2	Me	Me	88	66	23
DP-3	Et	Et	90	75	25
DP-4	Et	Me	85	73	20
DP-6	Me	*i*-Pr	83	81	23
DP-8	Me	CH_2CF_3	94	83	25
DP-9	Me	CH_2CH_2OMe	80	60	23
DP-10	Me	CH_2CH_2OEt	93	79	35
DP-11	Me	CH_2OEt	87	83	17
DP-12	Me	CH_2Ph	83	72	60
DP-13	Me	$(CH_2)_3F$	87	62	26
DP-14	Me	H	82	64	57

[1]Averaged % preemergent control of 6 broadleaf weeds (BLW) and 5 warm season grass weeds (GW). [2]Averaged % injury of three crops (maize, soybean and winter wheat) when applied preemergence.

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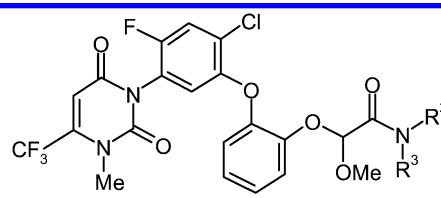
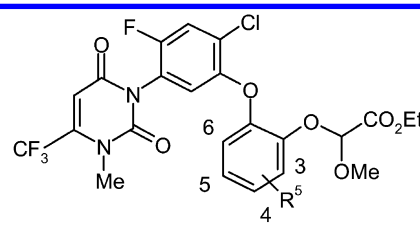
Table 3. Postemergent Weed Control by *N*-Phenoxyphenyluracil Acetal Amides at 8 g/Ha



Entry	*NR^2R^3*	*% BLW Control[1]*	*% GW Control[1]*
DP-19	NHEt	93	47
DP-21	NHPh	57	5
DP-20	NMe_2	20	20
DP-22	N(Me)OMe	100	75

[1]Averaged % postemergent control or injury of 3 broadleaf weeds (BLW) and 3 warm season grass weeds (GW).

Table 4. Postemergent Weed Control by *N*-Phenoxyphenyl Uracil Acetal Esters with Substitution on the Terminal Phenyl Ring



Entry	*Rate (g/Ha)*	*R^5*	*% BLW Control[1]*	*% GW Control[1]*
DP-1	8	H	100	90
DP-23	8	3-F	100	87
DP-24	8	5-F	97	80
DP-25	31	4-Me	100	87
DP-26	31	4-Cl	100	70
DP-27	31	6-OMe	87	43
DP-28[2]	31	4,5-benzo	70	20

[1]Averaged % postemergent control or injury of 3 broadleaf weeds (BLW) and 3 warm season grass weeds (GW). [2]Methyl ester of the above structure.

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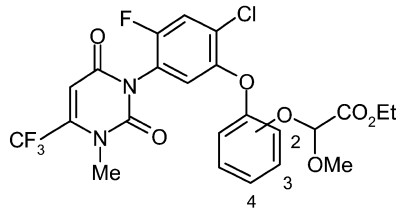
Herbicidal activity of the three positional regioisomers, where the acetal ester side-chain and ether linkage between the two phenyl rings of the diphenyl ether fragment are *ortho* (**DP-1**), *meta* (**DP-29**) and *para* (**DP-30**) to each other, is provided in Table 5. All three were active but optimum weed control was observed with the *ortho* substitution pattern on **DP-1**.

Table 5. Postemergent Weed Control and Crop Injury of *N*-Phenoxyphenyl Uracil Acetal Ester Regioisomers



Entry	*Rate (g/Ha)*	*Regioisomer*	*% BLW Control*[1]	*% GW Control*[1]
DP-1	31	2 (*ortho*)	100	95
DP-1	8	H	100	90
DP-29	31	3 (*meta*)	100	90
DP-30	31	4 (*para*)	100	65

[1]Averaged % postemergent control of 3 broadleaf weeds (BLW) and 3 warm season grass weeds (GW).

Instrinsic Activity

Inhibition of plant versus mammalian forms of the PPO enzyme by *N*-phenoxyphenyluracil **DP-1** versus the standard azafenidin is shown in Table 6. Both compounds are potent low nanomolar inhibitors of the PPO enzyme from Arabidopsis *thaliana* (mustard) and rice. Although having a slightly lower level of potency against the mammalian (human) form of the enzyme, there was not a dramatic difference in selectivity for the three enzyme forms.

Soil Degradation

Compounds of this chemistry class were found to breakdown readily in soil, both hydrolytically and by microbial pathways. Hydrolysis of the acetal ester to the acetal acid (also herbicidally active) tended to occur first, and rapidly in high pH soils, followed by further degradation involving loss of the acetal acid functionality and/or fragmentation of the uracil ring to a urea degradate. Half-lives of acetal acids resulting from ester hydrolysis were usually less than 20-25 days in soil. Although these soil half-lives were short, substantial levels of preemergence herbicidal activity were still observed with varying lengths of residual weed control, depending on use-rate, soil types and temperature.

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Table 6. Instrinsic Activity of *N*-Phenoxyphenyl Uracil Acetal Ester DP-1 against the Plant versus Mammalian Forms of the PPO Enzyme

Entry	*Arabidopsis*	*Rice*	*Human*
		IC_{50} Values (nM)	
DP-1	1.6	0.3	17
Azafenidin	2.8	2.7	16

Field Activity

Field testing of **DP-1** was carried out at multiple test sites. With insufficient POST crop safety, **DP-1** was evaluated in row crops as primarily a pre-plant "burndown" agent retaining some soil residual activity. At higher rates, **DP-1** was also looked at in vegetation management (VM) trials (i.e. permanent crops, sugarcane, orchards) for control of larger weeds. **DP-1** had very little systemic properties and required good coverage on plant foliage for optimum biological performance. In pre-plant trials in corn and soybeans, use rates of 35-70 g a.i./Ha gave excellent levels of broadleaf weed control (i.e. Amaranthus *retroflexus*, Ipomoea *hederacea* and Abutilon *theophrasti* Medik) with only suppression of most warm season grasses (i.e. Digitaria *sanguinalis* and Setaria *pumila*) in most cases. Sufficient levels of crop safety were usually observed on plant-back with corn and soybeans within 3-7 days. In VM field trials of more mature weeds, rates of 70-140 g a.i./Ha gave comparable weed control to that of glyphosate and paraquat at much higher rates (> 1 Kg/Ha). Field tests confirmed high activity against both glyphosate and ALS resistant Amaranthus *palmeri* (Palmer Amaranth), Amaranthus *rudis* (common waterhemp) and Conyza *canadenesis* (marestail).

Conclusion

Substituted N-phenoxyphenyluracil acetal esters represent a unique family of large molecular weight 3-ring containing PPO-inhibiting herbicides that were designed as potentially short-residual "burndown" agents that would perform similarly to that of paraquat or glyphosate but at lower application rates. Although possessing a derivatized acidic functionality, these compounds showed very little plant systemic capability. Nevertheless, **DP-1** gave excellent POST control of broadleaf weeds at low rates in field trials accompanied with some suppression of warm season grasses. Higher use rates generally translated to better overall weed control, especially in the case of grasses and more mature weeds. The soil dissipation profile was generally favorable but minor soil residual effects on emerging crops were sometimes observed following pre-plant applications, indicating a longer than expected soil half-life under certain test conditions. While possessing many positive attributes, further interest in this subclass of PPO-inhibiting herbicides was precluded by the constraints around crop

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safety, concerns of emerging PPO weed resistance in the marketplace and other considerations associated with the mode-of-action.

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Chapter 21

New Approaches to the Design and Synthesis of Inhibitors of Acetyl-CoA Carboxylase

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The inhibition of acetyl-CoA carboxylase (ACCase) is one of the most commercially important modes of action for the control of grass weeds. Three chemical classes of ACCase herbicides have been commercialized – FOPs (aryloxyphenoxypropionates), DIMs (cyclohexanedione oximes) and most recently DENs (2-aryl-1,3-diones). This chapter describes new synthetic methodologies which have been developed to prepare novel analogues of pinoxaden, the only current commercial product from the DEN chemical class.

Carbocyclic 2-aryl-1,3-diones have been of high interest to the agrochemical industry since they were first reported as herbicidal (and insecticidal) in the late 1970s (*1*, *2*). Until relatively recently most synthetic approaches were based on the ring-synthesis methodology developed originally by Union Carbide (*1*), for example Scheme 1. However, with more functionalized examples we have found these approaches to be lengthy and often low yielding. The most problematic steps were typically synthesis of the ketonitrile intermediate **1** (or related ketoester) and the final dione product **2** (Scheme 1).

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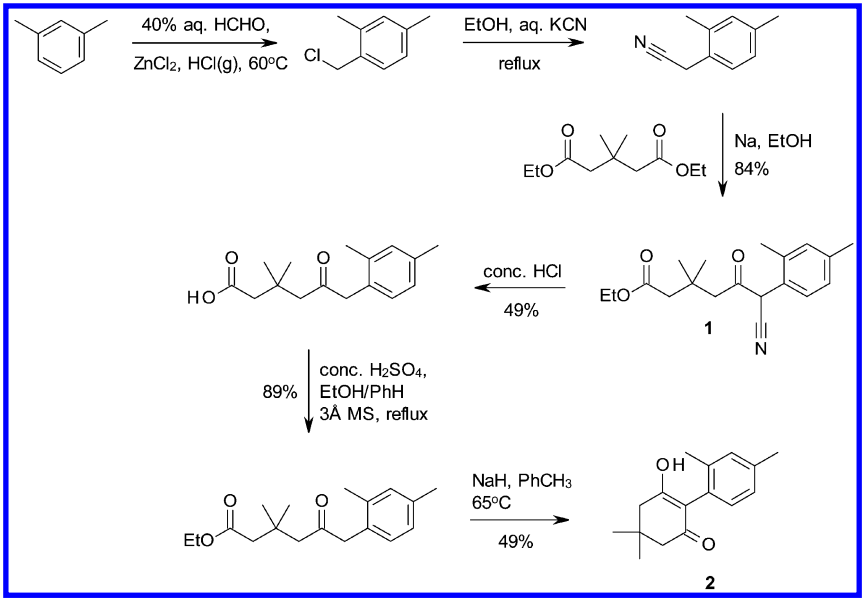

Scheme 1. Example ring-synthesis approach to carbocyclic 2-aryl-1,3-diones

As an alternative, we have investigated various late-stage aryl coupling approaches, including state of the art palladium-catalyzed conditions as reported by Buchwald and co-workers (*3*, *4*). In our hands these have proved only moderately successful and limited to examples with mono-*ortho* aryl substitution (*5*). A more reliable procedure, even for highly hindered aryl groups, was the palladium-catalyzed cross-coupling of iodonium ylides with aryl boronic acids (*6–9*), although even this approach was sometimes only moderate yielding.

The most robust method for the direct coupling of hindered aryls was found to be an unusual aryl lead cross-coupling reaction. Using conditions reported by Morgan and Pinhey (*10*), we observed that a wide variety of aryl lead reagents efficiently *C*-arylated a diverse range of cyclic 1,3-diones (*5*) *via* a proposed intermediate of type **3** (Scheme 2). To the best of our knowledge these are the first examples of mono *C*-arylation of such compounds. After further optimization of the original reaction conditions this method was used to successfully couple a wide variety of cyclic 1,3-diones and aryl fragments (*11–13*). Aryl lead reagents were prepared directly from the corresponding aryl boronic acids using lead tetraacetate and catalytic mercury(II) acetate in chloroform (*10*), followed by scavenging of the residual acetic acid over solid potassium carbonate. This acid scavenging step was critical to achieve high coupling yields. The arylation itself was performed using a large excess of *N,N*-dimethyl-4-aminopyridine in a 4:1 chloroform / toluene solvent mixture at 80 ºC. Representative iodonium ylide and aryl lead coupling approaches to the 2-aryl-1,3-dione **4** are shown in Scheme 2.

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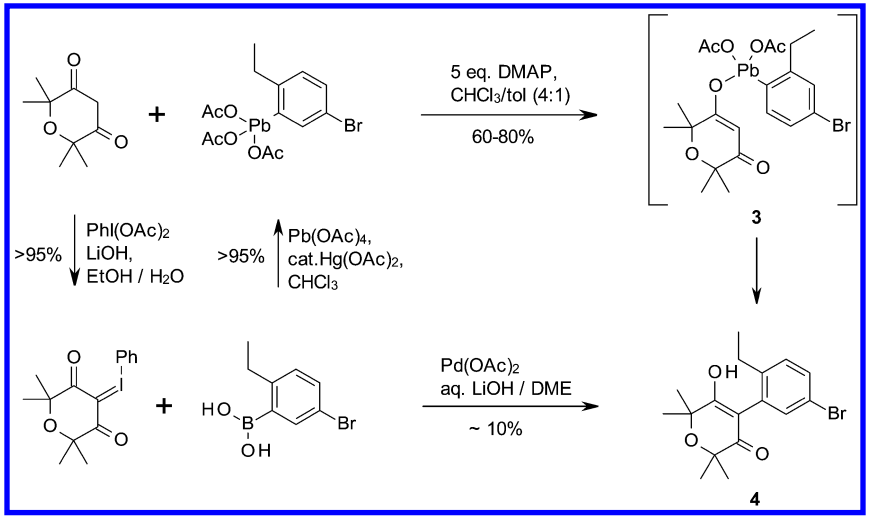

Scheme 2. Example iodonium ylide and aryl lead cross coupling reaction

Although the aryl lead coupling protocol was typically high yielding, scale up issues still remained. We therefore continued to investigate improved synthetic approaches, with a focus on novel intramolecular rearrangement chemistry. These are described in the following examples.

Claisen Rearrangement Approach

This methodology represents a novel synthesis of both five and six ring carbocyclic 2-heteroaryl-1,3-diones, inspired by the classical [3,3]-sigmatropic rearrangement of allyl phenyl ethers. In this instance the rearrangement was found to be more challenging due to competing 'benzyl shift' by-products, which usually predominated for phenyl substrates. Nevertheless, this approach worked well for the preparation of a diverse range of five-membered heterocycles, such as 2-(2-methyl-3-thienyl)cyclohexane-1,3-dione **5** (Scheme 3) (*14*).

Heteroaryl methyl ether precursors (for example, compound **6**) were prepared either by condensation of the required alcohol and cyclic 1,3-dione reaction partners (using catalytic sodium gold chloride as reported by Arcadi *et al.* (*15*)), or more generally by a conjugate addition to the specific 3-chloro enone. The 3-chloro enones were prepared from the corresponding 1,3-dione by treatment with phosphorus pentachloride in chloroform. Claisen rearrangements were performed at high temperature under microwave irradiation in dipolar aprotic solvents such as 1-methyl-2-pyrrolidinone, or alternatively in dimethoxyethane in the presence of an ionic liquid, e.g. 1-butyl-3-methyl-imidazolium bis(trifluoromethylsulfonyl)imide, to facilitate higher microwave irradiation temperatures (Scheme 3). Low reaction concentrations were found to increase yields by disfavoring the 'benzyl shift' pathway.

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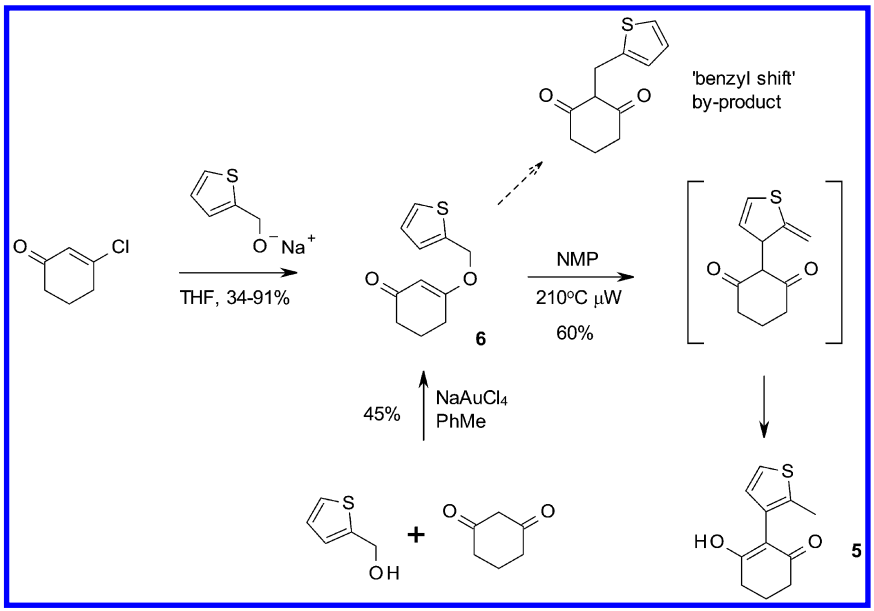

Scheme 3. Claisen rearrangement synthesis of 2-(2-methyl-3-thienyl)cyclohexane-1,3-dione

This methodology was used to successfully prepare a diverse range of *ortho*-methyl and *ortho*-ethyl 2-heteroaryl-1,3-diones. The reaction was found to be highest yielding for electron-deficient five-membered heterocycles – thiazole > thiophene > oxazole > furan – which also reflects a lower yield of the 'benzyl shift' by-product. We propose this is because of reduced stabilization of the benzyl cation intermediate which leads to the by-product. Representative applications of this methodology using highly functionalized reaction partners are shown in Scheme 4 (*16*).

Piancatelli Rearrangement Approach

Since the Claisen rearrangement approach had proven unsuccessful for the synthesis of 2-phenyl-1,3-diones we continued investigations into alternative routes. The most general method developed for the synthesis of five ring carbocyclic examples of this compound class was based on the Piancatelli rearrangement of furfuryl alcohols (*17*), such as compound **7** (Scheme 5). The key transformation was promoted using the aqueous polyphosphoric acid conditions as described by Saito and Yamachika for a related reaction (*18*). The yield of the rearrangement to the corresponding hydroxy-enone product (e.g. compound **8**) was usually very high, irrespective of the nature and number of aryl substituents (*19*).

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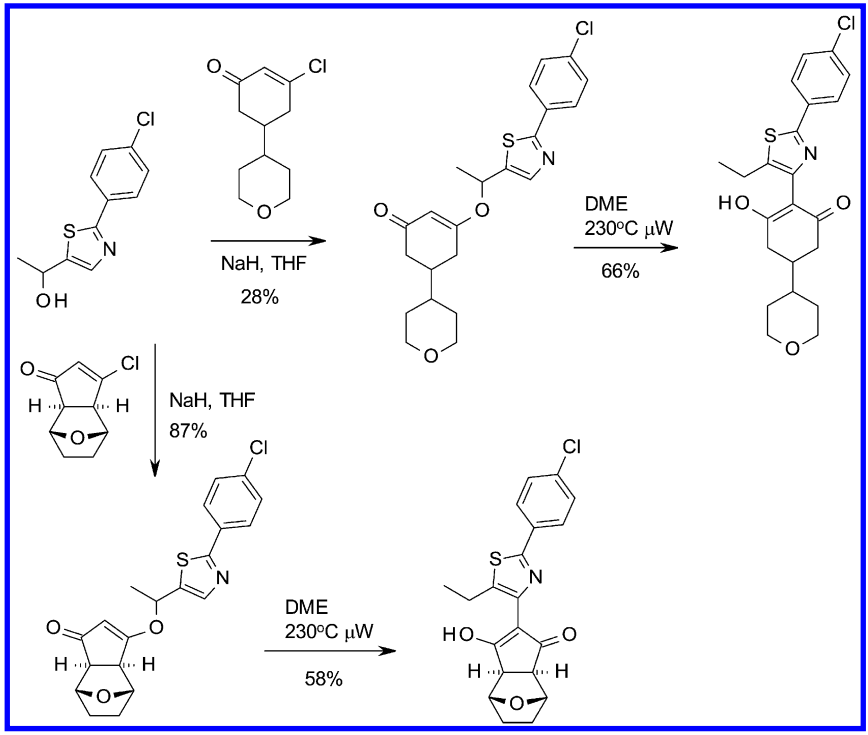
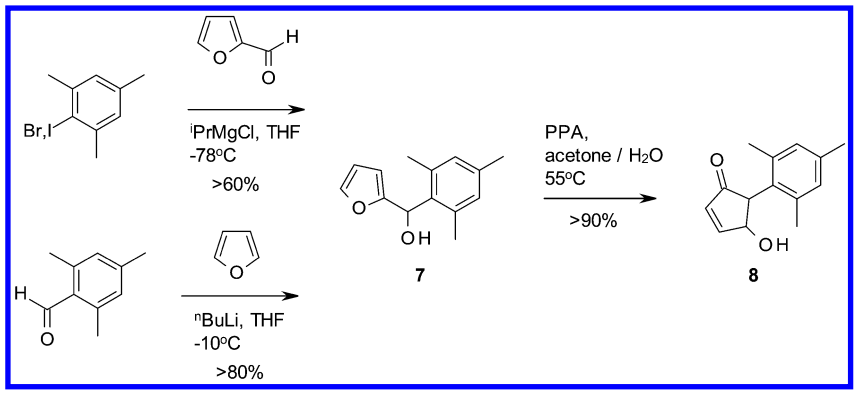


Scheme 4. Example applications of the novel Claisen rearrangement methodology



Scheme 5. Example application of Piancatelli rearrangement methodology

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Hydroxy-enone products (for example, compound **8**), were subsequently oxidized using Jones reagent, then further derivatized by either cycloaddition or C5-alkylation of the derived enol ether (Scheme 6). Cycloadditions were successfully carried out with furans (leading to compounds such as **9**) (*11*), cyclopentadienes (*19*), cyclohexadienes (*19*), nitrones, nitrile oxides and thiophene *S*-oxides (Figure 1), often in the presence of Lewis acids such as magnesium iodide. Alternative C5-alkylated 1,3-diones were accessed by the zinc / acetic acid reduction of the cyclopentene-1,3-dione precursor, followed by protection as the methyl enol ether. These intermediates were then deprotonated with strong base, such as potassium hexamethyldisilazide, and quenched with various aldehydes such as 2-pyridinecarboxaldehyde to afford the corresponding exocyclic enone. Synthesis of the final products, such as compound **10**, was completed by a second zinc reduction followed by acid catalyzed deprotection of the methyl enol ether (*20*).

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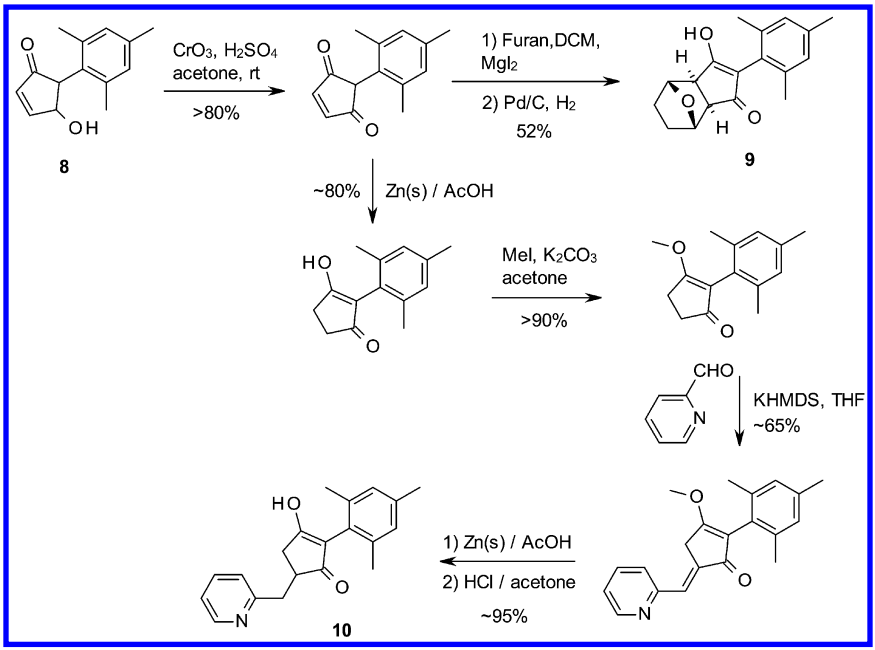

Scheme 6. Elaboration of an example hydroxy-enone intermediate by both cycloaddition and C5-alkylation chemistry

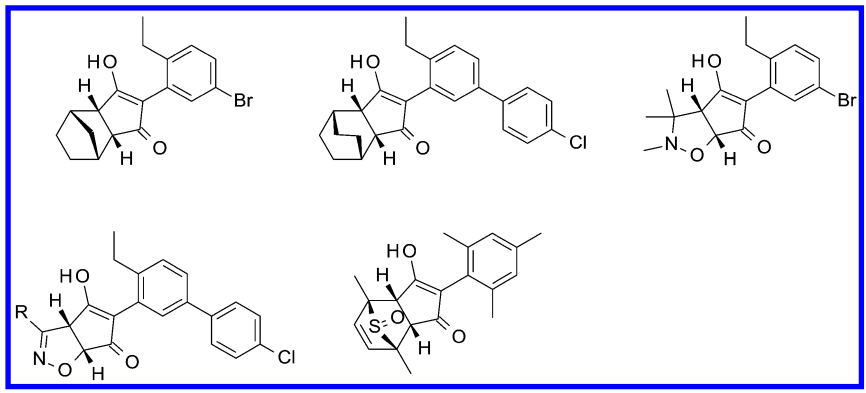

Figure 1. Examples of additional 2-phenyl-1,3-diones derived from cycloaddition chemistry

Enol Lactone Rearrangement Approach

In order to synthesize six ring carbocyclic 2-phenyl-1,3-diones additional synthetic methodology was required. The first approach was based on the intramolecular rearrangement of enol lactones (e.g. compounds **11** and **12**, Scheme 7). After substantial optimization two complementary sets of reaction conditions were identified. Rearrangement under acidic conditions was performed using Eaton's reagent (*21*) (7.7% phosphorus pentoxide in methane sulfonic acid) in toluene at 70 ºC, which very rapidly and reliably led to moderate to high yields of the desired products such as compound **13** (*13*). The same transformation was also successfully achieved under basic conditions using catalytic potassium cyanide and triethylamine in acetonitrile (*22*), although this alternative procedure was found to be slightly less general.

The enol lactone precursors were prepared by one of two general methods. The first approach involved condensation of a ketone and an aldehyde, followed by regioselective Baeyer-Villiger oxidation. The aldol step was performed under a variety of standard conditions, such as potassium hydroxide in ethanol, and was typically selective for the *E*-olefin. This methodology was high yielding when the α′-position was blocked, preventing a second condensation. The subsequent regioselective Baeyer-Villiger oxidation was performed using the selenium dioxide catalyzed conditions reported by Guzmán *et al.* (*23*), which afforded the *E*-enol lactone resulting from preservation of olefin geometry, as expected.

The second approach was a silver or gold catalyzed lactonisation of an appropriately functionalized aryl-acetylene precursor (e.g. compound **15**), *via* a formal *6-exo-dig* cyclisation. Near quantitative conversion was achieved using silver carbonate in acetonitrile (*22*), which afforded the *Z*-olefin **12**. Representative examples of both the Baeyer-Villiger and silver-catalyzed lactonisation strategies are shown in Scheme 7.

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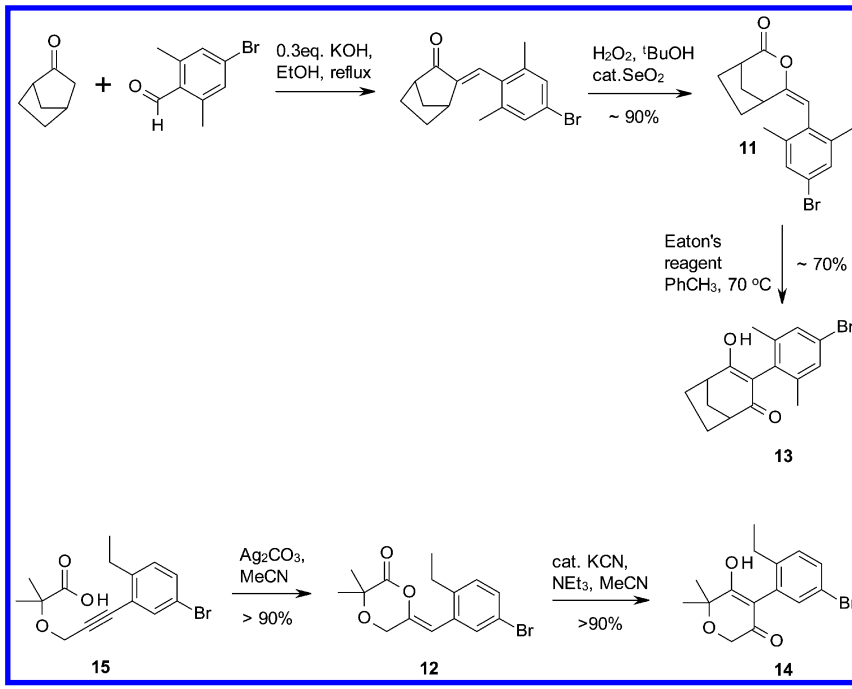

Scheme 7. Example of enol lactone rearrangement approaches to bicyclo[2.2.1]heptan-2-one and dimethylpyrandione 2-aryl-1,3-diones

Semi-Pinacol Rearrangement Approach

The final approach developed for the synthesis of carbocyclic 2-aryl-1,3-diones was the semi-pinacol rearrangement of epoxy-ketones (*24*, *25*). This method leads to 2-aryl-1,3-diones, rather than isomeric 1,2-diones, through a preferential acyl transfer mechanism (proceeding *via* proposed intermediates **16** and **17**, Scheme 8).

This ring expansion has proved very reliable and efficient across a diverse range of phenyl and heteroaryl substituted epoxides, for example compounds **18** and **19** (Scheme 8), using a variety of protic and Lewis acid catalysts. Preferred rearrangement conditions involve treatment with concentrated sulfuric acid in dichloroethane, or boron trifluoride diethyl etherate in dichloromethane (*25*).

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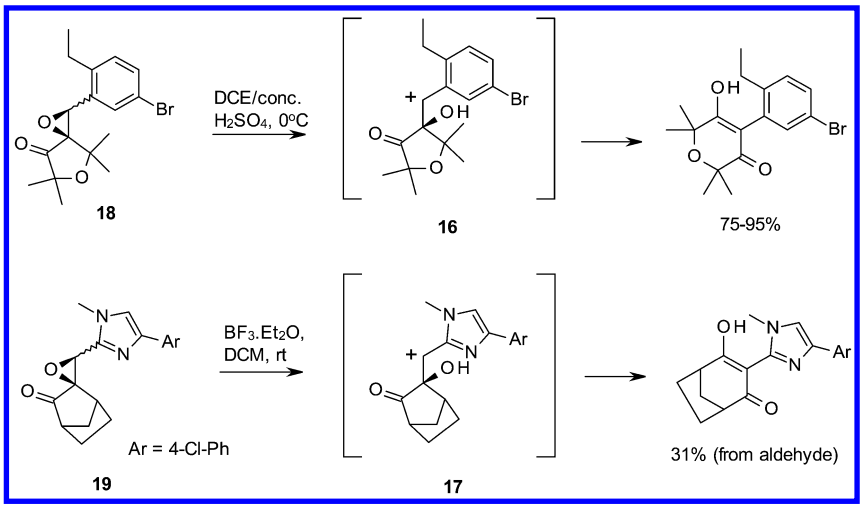

Scheme 8. Example of semi-pinacol rearrangement approaches to tetramethylpyrandione and bicyclo[2.2.1]heptan-2-one 2-aryl-1,3-diones

Epoxide starting materials (compounds **18** and **21**, Scheme 9) were synthesized by either addition of hydrogen peroxide to the enone precursor or Darzens epoxidation. Enone epoxidation using basic hydrogen peroxide was initially problematic due to over-oxidation to the carboxylic acid **20** (after hydrolysis), but this Baeyer-Villiger step was minimized by using catalytic rather than stoichiometric metal hydroxide, higher reaction temperatures and lower reaction concentrations. Lithium hydroxide was found to afford higher yields than either potassium hydroxide or sodium hydroxide (*25*). The alternative Darzens reaction was achieved by deprotonation of the α-bromo ketone (for example, compound **22**) using potassium *tert*-butoxide in the presence of the aryl aldehyde reaction partner (*25*). Highest yields were obtained in dipolar aprotic solvents such as dimethyl sulfoxide or *N,N*-dimethylformamide. This reaction was limited to substrates lacking an acidic α′-hydrogen, such as bicyclo[2.2.1]heptan-2-one, Scheme 9.

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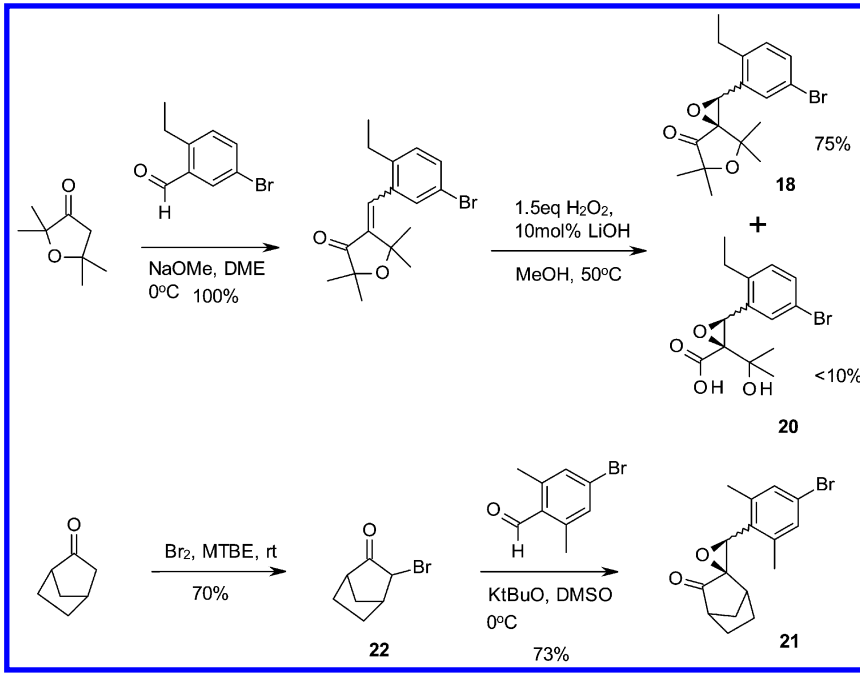

Scheme 9. Synthesis of example epoxides by hydrogen peroxide and Darzens approaches

Case Study - Meta-Biphenyl Tetramethylpyrandiones

During this work *meta*-biphenyl tetramethylpyrandiones were identified as a novel ACCase chemical class with outstanding herbicidal activity, particularly for the post-emergence control of warm climate grasses (*22*). The glasshouse biological profile of three example *meta*-biphenyl tetramethylpyrandiones **23**, **24** and **25** is shown in Table 1.

The original route to these compounds involved an aryl lead cross-coupling (as described earlier) to access compound **4** (Scheme 2), followed by a palladium-catalyzed Suzuki cross-coupling as the final step (*22*). The aryl lead reagent was prepared in four steps starting from 2-ethyl-5-bromonitrobenzene (*22*), and the 1,3-dione coupling partner 2,2,6,6-tetramethylpyran-3,5-dione was prepared in three steps from 2,2,5,5-tetramethyltetrahydrofuran-3-one **26** (*26*). The overall length of this chemical sequence and undesirable nature of certain reagents led us to investigate an improved large scale process which would also reduce the cost of goods. The optimized route to *meta*-biphenyl tetramethylpyrandiones is outlined in Scheme 10.

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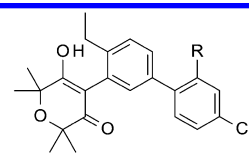
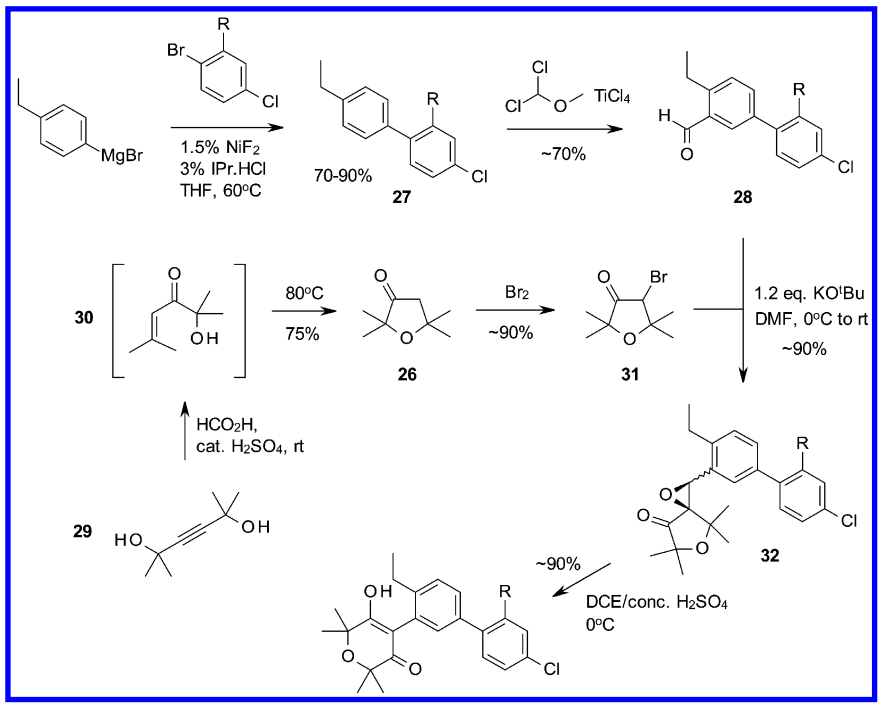
Table 1. Herbicidal activity of example *meta*-biphenyl tetramethylpyrandiones



Cmpd	*R*	*g/ha*	*ALOMY*	*AVEFA*	*BROTE*	*LOLPE*	*SETFA*	*PANMI*	*SORVU*	*DIGSA*	*ECHCG*
23	H	60	60	70	30	90	100	100	100	100	100
24	Cl	60	30	70	0	70	100	100	60	100	100
25	F	60	60	0	30	0	80	100	100	30	100

Percentage post-emergence control of grass weeds at 60g/ha application rate



Scheme 10. Optimized large scale route to meta-biphenyl tetramethylpyrandiones

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Synthesis of biphenyl **27** was achieved by a nickel-catalyzed Kumada cross-coupling of 4-ethylphenylmagnesium bromide with the required 4-chloro bromobenzene. This process was considered more attractive than the more conventional palladium-catalysed Suzuki cross-coupling due to the lower cost of the metal catalyst and shorter reaction sequence. Various catalytic systems were thoroughly explored before we identified the nickel(II) fluoride / 1,3-bis-(2,6-diisopropylphenyl)imidazolium chloride conditions, originally reported by Nakamura and co-workers (*27*), as highly efficient. This reaction was not only halogen selective (oxidative insertion into the carbon-bromine not carbon-chlorine bond), but also very selective for the desired cross-reaction product. Almost all other conditions evaluated afforded relatively high yields of both the Grignard and aryl halide homocoupling products, which were difficult to separate and, in the case of polyhalobiphenyls, also associated with toxicity alerts. The mechanism of this catalytic system is proposed to proceed *via* a nickel (II) – nickel (IV) cycle which promotes a fast reductive elimination, therefore minimizing ligand scrambling leading to homocoupling (*27*). Conversion of biphenyl **27** to benzaldehyde **28** was achieved by regioselective formylation using dichloromethyl methyl ether and titanium tetrachloride in dichloromethane in 70% yield.

During this program we also successfully developed a novel protic acid rearrangement of 2,5-dimethyl-3-hexyne-2,5-diol **29** to 2,2,5,5-tetramethyltetrahydrofuran-3-one **26**. All literature reports of this transformation use mercury catalysis (for example (*28*)), however we identified that specific formic acid / concentrated sulfuric acid conditions were very effective. The highest yields were achieved by an overall one pot, two-step process, in which compound **29** was stirred at room temperature to afford the intermediate enone **30** (*via* a presumed Meyer–Schuster type rearrangement), then briefly heated at 80 ºC to effect the formal *5-endo-trig* cyclisation (Scheme 10).

The final stages of this new synthesis relied upon the recently developed Darzens epoxidation – semi-pinacol rearrangement sequence outlined earlier in the chapter. In this instance the fully assembled biphenyl aldehyde **28** reacted very smoothly with the bromo ketone **31** using potassium *tert*-butoxide in *N,N*-dimethylformamide to provide epoxide **32** in excellent 90% yield. The subsequent acid-catalysed rearrangement using concentrated sulfuric acid / dichloroethane was also highly efficient, affording the final target compound(s) in approximately 90% yield (Scheme 10).

Conclusion

A range of novel synthetic methodologies have been developed and introduced into the discovery program of ACCase-inhibiting carbocyclic 2-aryl-1,3-diones. These new approaches have allowed the rapid SAR exploration of a diverse range of chemical classes, leading to the identification of *meta*-biphenyl tetramethylpyrandiones as highly active post-emergence graminicides. An extremely efficient and concise large scale synthesis of this exciting chemical class was also developed.

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Chapter 22

4-Azolyl-5-Hydroxy Pyridazinones: Potent Grass Herbicides

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We have found that 4-azolyl-5-hydroxy pyridazinones are potent grass herbicides which control sensitive species at rates as low as 4 g/ha in both pre- and post-emergence application. The linkage of the azole group to the pyridazinone can be either through nitrogen or through carbon. These compounds act by inhibiting Acetyl CoA Carboxylase (ACCase).

Introduction

One enduring facet of the discovery process for new crop protection agents is that synthetic intermediates and unexpected products often yield starting points for further work. During our work on the discovery and optimization of the chlorantraniliprole (Rynaxypyr®) we discovered some unexpected reactivity of azoles (*1*). As shown in Scheme 1 our synthetic route was based on displacement of 2,3-dichloropyridine with various 3-substituted pyrazoles. These intermediates were then lithiated and carboxylated to lead to 5-pyrazole carboxylic acids. Such intermediates were converted to anthranilic diamides and showed high levels of insecticidal activity.

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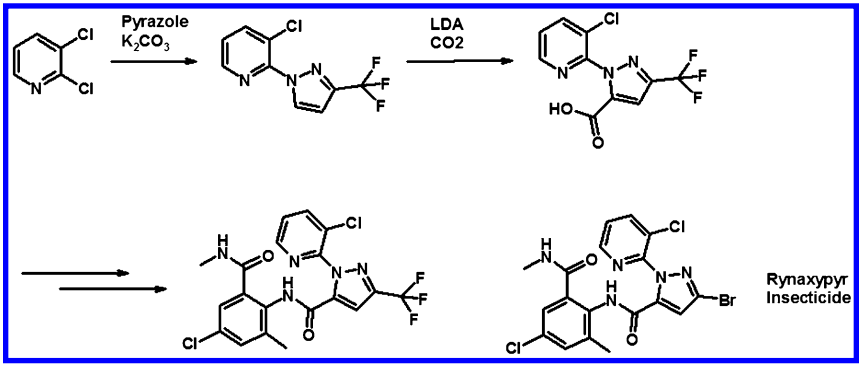
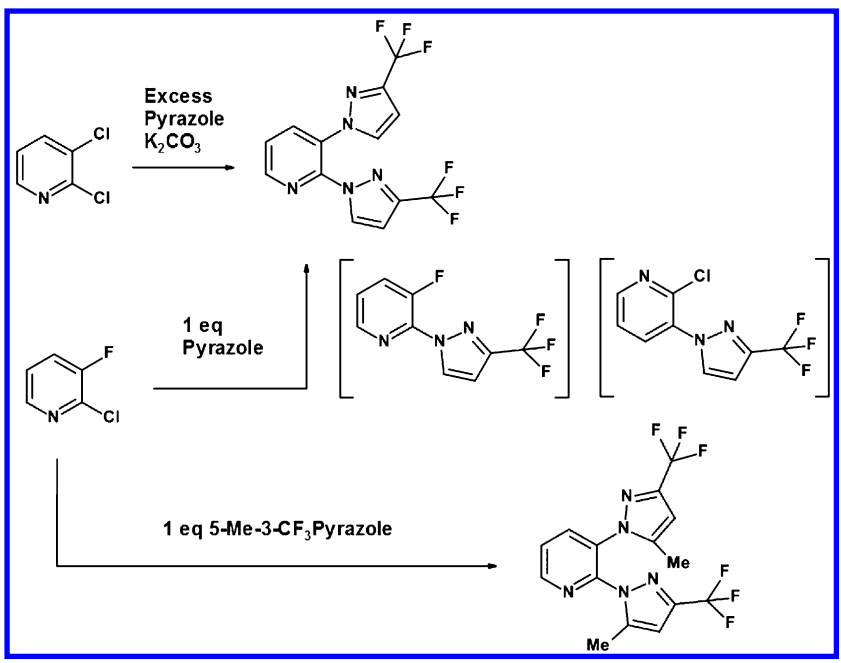


Scheme 1. Synthesis of AnthranilicDiamide Insecticides

One unexpected class of byproducts we saw was the displacement of both chlorines to give the 2,3-bis(3-substituted pyrazolyl)pyridines (Scheme 2). When we turned to 3-fluoro-2-chloropyridine, the disubstituted material was the sole product which we isolated even if we used only 1 equivalent of pyrazole. Because only one product was isolated, it was not clear which halogen was substituted first. This tendancy extended to more hindered substrates such as 3,5-disubstituted pyrazoles.



Scheme 2. Disubstitution of 2,3-Dihalopyridines

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We then turned to some more reactive dihaloheterocyclic systems such as 2,3-dichloropyrazine and 4,5-dichloropyridazinones and observed the same propensity to only form disubstituted adducts with pyrazoles (Scheme 3). This corroborated the report that hexafluorobenzene preferentially forms a hexa-substituted adduct with even decidedly sub-stoichiometric amounts of azoles (*2*). For the synthesis of analogs of anthranilic acid diamides, these bis-pyrazoles initially appeared to be synthetic dead ends. However, we wondered if they would react with nucleophiles to displace one of the azoles. This turned out to be the case, reaction with sodium methoxide smoothly displaced one of the azoles to give the *ortho*-methoxy azole products in good yield.

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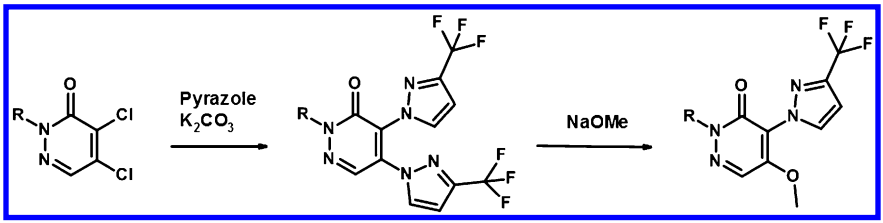

Scheme 3. Disubstitution and Nucleophilic Displacement on Pyridazinones

Chemistry

The discovery of the ability of pyrazoles in particular to activate nucleophilic substitution did not initially provide the impetus to use it to design new synthetic targets for crop protection utilities. However, in 2003 Bayer CropScience reported that 3-arylpyrazoles were effective substituents in an area of both herbicide and insecticide chemistry based on inhibition of Acetyl CoA Carboxylase (ACCase) (*3*). This was particularly surprising to us since we had previously prepared herbicidally inactive 3,5-dimethylpyrazole keto-enols. Bayer dubbed this class of compounds as the keto-enols based on the predominant tautomers of the dicarbonyl scaffolds for which they found activity (Figure 1). This discovery piqued our interest because we had previously discovered that pyridazinones with a 5-hydroxy group could be effective scaffolds to replace dicarbonyls in ACCase herbicides (*4*). Further motivation for work in this area stemmed from the fact that pinoxaden, an analog of the keto-enols, was commercialized as a herbicide by Syngenta as Axial (*5*). Bayer commercialized 3 insecticidal candidates in the keto-enol area, but none as herbicides.

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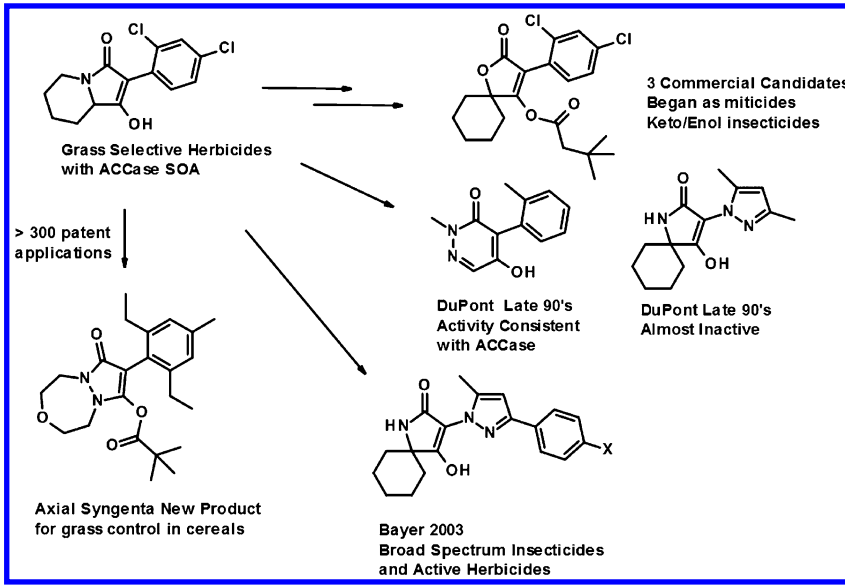

Figure 1. Keto-enol Inhibitors of ACCase

The unexpected nucleophilic displacement chemistry which we had discovered in our anthranilic diamide insecticide work seemed to provide a direct way to prepare nitrogen linked (*N*-linked) pyrazoles on the pyridazinone nucleus. Based on our earlier work on pyridazinones as ACCase inhibitors, exploring pyridazinones with *N*-linked 3-arylpyrazoles appeared to be a fertile area to investigate. In our initial experiment, we heated a 3-aryl-5-methylpyrazole with *N*-methyl-4,5-dichloropyridazinone in the presence of potassium carbonate in dimethylacetamide and isolated the desired bis-pyrazole in good to excellent yield (Scheme 4). Displacement with sodium methoxide proceeded in high yield to provide the 5-methoxy product. The product could be deprotected to unmask the keto-enol fragment with boron tribromide. Later work showed that the deprotection could also be performed with aqueous hydroxide in dioxane as well. Direct deprotection of the bis-pyrazole was also possible, but initially complicated isolation of the final product. To our delight, herbicidal testing revealed this compound to have excellent activity on grasses in both pre- and post-emergence testing (*6*). As expected testing of the compound against isolated ACCase enzyme revealed it to be a potent inhibitor.

The excellent activity for our intial target led us to undertake an extensive analoging program. In order to vary the substitution of the *N*-1 position of the pyridazinone we followed two general routes (Scheme 5). Alkylation of the commercially available NH compound could be accomplished with potassium carbonate in dimethylformamide with a variety of alkyl halides. In the case of *c*-propyl, a modified Chan-Lam coupling was employed (*7*). In order to obtain the NH compound itself we protected the N with the tetrahydropyranyl (THP) group then followed the general synthetic method and deprotected with acid in

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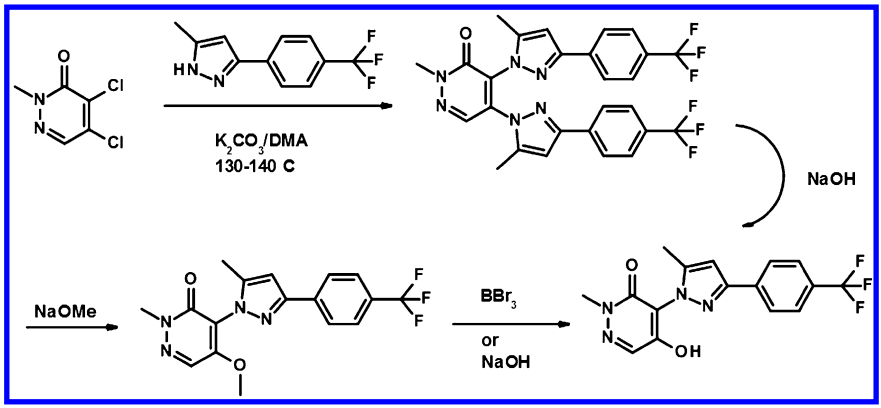
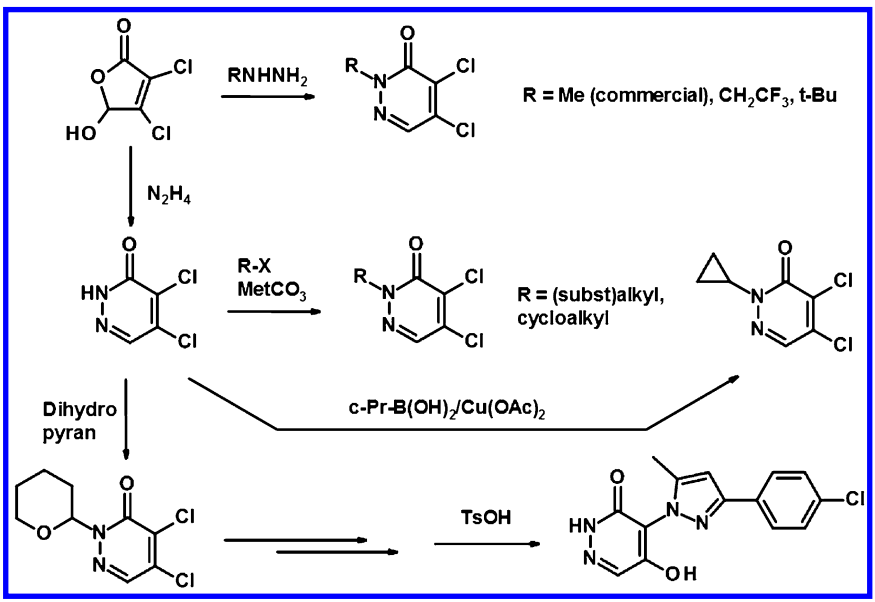
the final step. The second method was to react commercially available hydrazines with mucochloric acid by cyclization to provide the desired starting materials.

We also explored substitution at the 6-position of the pyridazinone ring (Scheme 6). Reaction of dihalomaleic anhydrides with hydrazine or substituted hydrazines provides 6-hydroxypyridazinones with 4,5-dihalo-substitution (*8*). Alkylation with alkyl halides or triflates provides 6-alkoxypyridazinones cleanly. In the case of the unsubstituted hydrazine adduct, the *N*-methyl, *O*-methyl product can be directly obtained by treatment with dimethyl sulfate. Under these conditions *O*-substitution greatly predominates over *N*-1 alkylation.



Scheme 4. Initial Synthesis of N-Pyrazolyl Pyridazinones



Scheme 5. Preparation of Various N-1 Substituted Compounds

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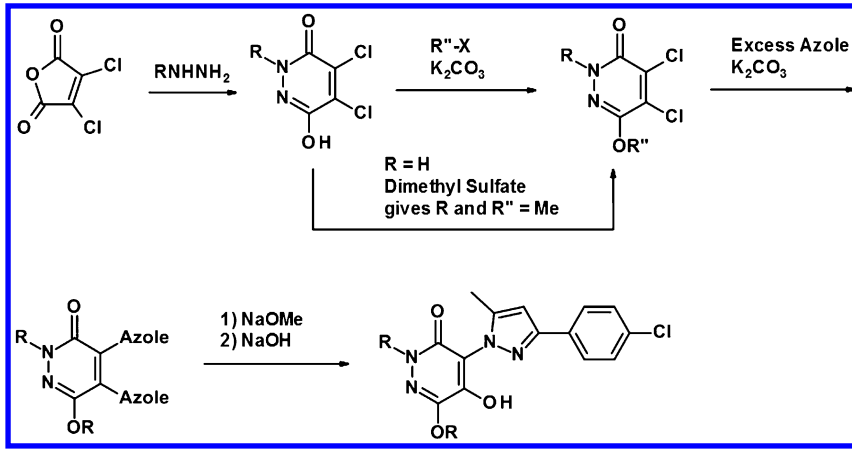
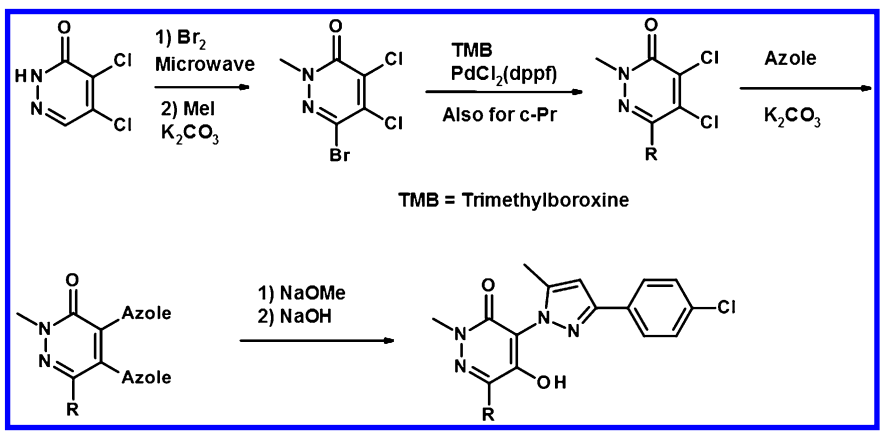


Scheme 6. Synthesis of 6-Alkoxy Targets

In order to prepare 6-alkyl targets we had to employ a different synthetic strategy as shown in Scheme 7. We discovered that it was possible to brominate the 6-position of the *N*-unsubstituted pyridazinone under forcing conditions in the microwave. The intermediate could be alkylated with methyl iodide to provide the starting material for coupling reactions. We were able to methylate the 6-position with trimethylboroxine in the presence of a palladium catalyst, but in addition to the desired product also recovered both 4,6- and 5,6-dimethyl side products. Use of dimethylzinc provided an even less desirable mixture of products. Other alkyl boronic acids such as *c*-propyl also gave 6-substituted products.



Scheme 7. Synthesis of 6-Alkylpyridazinones

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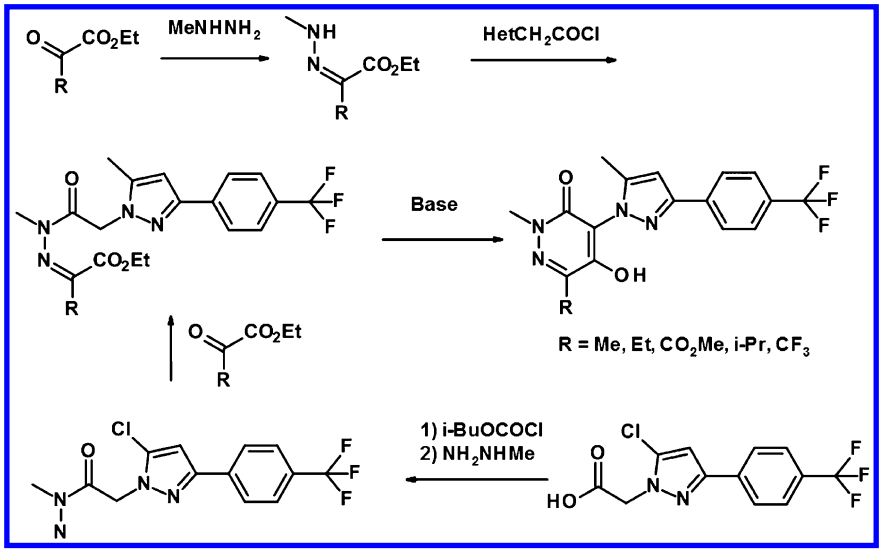
An alternative route to C-6 carbon substituents was based on related cyclization chemistry reported by Sumitomo as shown in Scheme 8 (*9*). Hydrazones of alpha-keto esters were alkylated on nitrogen with acid chlorides derived from *N*-pyrazolyl acetyl chlorides. Cyclization was accomplished by treatment with a strong base such as potassium t-butoxide. Alternatively, direct reaction of *N*-pyrazolyl acetyl hydrazides with the alpha-ketoesters provided the same intermediate.



Scheme 8. Synthesis of Variously 6-Substituted Pyridazinones

We also wished to synthesize azoles and other heterocyles that were linked through carbon in addition to nitrogen. Our original route to *C*-linked pyrazoles relied on a Sydnone cycloaddition (Scheme 9). Stille reaction of a known 4-iodopyridazinone with tributylstannylpropyne or acetylene followed by treatment of the resulting acetylene with *N*-4-chlorophenylsydnone at reflux in xylenes gave low to moderate yields of separable mixtures of the 3- and 4-substituted *C*-linked pyrazole products. Pyrazoles lacking 4-substitution could be halogenated with *N*-halosuccinimides or SelectFluor. The 3-substituted *C*-linked pyrazoles retained similar herbicidal activity to the *N*-linked pyrazoles, but the 4-substituted pyrazoles were generally less active.

We turned to a route we had previously used to introduce a wider variety of groups by Stille Coupling chemistry as shown in Scheme 10 (*4*). Reaction of 4-iodo or bromo pyridazinones with hexamethyl distannane in the presence of a palladium catalyst gave the 4-trimethylstannylpyridazinones. These were then coupled with substituted iodoazoles and other iodoheteroaromatics in good yield in a second Stille Coupling. The efficiency of the couplings was increased by the addition of CuI as a co-catalyst.

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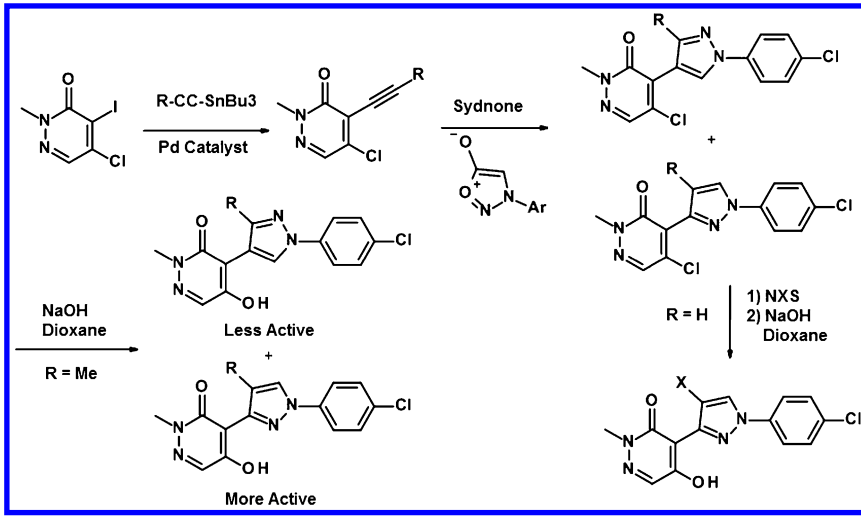
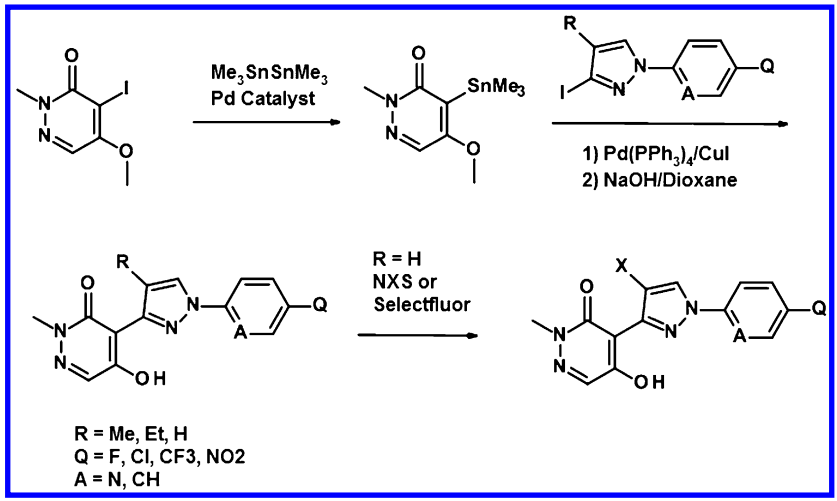


Scheme 9. Sydnone Cycloaddition Approach to C-Linked Pyrazoles



Scheme 10. Stille Coupling Approach to C-Linked Heterocycles

Despite the ready synthesis of the target molecules by the double-displacement route, we were quite interested in finding more cost and material effective ways to synthesize our final products (Scheme 11). We were intrigued by the reactions of dichloropyridazinones with sodium nitrite which were reported by BASF in the 1960s (*10*). After chlorination of the hydroxy intermediate, the 4-chloro-5-nitropyridazinones are much more reactive at the 4-position and can be readily mono-displaced with pyrazoles at room temperature. The nitro group can then be hydrolyzed with aqueous hydroxide to provide the desired materials. Alternatively, 4-methoxy-5-chloropyridazinones have been reported by Syngenta to react selectively at the 4-position with benzazoles (*11*).

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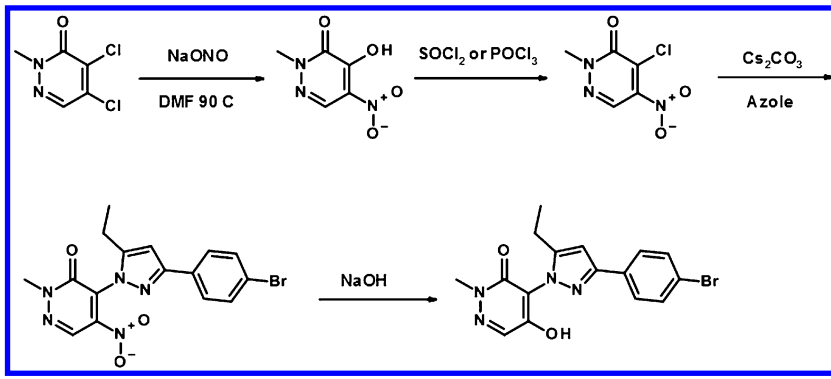

Scheme 11. Alternative Route to 4-Azolylpyridazinones

Biology

Azolylpyridazinones are very active herbicides in both post- and pre-emergence application. Activity on grass species of optimal compounds is apparent at rates as low as 4 g/ha. Post-emergence activity of some representative compounds is shown in Table 1. The data shown represents the average control of 14 grass weeds at 8 g/ha planted in sandy loam soil and evaluated 14 days after treatment. The weeds included in the average were bermudagrass, blackgrass, downy brome, giant foxtail, green foxtail, Italian ryegrass, johnsongrass, large crabgrass, Surinam grass, canarygrass, wild oats, windgrass, wooly cupgrass, and goosegrass. Pinoxaden showed only 61% average control of these weeds at this application rate.

Pre-emergence activity was also very strong for the area. The data shown in Table 2 represents the average control of 10 grass weeds at 8 g/ha planted in sandy loam soil and evaluated 14 days after treatment. The weeds included in the average were bermudagrass, downy brome, giant foxtail, green foxtail, Italian ryegrass, johnsongrass, large crabgrass, Surinam grass, wild oats, wooly cupgrass, and goosegrass. Pinoxaden showed only 9% average control of these weeds at this application rate.

The compounds studied showed safety to all broadleaf crops tested. Varying levels of safety to grass crops were observed. While safety to corn was not common, safety to wheat and especially to barley was observed. In particular, pyrazoles which were disubstituted at the 4- and 5-positions with alkyl groups were safest to cereals. The best safety was observed with 5-ethyl-4-methylpyrazoles. Cereal safety was observed both pre- and post-emergence, but was more pronounced in pre-emergence applications.

Because of the broad spectrum of grass control, field trials were carried out at a variety of locations. Considerable herbicidal activity both pre- and post-emergence was observed at rates between 35 and 70 g/ha for **DP-1**, **DP-2** and **DP-7**. Of these compounds, **DP-7** demonstrated the best residual control in field conditions with good control of grass weeds in soybeans at 35 g/ha to at least 60 days.

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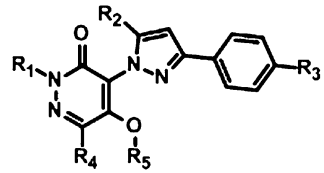
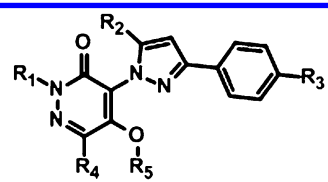
Table 1. Post-emergence Weed Control at 8 g/ha



Entry	*R1*	*R2*	*R3*	*R4*	*R5*	*% Control*
DP-1	Et	Et	I	H	H	95
DP-2	Me	Et	I	H	H	94
DP-3	*n*-Pr	Et	I	H	H	93
DP-4	Me	*c*-Pr	I	H	H	92
DP-5	Me	*c*-Pr	I	H	CO-*t*-Bu	92
DP-6	*i*-Pr	Et	I	H	H	91
DP-7	Me	Et	Br	H	H	90
DP-8	Me	Et	I	OMe	H	89
DP-9	Me	*n*-Pr	I	H	H	87
DP-10	Me	Et	CF_3	H	H	87
DP-11	Me	Me	I	H	H	84
DP-12	Et	Me	I	H	H	84

Table 2. Pre-emergence Weed Control at 8 g/ha



Entry	*R1*	*R2*	*R3*	*R4*	*R5*	*% Control*
DP-1	Et	Et	I	H	H	91
DP-2	Me	Et	I	H	H	82
DP-8	Me	Et	I	OMe	H	83
DP-10	Me	Et	CF_3	H	H	86
DP-11	Me	Me	I	H	H	95
DP-13	Me	Et	CF_3	H	CO-i-Pr	85

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Conclusions

The azolylpyridazinones proved to be a very potent family of grass herbicides. The serendipitous discovery during the optimization of anthranilic diamide insecticides that pyrazoles activate halogens in the ortho-position of heterocycles towards further nucleophilic substitution was the key to discovery of this area of chemistry. These compounds control a very broad range of grass weeds in both pre- and post-emergence testing. The excellent pre-emergence control of grasses at extremely low application rates is noteworthy in comparison to known areas of ACCase chemistry (dims, fops and dens). Field activity of the compounds was demonstrated at rates of between 35 and 70 g/ha.

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Chapter 23

Chemical Control of Root Parasitic Weeds

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Root parasitic weeds in Orobanchaceae family including broomrapes (*Orobanche* spp. and *Phelipanche* spp.) and witchweeds (*Striga* spp.) cause serious damage to worldwide agriculture affecting 300 million people and causing an annual loss of US$ 7 billion or more. Therefore, an effective strategy for control of the root parasitic weeds is desirable. Several potential methods for controlling root parasitic weeds have been proposed. However, because of their complicated lifecycle that is closely associated with host crops, few practical methods are currently available for control of these species. Agrochemicals have been conventionally used to manage agricultural fields and continue to be valid today. Extensive efforts are being made to develop novel methods for the selective control of root parasitic weeds. In the present review, strategies to control root parasitic weeds by using conventional herbicides and natural growth inhibitors, the so-called suicidal germination strategy, as well as new approaches emplying the "omics" technologies in recent years, are discussed along with the associated problems and future challenges.

Introduction

Weedy parasitic plants cause problems to agriculture and horticulture globally. They parasitize the host plants utilizing nutrients absorbed from them. Parasitic plants account for about 1% of all seed plants and comprise

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of approximately 4,500 species across 270–275 genera (*1*, *2*). A criterion for defining whether a parasitic plant is weedy or non-weedy is the importance of its host plant as a crop. If the host is an economically important crop species and is affected by the parasitic plant, then this parasitic species is certainly regarded as a weed (*3*). Root parasitic weeds in the family Orobanchaceae, which parasitize the roots of hosts and do not survive without them (obligate parasites), are among the most destructive agricultural weeds. Within this family, holoparasitic weedy broomrapes (*Orobanche* spp. and *Phelipanche* spp.) and hemiparasitic weedy witchweeds (*Striga* spp.) cause particularly devastating damages to agricultural crops.

The witchweeds, *Striga* spp., are mainly distributed in Africa. *Striga hermonthica* and *S. asiatica* parasitize staple crops, such as sorghum, millet, rice, and maize, and therefore, are considered the largest biological cause for serious crop losses on the continent. *S. hermonthica* is estimated to cause annual losses of at least US$ 7 billion in African agriculture. *S. gesnerioides* parasitizes dicotyledonous cash crops, such as cowpea, tobacco, and sweet potato. Under such serious situations, many international programs have been implemented to establish effective methods for the control of parasitic weeds. For example, The Bill & Melinda Gates Foundation supported a project to help maize and cowpea farmers in African countries. The broomrapes, *Orobanche* spp. and *Phelipanche* spp., are mainly distributed in the Mediterranean region, Southern and Eastern Europe, and West Asia, and cause damage to a wide range of agricultural crops. *Orobanche crenata* parasitizes important legumes such as faba bean, chickpea, lentil, and pea. Carrot is also heavily damaged by *O. crenata*. *Phelipanche aegyptiaca* causes serious damage to tomato. In addition to food crops, oil crops such as sunflower and rapeseed are damaged by *P. ramosa* and *O. cumana*, respectively. Other species like *O. cernua*, *O. foetida*, and *O. minor* also affect agriculture (*3*–*7*).

These parasitic weeds draw away water and nutrients from their host crops causing serious reductions in their growth and yield. The seeds produced by mature flowers are numerous (10,000–50,000 seeds, annually) and tiny (around 0.2 mm) aiding their expansive dispersal (*8*, *9*). These characteristics also make it difficult for farmers to detect the seeds and remove them from the crop fields. Moreover, the long-lived seeds that can survive for several decades have increased chances of parasitism, causing long-lasting damage to the crops upon infestation (*10*).

Though several potential methods for controlling root parasitic weeds have been proposed (*5*, *9*, *11*, *12*), there are few practical methods currently available to control these species because of their complicated lifecycle that is closely associated with host crops. The most effective strategy proposed for the control is to reduce the soil seed bank and/or inhibit the parasitic weeds at early growth stages (e.g., during germination and radicle elongation) before their attachment to the hosts because most of the damage to the hosts occurrs underground. Agrochemicals have been conventionally used to manage agricultural fields and continue to be valid today. Extensive efforts have been invested in developing novel methods for the selective control of root parasitic weeds. This review discusses the chemical control strategies to control root parasitic weeds from

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conventional herbicides to recent approaches utilizing the "omics"-based technologies.

Herbicides

Herbicides can reduce, to some extent, the damage to crops caused by the root parasitic weeds (*5*, *13*). However, damage to the crops themselves by herbicides sometimes becomes a problem, because there is no parasite-selective herbicide in the real sense. There is also a difficulty in controlling the root parasitic weeds by herbicides because damage to hosts occurs before the emergence of parasitic weeds above the ground as mentioned above, especially in the case of broomrapes (*13*). Nonetheless, extensive efforts have been made to control the parasitic weeds by herbicides since 1956 when *S. asiatica* was identified in the United States (*14*).

For *Striga*, dicamba and 2,4-D are used as feasible herbicides in African countries, although there are associated economical and technical problems (*15*). In fact, 2,4-D was used in the early program to eradicate *S. asiatica* in the United States. Then, dinitroaniline herbicides, such as trifluralin, benefin, fluchloralin, and pendimethalin, were used in the program, but some *Striga* could survive underground and caused damage to the host crops. Diphenyl ethers, such as oxyfluorfen and fomesafen, were shown to control *Striga* effectively in the program (*14*). Finally, ethylene gas used as a germination stimulant in the suicidal germination strategy successfully eradicated *S. asiatica* in the United States as described later in this review (*14*, *16*). In African and Mediterranean countries, many other modern herbicides were evaluated for their ability in controlling root parasitic weeds effectively (*13*). For example, seed coating of cowpea with an imidazolinone herbicide, imazaquin, was shown to effectively reduce the numbers of attached *S. gesnerioides* and *Alectra vogelii*. However, high-dose of imizaquin reduced the germination rate and delayed the flowering in cowpea (*17*). Imidazolinones also can effectively control *O. crenata* (*18–21*), *O. cumana* (*22*), *S. hermonthica* (*23*) and others.

Application of those herbicides having mutant or transgenic hosts, with built-in target-site resistance against the corresponding herbicides, successfully controlled the root parasitic weeds (*24*). Single foliar application of chlorsulfuron on tobacco plants bearing acetolactate synthase (ALS) target-site for resistance resulted in 95% normal growth in broomrape-infested soil. Glyphosate treatment on rapeseeds, bearing 5-enolpyruvylshikimate-3-phosphate synthase target-site for resistance, infected by *Orobanche* prevented emergence of the broomrape resulting in the normal growth of the hosts. Foliar application of asulam on tobacco bearing 7,8-dihydropteroate synthase target-site for resistance reduced the number of broomrapes parasitizing a host by 70% (*25*). Seed coating of herbicide-resistant crops with corresponding herbicides has even higher potential for preventing damage from root parasitic weeds because germination and attachment to hosts occur underground near the hosts (*23*). Field trials of this approach were conducted in several countries in Africa. Imazaphyr and pyrithiobac were coated on seeds of maize bearing ALS target-site resistance resulting in a 3–4-fold increased yield even when *Striga* density was high (*26*).

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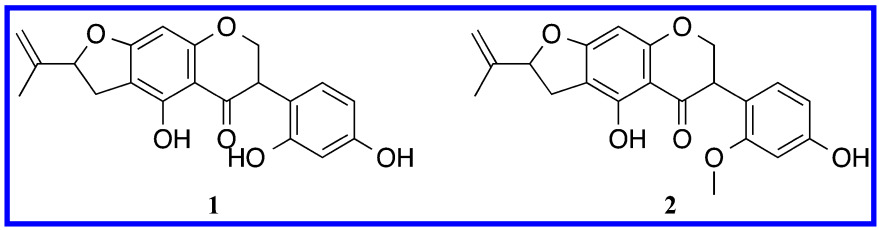
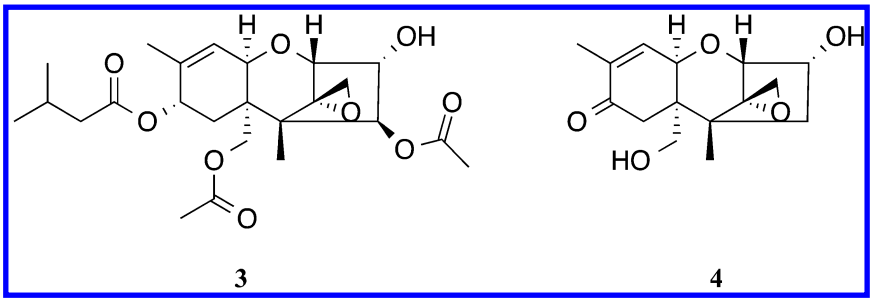
Additionally, reduction of *Striga* seedbank was confirmed after five cropping seasons using this method (*27*). Therefore, if herbicide-resistant crops are available, herbicide treatments can effectively control the root parasitic weeds. A concern about herbicide application is the emergence of herbicide-resistant parasitic weeds as in the cases of many other weeds. Fortunately, no such herbicide-resistant parasitic weed is reported as an agricultural menace until now.

Natural Growth Inhibitors

Some plant species are known to prevent *Striga* infestation and are intercropped alongside the host crops of *Striga* such as maize. *Desmodium uncinatum* cultivated as cattle forage significantly reduced the damage by *Striga* on maize in farmers' fields (*28*). It was shown that root exudates from *D. unicinatum* have germination stimulating and radicle growth inhibitory activities. Isolation and structure elucidation of the active compounds in the exudates revealed the isoflavones, uncinanone B (**1**) and uncinanone C (**2**). Uncinanone B has germination stimulating activity while unicinanone C has moderate radicle growth inhibitory activity (*29*). Since this combination is suitable for reducing the seed bank, biotechnological approaches to increase the amounts of these isoflavones in the root exudates will be expected to contriubute to a solution for the *Striga* problem.



Several fungal phytotoxins have also been screened and some toxins, such as T-2 toxin (**3**) and deoxynivalenol (**4**) isolated from *Fusarium* spp., were found to inhibit the germination of *S. hermonthica* (*30*). Isolation and evaluation of metabolites from fungi, *Myrothecium verrucaria* and *Fusarium compactum*, isolated from diseased *P. ramosa* revealed some other trichothecenes have strong inhibitory activity against seed germination of *P. ramosa*. However, as these compounds are highly toxic also to animals, there is a great risk in the use of these compounds in the fields (*31*).



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Suicidal Germination

The obligate root parasitic weeds have evolved a unique germination process to infallibly parasitize host plants. The root parasitic weeds require host-derived germination stimulants such as strigolactones for seed germination. These stimulants from the host roots enable the parasites to recognize the adjacent host plants (*32*, *33*). Because of small storage substances in the seeds, they cannot survive without immediate association with the hosts after germination. Therefore, if the seeds germinate without hosts by artificial application of germination stimulants, they will die in short time. This strategy is called "suicidal germination" (*34*) and might be the most effective strategy to reduce the soil seed bank of parasitic weeds.

Unlike *Orobanche* and *Phelipanche*, seeds of *Striga* spp. germinate with ethylene treatment (*16*, *35*). Injection of ethylene gas into soil successfully eradicated *S. asiatica* in the United States (*36*). However, using ethylene gas requires costly special equipments, and thus, is unsuitable for African countries where huge areas are infested by *Striga*. Instead, using ethylene-producing bacterium spieces *Pseudomonas synringae* for suicidel germination was proposed. Several strains of *P. synringae* induced the germination of *S. aspera*, *S. gesnerioides* and *S. hermonthica* more effectively as compared to ethylene gas (*37*). These bacteria may, therefore, provide a new strategy for *Striga* control. However, since ethylene is a gaseous plant hormone promoting senescence, release of large amount of these bacteria should be cautiously conducted.

Researchers have been investing a lot of effort in the design and synthesis of analogs of the common natural germination stimulants, strigolactones (*38*). An array of compounds containing α-formyl-γ-lactone were synthesized and their activities were evaluated (*38*, *39*). Among them GR24 (**5**) was one of the most active and stable compounds. A single application of GR24 reduced the seed bank of *S. asiatica* by 65% in India (*38*), and by 50% in the United States (*14*). However, these analogs could not find practical applicability as they were easily degraded in alkaline soil and, thus, were not very effective in controlling the parasitic weeds. The strigolactones are now recognized as a class of molecules involved in signling not only between parasitic plants and hosts but also between symbiotic arbuscular mycorrhizal fungi and hosts (*40*). They also form a class of plant hormones regulating plant architecture (*41*, *42*). GR24 is now widely used in experimental studies to unravel the physiological roles of strigolactones.

One of the bottlenecks in utilization of strigolactones as agrochemicals is the high cost of their synthesis owing to complicated structures. Using the information about structure-activity relationship of strigolactones, analogs with high activity and low cost of synthesis were synthesized. Nijmegen-1 (**6**) is a representative analog possessing slightly less activity compared to GR24 (*43*). Field trials of Nijmegen-1 in a tobacco field infested by *P. ramosa* resulted reduction of 77% of seed bank with no *P. ramosa* found in the treated field (*34*). These results demonstrate that suicidal germination strategy is a valid way to control the parasitic weeds; the application of stirigolactones however, could have adverse environmental effects, since they function as hormones and signaling molecules in plants and symbiotic fungi.

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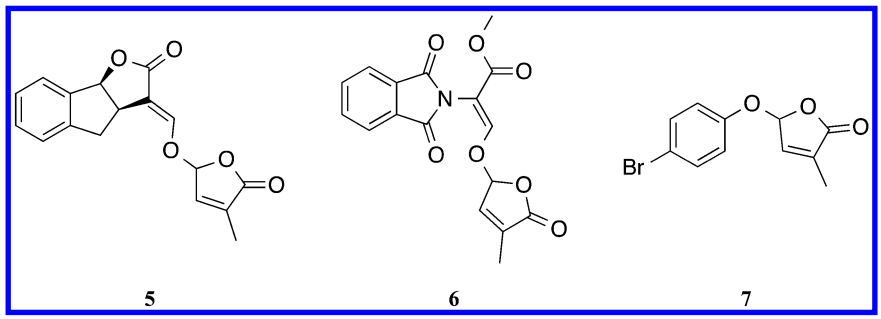
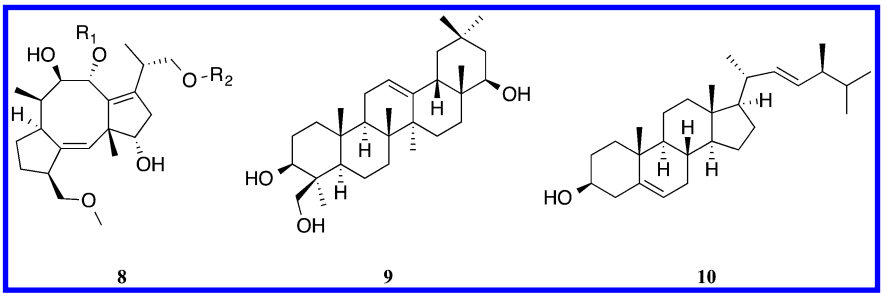
Considering the varied bioactivities of strigolactones, new compounds that can selectively induce one of the activities of strigolactones are being researched. A strigolactone mimic 4-Br debranone (**7**) was found to have only inhibitory activity on shoot branching but had feeble stimulating activity on germination of *S. hermonthica* (*44*). Similarly, there may be a compound showing activity only on germination of root parasitic weeds but not as a plant hormone. Such kinds of selective compounds are required to formulate a practicable suicidal germination strategy.



5 **6** **7**

Some natural compounds can induce the germination of parasitic weeds. Fusicoccins (**8**) and their related compounds induced the germination of *S. hermonthica*, *O. minor* and *P. ramosa* at concentrations as low as 10^{-5} M (*45*, *46*), and therefore, have been proposed as candidate inducers for suicidal germination (*47*). However, the effects on the hosts and/or other plants should be evaluated because fusicoccins are fungal phytotoxins possesing various physiological activities (*48*).

Like uncinanone B (**1**) in *D. uncinatum*, some polyphenols in pea root exudates strongly and selectively stimulated the germination of *O. foetida* but not of *P. aegyptiaca*, *O. crenata*, or *O. minor* (*49*). Similarly, triterpenoide soyasapogenol B (**9**) in the root exudates of the common vetch selectively stimulated the germination of *O. minor*, while *trans*-22-dehydrocampesterol (**10**) in the exudates stimulated the germination of *P. aegyptiaca*, *O. crenata*, *O. foetida* and *O. minor* (*50*). These compounds may be involved in the host specificity of those parasitic species and have potential to be used as species-specific inducers of suicidal germination.



8 **9** **10**

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New Approaches Based on Understanding of Biological Processes in Parasitic Weeds

Biological processes existing only in the root parasitic weeds could be the potential targets for selective control. In the search for such processes to establish a novel control strategy, we have focused on the unique germination process of root parasitic weeds in Orobanchaceae.

A metabolomics study conducted with an early stage of germination process of *O. minor* determined that a trisaccharide planteose (α-d-galactopyranosyl-(1→6)-β-d-fructofuranosyl-(2→1)-α-d-glucopyranoside) is involved in the germination. By sugar profiling during germination of seeds of *O. minor*, a metabolic pathway of planteose was predicted as follows: hydrolysis of the galactose moiety in planteose produces sucrose, which is hydrolyzed to glucose and fructose (Figure 1). Planteose metabolism was also evaluated in *O. crenata*, *P. aegyptiaca*, and *S. hermonthica*. The study detected planteose to be universally present in the seeds of these species (*51*).

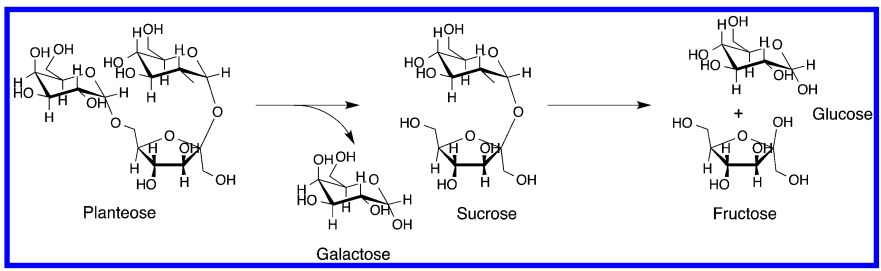

Figure 1. Predicted metabolic pathway of planteose in the germinating seeds of O. minor.

The importance of planteose in the seed germination of *O. minor* was studied by verifying whether suppression of the planteose metabolism leads to inhibition of germination. Some glycosidase inhibitors were applied together with GR24. Among the tested inhibitors, nojirimycin bisulfite (NJ) (**11**) showed a strong and selective inhibitory effect on the seed germination of *O. minor* in a dose-dependent manner without an effect on seed germination of *Arabidopsis thaliana* and red clover (*Trifolium pratense*), a host of *O. minor*. In the presence of NJ, sucrose accumulated to high levels whereas the amounts of glucose and fructose were very low in the seeds. Furthermore, addition of exogenous glucose recovered the inhibited germination rate, which suggests that NJ inhibits sucrose degradation in the planteose metabolic pathway and the supply of glucose, resulting in inhibition of germination (*51*).

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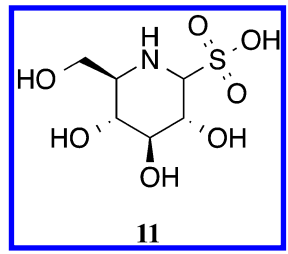

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The effect of NJ on germination was also tested on other root parasitic plants in Orobanchacae. In the case of *S. hermonthica*, NJ did not inhibit the seed germination, but caused a dose-dependent reduction in radicle elongation. The effect of NJ on *Phtheirospermum japonicum*, which is a facultative hemiparasite closely related to *Orobanche* and *Striga* (*52*) but not a weedy parasite, was also analyzed. Similar to the case in *S. hermonthica*, NJ did not affect the germination rate of *P. japonicum* seeds but it inhibited root elongation at concentrations of 10 μM or higher. There was also a possibility that NJ affects seed germination or root elongation of other non-parasitic plants that contain the planteose metabolic pathway. To investigate the effects of NJ on planteose containing seeds, we conducted germination assays and root length measurements using seeds of tomato (*Solanum lycopersicum*) (*53*, *54*), sesame (*Sesamum indicum*) (*55*), and spearmint (*Mentha spicata*) (*56*). The germination rates in these seeds were not affected by NJ, even at 1 mM, but it inhibited root elongation of sesame and spearmint. However, the concentration of NJ required to inhibit the root elongation was much higher for sesame and spearmint than for *S. hermonthica* and *P. japonicum* (*51*).

This was the first report of broomrape-specific chemical inhibition of germination. NJ did not inhibit germination except for *O. minor*, but it inhibited the radicle or root elongations in some plants. The plant species could be arranged in descending order of their sensitivity to NJ as follows: *O. minor* (obligate holoparasite), *S. hermonthica* (obligate hemiparasite), *P. japonicum* (facultative hemiparasite), spearmint (Lamiaceae), sesame (Pedaliaceae). Additionally, seedling growth of Arabidopsis (Brassicaceae), red clover (Fabaceae) and tomato (Solanaceae) was not affected by NJ treatment. Interestingly, this is the reverse order of the evolutionary process of parasitism (*57*, *58*). Therefore, it was suggested that sugar metabolism or a regulatory mechanism of sugar metabolism targeted by NJ is changed with the acquisition of parasitism. Considering the strong inhibitory effect of NJ on germination of *O. minor* and the weak effect on non-parasitic plants at low concentrations, the site of action of NJ is a promising target for selective control of root parasitic weeds.

Unique responses of the root parasitic weeds to plant hormones were also reported. Gibberellin (GA) is assumed to be synthesized during conditioning of *Orobanche* and is required for perception of germination stimulants because treatment with a GA biosynthesis inhibitor, pacrobutrazol, during conditioning reduced the sensitivity toward germination stimulants and so the germination rates (*8*). In fact, soil application of uniconazole reduced the broomrape infection on sunflower (*59*). Interestingly degradation of abscisic acid (ABA), another important plant hormone regulating seed germination, was initiated by the

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perception of germination stimulants through the expression of an ABA catabolic gene, *CYP707A1*, resulting in the release of seed dormancy (*60*). Moreover, it was revealed recently that a DNA methylation status in the promoter of *CYP707A1* is involved in the regulatory mechanism of its expression (*61*). These kinds of molecular and biochemical studies to decipher the precise molecular mechanism of germination of root parasitic weeds will provide us with new concepts and targets for their selective control.

Conclusion

Crop protection by agrochemicals is a conventional but still an effective approach to produce sufficient amount of foods for the increasing world population. Many trials showed that modern herbicides, such as imidazolinones and sulfonylureas, can control the root parasitic weeds to some extent. On the other hand, biotechnology is becoming more and more important in food production. The combination of agrochemicals with genetically modified crops, i.e. herbicides with the herbicide-resistant transgenic crops, has been proved a powerful and cost-effective method for food production. However, continuing use of a certain herbicide will promote appearance of herbicide-resistant weeds. To prevent the risk, multiple methods should be investigated and applied to control the parasitic weeds. In this regard, target-based screening of agrochemicals, as in the case of development of medicinal drugs, could provide a novel class of compounds to control the parasitic weeds selectively. "Omics" technologies are powerful tools to identify specific biological events or molecules of interest. One example is provided in this review, wherein metabolomics revealed the planteose metabolism as a possible target for selective control of broomrapes (*51*). Since new-generation sequencers are being widely used, a genome project was launched for parasitic plants (*62*). Transcriptome of the haustorium of a facultative parasitic plant, *Triphysaria versicolor*, was analyzed and several genes expressed in a host-specifically manner, at the host-parasite interface were identified (*63*). Such large sets of genetic information will also be of great help in the identification of suitable targets for selective control of the root parasitic weeds (*64*). Ultimately, a series of parasite-specific herbicides with diverse modes of action is expected to be developed by means of the multi-omics approach.

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Chapter 24

Sivanto® - A Novel Insecticide with a Sustainable Profile

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Sivanto® (common name: flupyradifurone, FPF) is a member of the new class of butenolide insecticides. Inspired by the stemofoline lactone *"head group"* as pharmacophore pattern and structural features of relevant *n*AChR agonists, its bioactive scaffold was identified. As partial agonist FPF reversible binds to insect nicotinic acetylcholine receptors and provides a favorable pharmacokinetic and safety profile. Sivanto® prime is very versatile in terms of application methods, exhibits fast contact and feeding efficacy, particularly useful for efficient virus vector control. The ready-to-use SL-formulation demonstrates excellent adhesion, spreading and penetration properties on leaves with improved translaminar activity and rainfastness. Target markets are primarily fruits and vegetable crops as well as plantation (citrus, coffee, cocoa) and tropical fruits. As it is non-hazardous to honey and bumble-bees as well as to most beneficial insects, Sivanto® prime perfectly fits into IPM systems and will be a sustainable tool to control sucking pests in many agricultural and horticultural settings.

Today, there are global chalenges to a product development in modern crop protection. The requirements to develop plant protection products have increased from pure efficacy aspects to multifold parameters to be considered. These include *e.g.* the *(i) product profile* (mode of action, safety profile, efficacy and

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resistance management of the active ingredient), *(ii) food security* (yield and quality, Minimal Risk Levels (MRLs) and import tolerances, Good Agriculture Practice), *(iii) biodiversity* (stewardship, product selectivity), *(iv) climate change* (adaption and mitigation) and the *(v) rural development* (education and knowledge transfer, easy to use crop protection products), respectively.

As a modern insecticide and based on the results at manufacturer recommended field-rates Sivanto® prime (common name: flupyradifurone, FPF) matches the global challenges to product development in a sustainable way in respect of its:

- outstanding safety profile for an uncomplicated control of major sucking pests,
- high compatibility with honey and bumble-bees allowing wide application windows and treatments during flowering,
- selectivity to most beneficials in fruit and outdoor grown vegetable crops, providing a perfect fit for Integrated Pest Management (IPM),
- use for resistance management against whiteflies and selected aphids, and
- significant benefit to growers, such as excellent speed of action leading to quick feeding cessation which provides an effective virus vector control and flexible applicability at any crop stage (http://www.sivanto.bayer.com).

Because of its excellent profile concerning human- and environmental safety, Sivanto® is a designated United States Environmental Protection Agency (U.S. EPA, 2013) reduced risk candidate for seven horticultural crop groups (e.g. citrus, curcurbit vegetables, fruiting vegetables, pome fruit) and cotton.

A New Dimension to an Established Mode of Action

The selection of butenolide chemistry was inspired by the natural occurring and complex stemofoline alkaloids (isolated from the Asian medicinal plant *Stemona japonica*; *Stemonaceae* family) (*1*), considered as privileged structures to design simplified, insecticidally active nicotinic acetylcholine receptor (*n*AChR) agonists (*2*). Based on the stemofoline lactone *"head group"* as a topological pharmacophore pattern, and in combination with molecular modelling studies using structural features of relevant *n*AChR agonists, a new bioactive scaffold was identified, which finally resulted in the identification of butenolide insecticides. Finally, the stepwise optimization of 5,6-disubstituted pyridin-3-yl (R^1)-containing butenolides (I; R^2, R^3 = H) resulted in the discovery of FPF containing the *N*-2,2-difluoroethyl residue (R^4) as specific substitution pattern (*cf.* Figure 1) (*3*).

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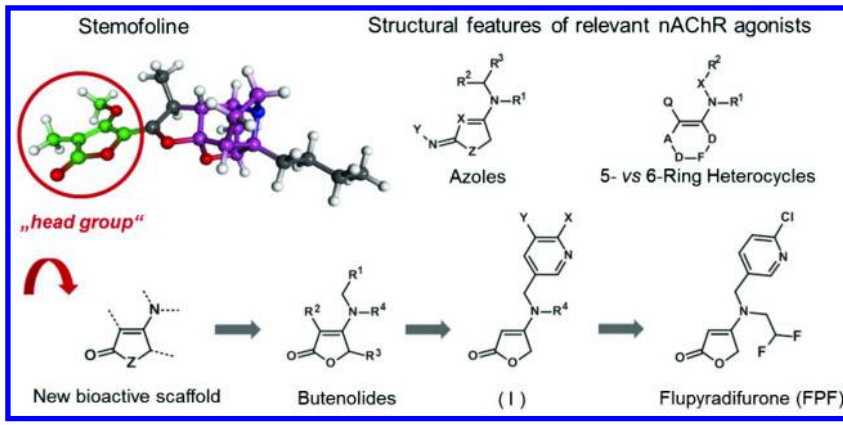

Figure 1. Stemofoline lactone "head group" and structural features of relevant nAChR agonists as the starting point for the selection of the butenolide chemistry (Z=O), which resulted in the butenolide subclass (I) and finally in FPF.

It was found that the structure-activity-relationship (SAR) of butenolide insecticides is distinct from the class of neonicotinoids. For butenolides a preference of the hetaryl moiety *6-chloro-pyridin-3-yl* (R^1) could be observed, which results in a different *in-vitro* and *in-vivo* SAR as known from commer-cialized neonicotinoid insecticides. In addition, it could be demonstrated that the *N*-2,2-difluoroethyl group (R^4) is a specific substitution pattern for the butenolide class (*4*).

FPF acts selectively on the insect central nervous system (CNS) as a partial agonist of postsynaptic *n*AChRs and binds with a different pharmacophore than neonicotinoids (*5*) and sulfoxaflor (*6*) to the ACh binding site (*7*). In addition, FPF presents little to no metabolism-based cross-resistance with neonicotinoids in targeted pest species.

Because of its clear structural differentiation (new pharmacophore system for commercial *n*AChR agonsists, unique difluorinated side chain) from active ingredients of the:

- **IRAC MoA Sub-group 4A, i.e.** neonicotinoid insecticides containing *N*-nitro-guanidine- [imidacloprid (IMD), thiamethoxam (TMX), clothianidin (CLO), dinotefuran (DNF)], *N*-cyano-amidine- [acetamiprid (ACT), thiacloprid (TCL)] or nitromethylene [nitenpyram (NPR)] pharmacophors, and
- **IRAC MoA Sub-group 4C, i.e.** *N*-cyano-sulfoximines [sulfoxaflor (SFX)],

flupyradifurone was classified in 2013 as a new butenolide **IRAC MoA Sub-group 4D**.

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Systemicity and Translocation

The active ingredient FPF contains a new butenolide pharmacophore system as a bioactive scaffold which is responsible for its specific physico-chemical and environmental fate properties (hydrolysis stable; aqueous photolysis: 33 to 228 d half-life; absorption in soil: K_{OC} of 98 ml/g mean; half-life: 8.5 and 66.2 d).

Sivanto® prime (SL 200, soluble liquid) is taken up into the leaves and stems with spray application and via the roots when applied to soil or alternative substrates.

After absorption into the plant, Sivanto® prime is systemically translocated acropetally in the xylem, in direction of the transpiration stream, and is translaminarily distributed into the adjacent plant cells. Due to the translaminar distribution throughout the leaf (leaf cross-section), FPF is active against insect feeding on the underside, even when applied only on the upper leaf surface (Figure 2).

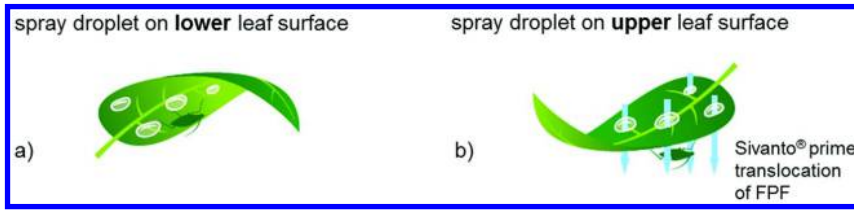

Figure 2. Efficiency of FPF after spray application: a) contact and feeding, b) translaminar, measured by honeydew excretion.

Finally, insects can even be controlled whilst feeding at the distal end on the lower surface of the leaf. Sivanto® prime provides contact, a very fast feeding cessation (measured by honeydew excretion) and translaminar efficacy even on hidden pests like aphids (*Myzus persicae*, *Aphis gossypii*) feeding on the lower surface of the leaves (Table 1).

Table 1. Contact, feeding and translaminar efficacy of Sivanto® prime against *Myzus persicae* and *Aphis gossypii* based on suppression of honeydew excretion.

Efficacy via	*Crop*	*Pest*	*Rate [g a.i./ha]*	*Efficacy*[a]
Contact & Feeding	Pepper	*M. persicae*	60	++++
	Pepper	*M. persicae*	12	++++
Translaminar	Cotton	*A. gossypii*	75	+++
	Cabbagge	*M. persicae*	60	++++

[a] ++++ excellent, +++ good, ++ satisfactory, + marginal, - insufficient, 0 no activity.

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Finally, due to the up-take through the roots and rapid distribution within the entire plant into the stem and leaves over a long distance, Sivanto® prime is ideal for drench application.

Crop-Pest Spectrum

Sivanto® prime can reach a large spectrum of sucking pests in a wide variety of fruit, vegetable, plantation and defined broadacre crops (Table 2).

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Table 2. Overview of the crop-pest spectrum of Sivanto® prime.

Crop	*Pest (Binomial name)*
Vegetables, Potatoes	Whiteflies (*Bemisia tabaci*, *B. argentifolii, Trialeurodes vaporariorum)* Aphids (*Myzus persicae, Nasonovia ribisnigri*) Potato Psyllid (*Bactericera cockerelli*)
Fruits	Aphids (*Dysaphis plantaginea, Aphis pomi*) Scales (*Quadraspidiotus perniciosus*) Psyllids (*Psylla pyri*)
Grapes	Leafhoppers (*Empoasca vitis; Scaphoideus titanus, Erythroneura* sp) Grapevine Mealybug (*Planococcus ficus*)
Citrus	Asian Citrus Psyllid (*Diaphorina citri*) Citricola scale (*Coccus pseudomagnoliarum*) Citrus Thrips (*Scirtothrips citri*)
Cotton	Cotton Aphid (*Aphis gossypii*) Lygus Bugs (*Lygus* sp.)
Coffee	Coffee Leafminer (*Leucoptera coffeella)*
Cocoa	Cocoa Mirids (*Sahlbergella singularis, Distantiella theobroma)*

It is a systemic insecticide that is flexible for different types of application (foliar, drench, drip irrigation), provides adaptable application timing (including during blooming) and is highly target-selective against a broad range of key adult and immature sucking pests, such as aphids, selected hoppers and whiteflies. However, it is also active against mealybugs, coffee leafminers, sawflies, cocoa myrids, soft scales, citrus psyllids as well as some weevils, thrips and beetles.

In most cases, the efficacy of Sivanto® prime is better than neonicotinoid insecticides (Table 3).

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Table 3. Application spectrum of Sivanto® prime in comparison with neonicotinoid insecticides.

Species	*Sivanto® prime Rate [g a.i./ha]/Efficacy*[a]	*Neonicotinoids' Rates [g a.i./ha]/Efficacy*[a]
B. tabaci	100-205 / +++(+)	150 IMD, 196 DNF / ++
T. vaporariorum	75-270 / +++	140-280 IMD / +
B. argentifolii	90-205 / +++	196 DNF / ++
A. gossypii	50-75 / ++++	100 IMD, 30 DNF / ++(+)
M. persicae	90-180 / +++	150 IMD / +++
B. brassicae	75-125 / +++	100 IMD, 63 ACT / +++
N. ribisnigri	90-125 / +++	100 IMD, 96 TCP / ++
Aphis spp.	50-125 / +++	100 IMD, TMX / +++
A. fabae	150-207/ +++	83 ACT, 200[b] / ++(+)

[a] ++++ excellent, +++ good, ++ satisfactory, + marginal, - insufficient, 0 no activity; activities in between are indicated by brackets. [b] Various neonicotinoids.

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Its fast activity against *B. tabaci*, *B. argentifolii* and *A. gossypii* is a clear benefit compared with current standards like spirotetramat and neonicotinoids/ pymetrozine. In addition, Sivanto® prime fits perfectly into integrated spray programs in sustainable perennial (e.g. apple) and annual crop (e.g. tomato) solutions (*8*).

Control of Neonicotinoid-Resistant Whitefly Populations

B- and Q-type strains of *B. tabaci* showing resistance ratios against IMD of >1300- and 250-fold, exhibit a three and seven-fold lower susceptibility to FPF, respectively when compared to a susceptible reference strain in leaf-dip bioassays. Recombinantly expressed CYP6CM1, a cytochrome P450 highly overexpressed in resistant cotton whitefly populations, hydroxylates IMD and pymetrozine (*9*), but not FPF.

Based on the results at manufacturer recommended field-rates, Sivanto® prime demonstrates a good control of whiteflies resistant to neonicotinoids (IRAC MoA Group 4A) (Table 4).

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Table 4. Efficacy of Sivanto® prime against neonicotinoid-resistant *Bemisia* sp. and -tolerant *Trialeurodes vaporariorum* in various countries.

Species	*(Country)*	*Efficacy*[a]	*Rate [g a.i./ha]*
Neonicotinoid-resistant *Bemisia* sp.	*Bemisia* sp., (Brazil)	+++	90-125
	B. argentifolii (USA)	+++	205
	B. tabaci (Spain)	++++	112.5-150
	B. tabaci (Japan)	+++	200-400
	B. tabaci (China)	+++(+)	125-150
Neonicotinoid-tolerant *T. vaporariorum*	*T. vaporariorum* (Italy)	+++	112.5
	T. vaporariorum (Brazil)	++(+)	270

[a] ++++ excellent, +++ good, ++ satisfactory, + marginal, - insufficient, 0 no activity; activities in between are indicated by brackets.

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Virus Vector Control

Whiteflies are one of the major pests on tomatoes that also transmit several viruses such as the Tomato Yellow Leaf Curl Virus (TYLCV) or the Pepper Huasteco Yellow Vein Virus (PHYVV) and permanent yellowing diseases.

In comparison with untreated controls, IMD and TMX, FPF (applied as Sivanto® prime to the foliage) provides rapid knockdown of whiteflies and protected tomatoes from plant virus transmission, as it shows a rapid control of the vector (Figures 3 and 4).

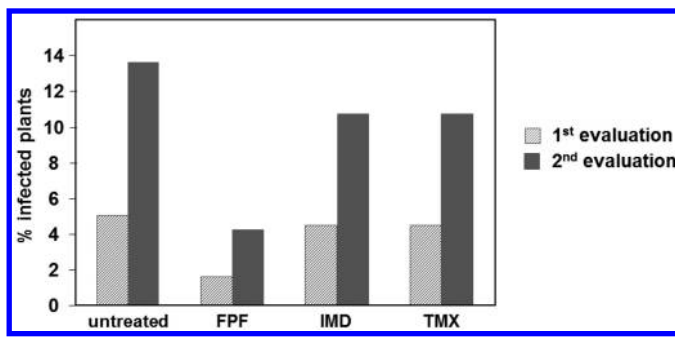

Figure 3. Tomato Yellow Leaf Curl Virus (TYLCV, transmitted by whiteflies) on tomatoes: virus vector control of Sivanto® prime (FPF) in comparison with IMD and TMX; Mexico 2012.

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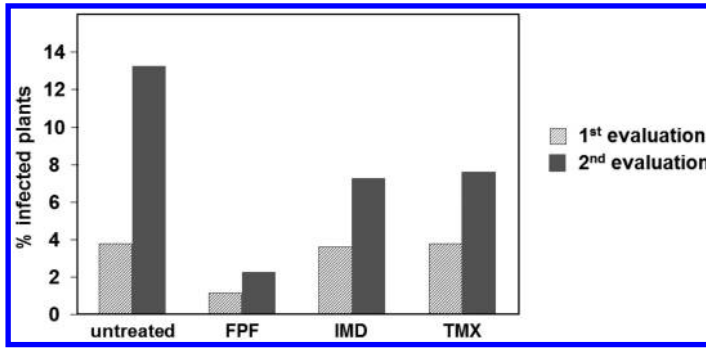

Figure 4. Pepper Huasteco Yellow Vein Virus (PHYVV, transmitted by whiteflies) on tomatoes: virus vector control of Sivanto® prime (FPF) in comparison with IMD and TMX; Mexico 2012.

This effect could be demonstrated in Mexican field trials against neonicotinoid-resistant whiteflies. It has been shown in various trials that also by applying Sivanto® prime (400 g a.i./ha) as drench after transplanting, a lower number of plants with diseases transmitted by sucking pests such as whiteflies was observed. In addition, more tomatoes with greater overall fruit quality were harvested.

Furthermore, a major concern in cantaloupe melon production is the whitefly species (*B. tabaci*) transmitting Curcurbit Yellow Stunting Disorder Virus (CYSDV). Sivanto® prime directly manages the problem of both whiteflies and CYSDV, while allowing production of cantaloupe melons to thrive.

In comparison with newer active ingredients recently developed for this segment (e.g. cyazypyr, 110 g a.i./ha or sulfoxaflor, 100 g a.i./ha), Sivanto® prime (application rate: 205 g a.i./ha) is clearly the most effective compound and has the best fit in desert melon IPM programs (*10*).

Feeding Cessation

Sivanto® prime shows excellent and fast feeding cessation of target pests such as whiteflies, psyllids as well as aphids, and thus reducing the risk of virus and bacteria transmission.

As demonstrated by Electrical Penetration Graph (EPG) measurements, during the feeding phases of adult Asian citrus psyllids (*Diaphorina citri*) of phloem ingestion and penetration the potential for pathogen transmission is given (marked with* in Figure 5; standard is Admire Pro®).

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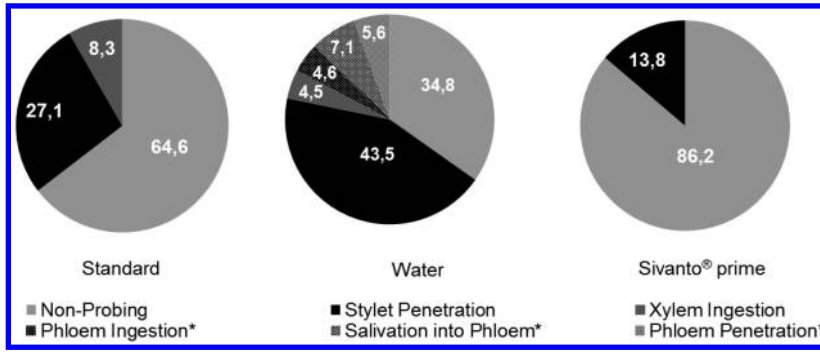

Figure 5. Frequency of Asian citrus psyllid (D. citri) adult feeding phases measured by EPG technique; adult's feeding phases [in %].

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Molecular Docking Studies of Flupyradifurone within the CYP6CM1vQ (*B. tabaci*)

The overexpression of CYP6CM1 is considered as one of the major mechanisms of IMD resistance in *B. tabaci*.

Recently, a possible explanation for the lack of metabolization of FPF in comparison with IMD could be given by molecular docking studies. Based on a structural model of CYP6CM1vQ from whitefly (*B. tabaci*) which resulted from a previous homology modelling study (including a subsequent molecular dynamics refinement) conducted by Karunker cit. (*11*) molecular docking investigations (LeadIT/FlexX) with both IMD and FPF were carried out.

Due to the large volume of the CYP6CM1vQ catalytic site, only ligand poses were taken into account accommodating one or more heavy atoms within 3.8 Å distance from the heme iron-oxygen centre, using FlexX-Pharm as post-filtering tool (*12*, *13*).

The docking poses underwent a positional clustering. In contrast to IMD (35 possible clusters), for FPF only 23 clusters of interaction poses within the CYP6CM1vQ cavity model were identified. It was found, that most of these clusters revealed an orientation of the rather non-reactive difluoro-methyl moiety of the *N*-2,2-difluoroethyl side chain of FPF towards the active heme-iron center of the CYP6CM1vQ cavity model (Figure 6).

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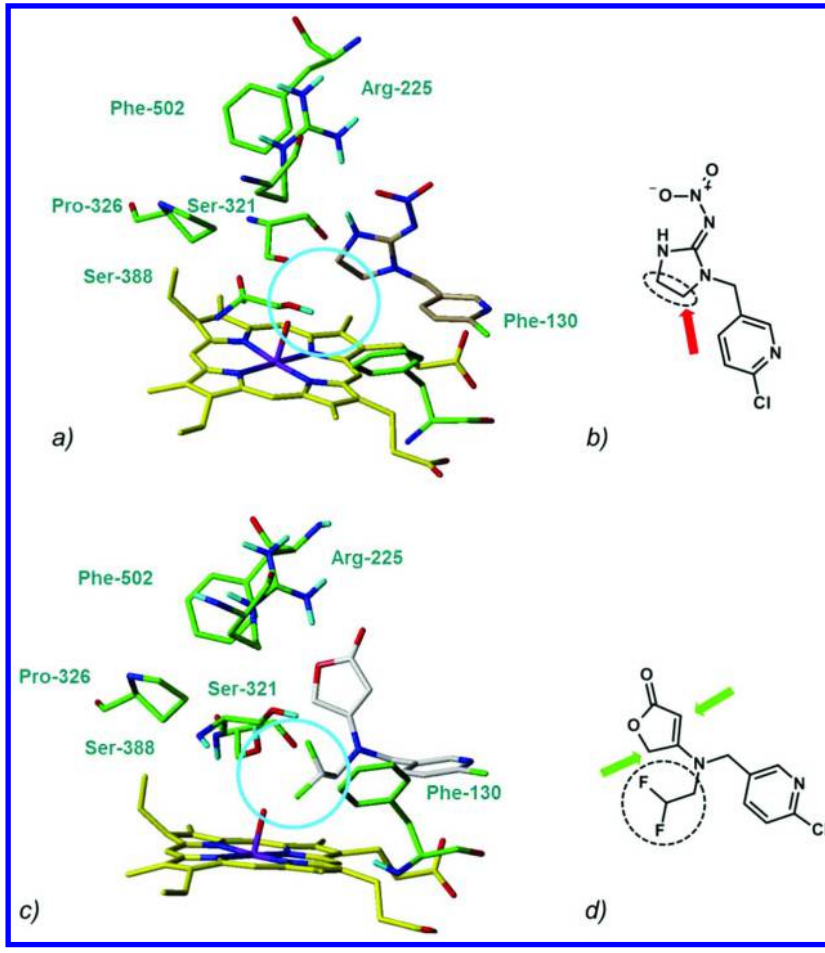

Figure 6. Molecular docking studies of IMD and FPF within the CYP6CM1vQ (B. tabaci) cavity model: a) representative IMD pose in surrounding of amino acids; b) oxidative metabolic attack (red arrow) of the 2-N-nitro-imidazolidine ring system of IMD; c) FPF pose in surrounding of amino acids; d) possible positions of oxidative metabolic attacks (green arrows) of the butenolide system of FPF.

In summary, the 23 docking poses of FPF appear quite similar with respect to the overall orientation within the active site, *i.e.* in sharp contrast to IMD. According to the modelling study, none of these poses suggested a metabolism in the corresponsing butenolide ring system, *e.g.* at the CH-position of the α,β-unsatturated carbonyl [C=C<u>H</u>-CO]-fragment or at the [-O-C<u>H</u>$_2$-]-group. The presence of the *N*-2,2-difluoroethyl residue in FPF increases its insecticidal activity and effectively prevents the butenolide moiety to be placed in an orientation suitable for oxidative metabolization by CYP6CM1vQ.

Safety Profile

It could be demonstrated, that Sivanto® prime meets the diverse safety needs of modern agriculture. Its active ingredient FPF has a low acute toxicity (LD_{50} (rat): ≥ 5000 mg/kg) when exposed through ingestion, inhalation and dermal absorption (LD_{50} (rat): > 2000 mg/kg) and therefore it is non-hazardous for humans and mammals.

Furthermore, by using under practical conditions in the field, there are no adverse effects on non-target arthropod populations.

Selectivity to Beneficials

Based on the recommended field-rates and positioning of Sivanto® prime, the results show that the insecticide is selective to most beneficials in fruits and outdoor vegetable crops (Table 5).

Table 5. Selectivity of Sivanto® prime against various beneficial groups and species after foliar vs drench application in relevant crops.

Beneficials	*Species*	*Stage*	*Crop*	*Foliar*[a]	*Drench*[a]
Predatory mites	*Amblyseius swirski*	motile	Bell pepper	2	1-2
	Typphlodromus pyri	motile	Apple	1	n.a.
	Kampimodromus aberrans	motile	Apple/grape	1	n.a.
Predatory bugs	*Orius laevigatus*	mixed	Bell pepper	3	1-2
	Anthocoris nemoralis	mixed	Pear/corn	3	1
Coccinellidae	*Coccinella septempunctata*	larvae	Apple	1	n.a.
Hover flies	*Episyrphus balteatus*	larvae	Cabbage	1	
Lacewing	*Chrysoperla spp.*	adult		1-2	1
Parasitoid wasp	*Encarsia formosa*	mixed/ mumies		1-3	1
	Eretmocerus	mixed		1	n.a.
	Aphidius colemani	mixed	Apple	1-2	n.a.
	Aphelinus mali				

[a] IOBC rating = International Organization for biological and Integrated Control 1 = non-hazardous, 2 = slightly harmful, 3 = moderately harmful, 4 = harmful, n.a. = not applicable.

In Table 5, the rating given for foliar and drench application is in accordance with the International Organization for Biological and Integrated Control (IOBC).

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Its positioning in IPM systems can be exemplified on pome fruits which are important for growers in Belgium. Two IPM-compatible foliar applications of Sivanto® prime to control rosy apple aphids and pear suckers have demonstrated a highly effective protection of pome fruits against these pests, responsible for significant damage and quality issues each year, and no adverse effects on predatory mites and coccinellidae.

No Adverse Effects to Honey and Bumble-bees

As part of the risk assessment study package prepared during the development and registration of Sivanto® prime, several studies were conducted to demonstrate the safety profile to honey bees. The studies indicate that Sivanto® prime has no adverse effects on foraging honey bees, foraging activity, brood and colony development, colony vitality and honey bee health or on over-wintering success when used in accordance with the proposed label instructions.

Sivanto® prime has been tested in laboratory and extensive *semi*-field and field studies using crops that are highly attractive to bees. The studies were conducted following a tiered approach, starting with acute and chronic laboratory studies that show that FPF is more toxic to adult bees when exposed orally than when exposed on a contact basis (oral LD_{50} = 1.2 µg a.i./bee and contact LD_{50} = 122.8 µg a.i./bee) (*14*). The latter way of exposure can be considered as practically non-toxic for adult bees. Furthermore, when individual bees are exposed in the laboratory to diet containing high concentrations of FPF, no effects were observed in adult and larva bees (chronic oral *No Observed Effect Concentration* (NOEC) to adult bees as well as the dietary NOEC for larvae > 10,000 µg/kg).

The data generated under laboratory conditions are an indication of the intrinsic toxicity of Sivanto® to individual bees under unrealistic worst-case exposure conditions. The next higher tier in the testing approach is the studies conducted with Sivanto® prime under *semi*-field with highly attractive surrogate crops such as Phacelia. The results show that even exposures in a crop under worst-case conditions causes no adverse effects on honey bees and honey bee colonies when applied at highest registered application rate during full bloom and when bees were actively foraging. Likewise, a study under confined and forced worst-case exposure to spiked diet with FPF demonstrated that no adverse effects on honey bees and honey bee colonies are caused, including overwintering at concentrations up to at least 10,000 µg/kg diet, a concentration that is higher than the concentration levels found in bee-relevant matrices of treated crops.

Residues were measured in many bee-attractive crops under realistic agronomic field conditions (*14*). The highest tier evidence, the field studies, demonstrate as well that under realistic exposure and field agronomic conditions that when applied at the maximum proposed label rates, Sivanto® prime causes no adverse effects on honey bees and bumble-bees in any of the tested endpoints even when applied to flowering crops.

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Concluding Remarks

Sivanto® prime (common name: flupyradifurone, FPF) is a modern butenolide insecticide with an outstanding safety profile for the control of major sucking pests like aphids, whiteflies, and other key insect pests including larval and adult stages. The SAR of butenolides containing the new butenolide scaffold for insect *n*AChR interaction is distinct from the class of neonicotinoid insecticides. Sivanto® prime has excellent safety characteristics: *(i)* it is highly compatible with honey and bumble-bees allowing wide application windows and treatments during flowering, and *(ii)* its selectivity to most beneficials in fruit and field-grown vegetable crops provides a perfect fit for most Integrated Pest Management (IPM) programs. FPF is a valuable tool for resistance management, in particular for selective neonicotinoid-resistant pests like whiteflies and selected aphids. Its *N*-2,2-difluoroethyl residue increases the insecticidal activity and prevents the oxidative metabolization by CYP6CM1 in whiteflies like *B. tabaci*.

Additionally, Sivanto® prime provides a wide range of significant benefits to the growers, such as excellent speed of action, quick feeding cessation, effective virus vector control and flexible application at any crop stage.

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Chapter 25

Pharmacophore Based Design and Synthesis of Novel Neonicotinic Insecticides

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Pharmacophore based design was used effectively to increase the probability of synthesizing biologically active neonicotinic insecticides. The pharmacophore was derived from a set of compounds known to interact with insect nicotinic acetylcholine receptors (*n*AChRs) by using a computer modeling technique known as the active analog method and data from a [^{3}H]imidacloprid binding assay. Statistical models were then constructed based on this pharmacophore, binding data and several insect assays. Three of these models were validated and used to evaluate proposed novel structures, some of which incorporated moieties that would be considered atypical for neonicotinic compounds. The design, synthesis and structure activity relationships (SARs) of several such molecules are discussed.

One of the most challenging questions faced today by researchers working on biologically active molecules is how to discover new structural motifs with improved efficacy. The traditional approach of making small modifications and looking for incremental improvements is reliable, but eventually, the need to move beyond known structural scaffolds asserts itself. Large changes, however, often lead to dramatic losses in activity. The advent of efficient and reliable computer modeling techniques such as comparative molecular field analysis (CoMFA) (*1–3*) could provide a potential solution.

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In the pursuit of improved biological activity, knowledgeable chemists and biologists regularly propose targets for synthesis. Intuition plays a large role in this process, but personal biases can have a substantial negative impact. New target ideas are often based on extrapolations from single data points, and ignore large bodies of SAR data. Furthermore, new target ideas are frequently derived from two dimensional representations of molecules or simple visual inspection of three dimensional structures. Tools that provide objective, mathematical rigor and comprehensively consider all available data are more likely to improve the selection process and increase the probability of success.

In an effort to discover a novel neonicotinic insecticide, three-dimensional quantitative structure activity relationship (3D-QSAR) methods were used to sort target molecules suggested by a team of experienced researchers. Several targets that passed threshold criteria were then prepared and tested. The results from five representative examples are reported in this chapter, which highlights both the strengths and weaknesses of this approach. A comparison of target molecules synthesized with and without the benefit of modeling is also included, providing clear evidence that a properly derived pharmacophore and validated statistical models can offer a significant advantage when exploring diverse structural modifications.

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Background

Safe and effective control of insect pests is vital to agriculture and the protection of the world food supply. The development of the neonicotinic class of insecticides (*4*) in the early 1980s was an important advance, and recognizing this, several companies launched commercial compounds in the following years (Figure 1). Although nithiazine might be considered the first neonicotinic insecticide, it was the addition of a heterocyclic tail to the nithiazine head that propelled the area to prominence and led to the discovery of imidacloprid (IMI) (*4*). This highly active molecule continues to be an important control option for sap feeding insects today.

The nicotinic acetylcholine receptor (nAChR) binding site is the target site of the neonicotinic insecticides (*4*). Many of the current commercial products are also agonists, in that they have an excitatory effect on nerves when bound to the receptor. Although mammals have nAChRs as well, target site differences allow the neonicotinic compounds to be highly selective, affecting insect nAChRs much more potently than mammalian nAChRs (*5*). This insect selectivity and low use rates make neonicotinic insecticides very attractive for the control of insect pests.

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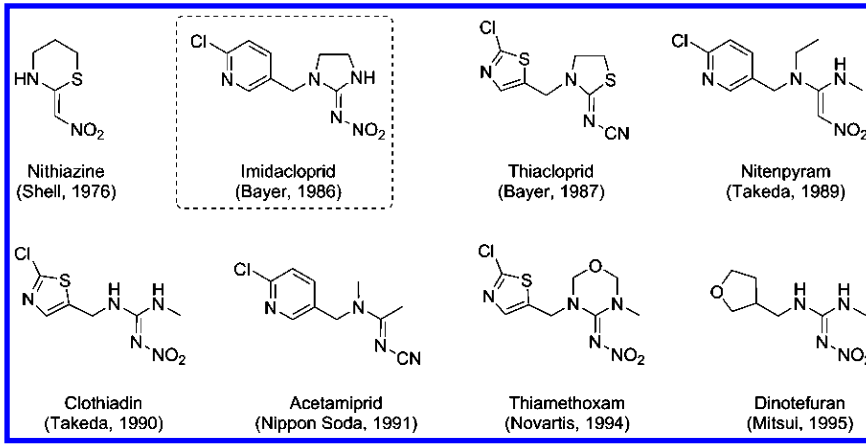

Figure 1. Commercial neonicotinic insecticides and year of initial patent.

Early investigations at Dow AgroSciences explored modest variations in the general structure of neonicotinic insecticides (Figure 2). The need to find novel molecules with superior activity, broader spectrum and an improved environmental profile led to the consideration of more diverse structures. In an effort to focus on the most promising candidates, we envisioned leveraging the established SAR with the aid of computer assisted molecular modeling to define the relevant pharmacophore and map the target site. If the critical spatial and electronic features necessary for binding at the nAChR could be deduced, it would be possible to assess proposed structures and only choose molecules for synthesis that fit the target site. Although such a pharmacophore based approach would only address binding, additional statistical models describing biological efficacy could account for factors that affect receptor function. Any improvement in the probability of preparing active compounds, especially agonists, was of great interest.

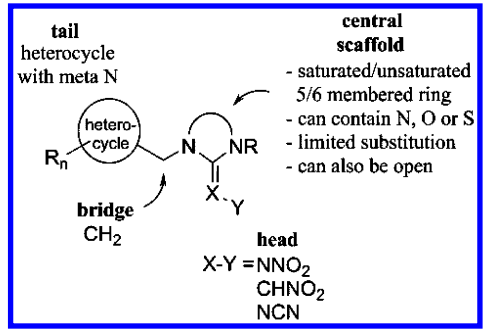

Figure 2. Generalized structure of known neonicotinic insecticides.

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Active Analog Method

Biological data for more than two hundred neonicotinic analogs with a wide range of efficacy were readily available. This information was used to derive a nAChR pharmacophore using the active analog (*6*, *7*) method. This approach involved overlaying 236 geometry optimized structures based on measurements from an [^{3}H]IMI displacement assay to determine the features required for high affinity binding (Figure 3). It was hypothesized that the neonicotinic target site is largely conserved over insect species so the binding assay based on a housefly head preparation was considered indicative of general intrinsic potency. In brief, the pharmacophore model was constructed in Sybyl 6.5 (*8*) and the molecules were minimized using the Tripos force field (*9*, *10*) and Gästeiger-Hückel charges (*11–15*) with a distance dependent dielectric of 2.0*R. After the alignment of all the structures to the pharmacophore model, single point AM1 (*16*) charges were calculated using MOPAC 93 (*17*) for use in the 3D-QSAR models. CoMFA[1] models using both PLS (*18*) and SIMCA (*19*) methodologies were used for making quantitative and categorical predictions, respectively.

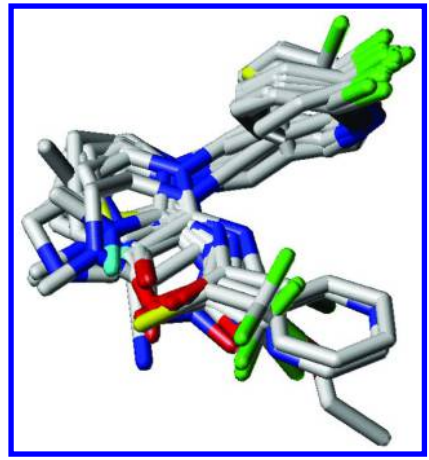

Figure 3. Minimized structure overlay of all compounds (25) with $K_i \leq 30$ nM (see color insert)

The nAChR pharmacophore was determined to consist of a pair of hydrogen bond acceptors 3.0 angstroms apart and separated by an angle of 60° (Figure 4). A further restriction was the total volume circumscribed by the overlay of all the component structures. Only target molecules that fit entirely within this space were considered.

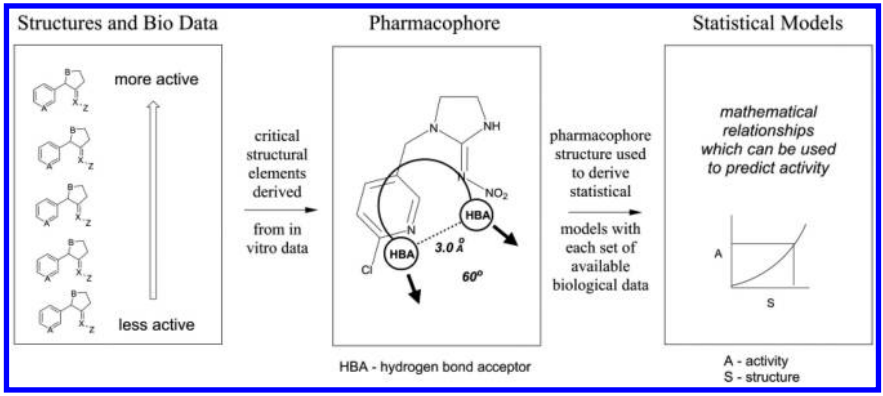

Figure 4. Derivation of a pharmacophore and statistical models

CoMFA models relate molecular structure to biological activity and can be used to predict the biological effects of yet to be synthesized target compounds. Individual CoMFA models were built, therefore, from the newly derived neonicotinic pharmacophore and data from several different insect assays. Such models, however, must be validated before they can be used for evaluating structures, meaning that they have to predict biological activity correctly at a higher than random rate. Three of the fourteen models constructed were confirmed in this way, as they did indeed predict insecticidal activity correctly 3 out of 4 times. These validated statistical models were a [^{3}H]IMI binding model, a cockroach symptomology model representing electrophysiological measurements of agonist response relative to nicotine (*20*) and a cotton aphid (*Aphis gossypii*) insecticidal assay model.

Compared to predictions for high affinity molecules with a K_i[IMI] < 1000 nM, the success rate was even higher (19 out of 20 times) for the binding assay model when predicting K_i[IMI] >1000 nM. This means that the model was particularly good at identifying very weak or inactive structures, which allowed focus on more promising targets.

Biological Assays

The three validated neonicotinic CoMFA models derived from the pharmacophore model were based on 1) the [^{3}H]IMI binding assay, 2) a set of electrophysiological measurements of agonist response relative to nicotine and 3) a cotton aphid (*Aphis gossypii*) insecticidal assay (Figure 5).

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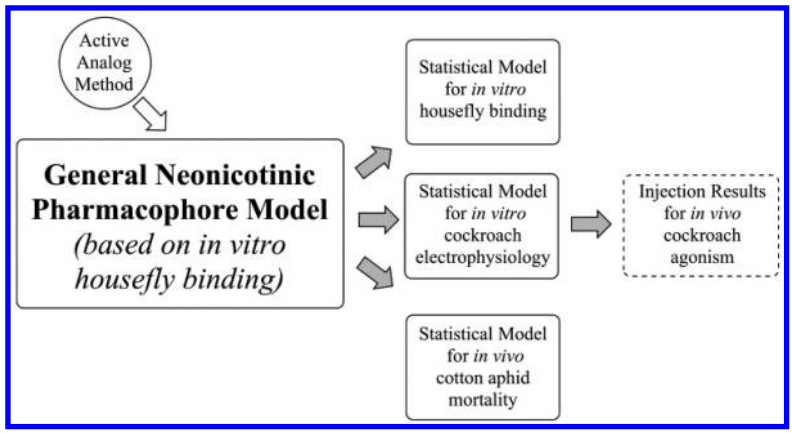

Figure 5. Diagram of molecular mechanics and statistical models

Housefly Head in Vitro Binding Assay

A binding assay based on radiolabeled IMI in a housefly head preparation was used to measure the intrinsic activity of each molecule (*21*). Compounds were tested in triplicate, typically in 10 fold dilutions. Assay volume was 200 µL and the assay was allowed to proceed for 1 hour. Data were analyzed and inhibition constants (K_i) calculated using Graph Pad Prism. A K_i[^{3}H]IMI displacement value of < 10 nM was considered excellent and such molecules were expected to show whole insect mortality.

Electrophysiological Measurements and the Cockroach Symptomology Assay

Blocking of nAChRs with antagonists does lead to insecticidal effects, but the insecticidal potency is generally inferior to that of agonists (*22*). Not surprisingly, most commercial neonicotinic insecticides are nAChR agonists. Electrophysiological measurements are needed to determine if a molecule binding at the target site is behaving as an agonist or antagonist.

Using a published method (*20*), the electrophysiological response of 28 neonicotinic compounds was measured relative to nicotine (*23*), which is an agonist of the nAChR. Although the set of compounds tested was small, it was possible to generate a valid model for predicting agonism. Unfortunately, measuring electric currents in nerve tissue is labor intensive and so not very practical for evaluating analogs on a regular basis. However, there is an excellent correlation between these measured values and the very distinctive tremor elicited in a cockroach when it is injected with an agonist. When 20 neonicotinic compounds were tested in this manner, the molecules that demonstrated an agonist response ≥80% of the amplitude of nicotine in the electrophysiological test all caused visible tremors. The other compounds tested did not cause tremors. Although conceptually a simple test, this new assay provided a powerful,

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straightforward method for identifying agonists. If 3 out of 4 cockroaches exhibited tremors upon being injected with up to 10 μg of a material, the compound was considered an agonist.

Finding a predictive model for agonism was quite remarkable, as recognizing an agonist by visual inspection of the structure is extremely difficult. Agonists do not look very different from antagonists. This point is well illustrated by considering a series of IMI analogs where chlorine substitution is varied around the pyridyl tail (Figure 6). Note how it is possible to have good intrinsic activity without generating an agonist response.

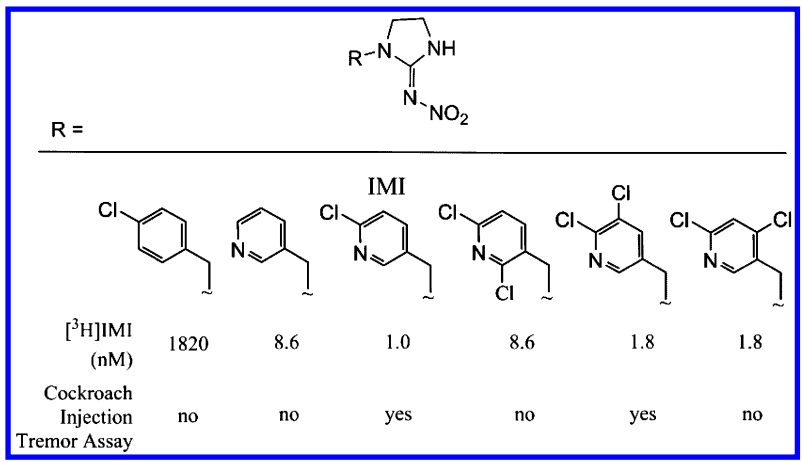

Figure 6. Intrinsic activity and agonist response of IMI tail analogs.

Cotton Aphid Whole Insect Assay

An important first test to see if a compound is insecticidal is to directly treat live insects with the material. Activity in such a test demonstrates not only that a compound is able interact with the target site, but that it can move through the host plant and insect pest before it is metabolized or excreted. This is arguably the most important evaluation in terms of utility.

Neonicotinic insecticides are most effective on sap-feeding insects, and therefore screening was done using cotton aphids. Commercial neonicotinic compounds control this insect at rates well below 1 ppm, but for an initial assessment of novel analogs, testing at 5 ppm was considered a good place to start. For simplicity, the SIMCA method (*19*) (soft independent modeling of class analogy) was used to quantify predictions of activity on cotton aphids. This provided a bimodal mortality score, where ≥ 50% = 1 and < 50% = 0.

Threshold Criteria and Prediction of Activity

Based on predictions from the validated CoMFA models for *in vitro* [^{3}H]IMI binding, *in vivo* electrophysiological measurements of agonist response relative

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to nicotine and *in vivo* cotton aphid mortality, proposed targets were evaluated *prior to synthesis*. Taken together, these models provided a useful appraisal of all proposed structures with respect to intrinsic binding, agonist response and whole insect activity. Threshold criteria were established for each of these assessments and only targets that passed all three prediction criteria were considered for synthesis.

Predicted Threshold Criteria:

- ^{3}H[IMI] K_i < 100 nM
- Agonist efficacy amplitude ≥ 80% that of nicotine
- Cotton Aphid @ 5 ppm mortality score ≥ 50%

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At first glance, the threshold for intrinsic binding may appear high, but it was set that way because the binding model tended to under predict the potency of the very best compounds and we did not wish to miss any. Even with this rather lenient threshold, however, most of the suggested targets were filtered out. Only 13% of all proposed structures (329 out of 2462) from a team of experienced chemists and biologists met all three of the threshold requirements.

Several proposed targets that met these criteria were synthesized and evaluated. A number of the compounds prepared were active, but not all. Five of these synthesized molecules have been selected to represent our results (Table 1) and illustrate the benefits and limitations of the pharmacophore based approach. Table 1 shows the targets, along with a three dimensional overlay of each with IMI. One of the degenerate minimum energy conformations of this important insecticide was selected to represent its bioactive shape, and so the superimposed structures provide a good visualization of how well a proposed new structure might fit the target site. The next two columns give the *predicted* values for the intrinsic binding model and the agonist model. All were expected to have ≥ 50% mortality in the 5 ppm cotton aphid assay. The corresponding *measured* values are reported next. The last two columns evaluate the performance of the three models in terms of how well the measured results matched the predicted activity and the quality of the insecticidal potency achieved.

Target Design and Synthesis

All proposed targets synthesized (Table 1) met the threshold criteria of all three validated CoMFA models derived from the pharmacophore model. The hypothesis and rationale for each target is described in this section, followed by the synthesis of the molecule and the resulting biological activity. Targets were often inspired by existing active molecules from the known neonicotinic SAR.

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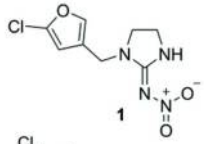
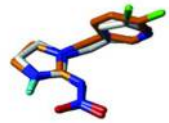
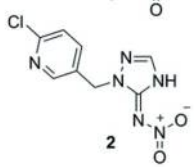
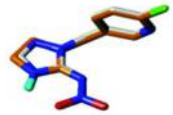
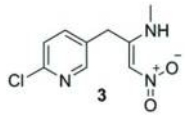
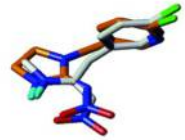
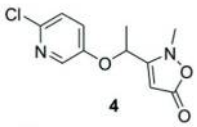
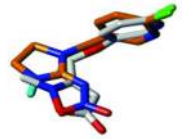
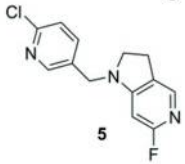
Table 1. Predicted and Measured Activity of Target Molecules (see color insert)

proposed target	3-D overlay with imidacloprid[a]	Predictions		Measurements			Results		
		pK_i[b] (nM)	pRel Eff[c] (%)	K_i[d] (nM)	Tremor Assay[e]	Aphid LC_{50}[f] (ppm)	Str Div[g]	Predict-ivity[h]	Acti-vity[i]
 1		15.1	125	101	yes	0.45	S	YYY	A
 2		7.5	141	NSD	no	not active	S	NNN	F
 3		36.8	122	14	yes	0.17	M	YYY	A
 4		80.1	89	255	yes (rev)	0.90	L	YYY	A
 5		11.3	86	42	---	4.8	L	Y-Y	B

a Minimized energy conforrmations. IMI is the orange structure. b Predicted binding in housefly head [^{3}H]IMI displacement assay (pass criterion: < 100 nM). c Predicted % efficacy relative to nicotine (pass criteria: ≥ 80%). d Measured binding in housefly head [^{3}H]IMI displacement assay (IMI K_i = 1 nM). e Cockroach injection assay: tremor indicates agonist response (yes or no; rev = reversible). f Cotton Aphid LC_{50} (IMI LC_{50} = 0.03 ppm). g Structural difference with respect to IMI: small (S), medium (M) or large (L). h Predictivity of 3 Models: yes (Y) no (N); first letter refers to housefly in vitro assay ± one order of magnitude (based on the standard error of the CoMFA binding model); second letter refers to whether tremor was observed in the cockroach injection assay when the % efficacy with respect to nicotine was predicted to be ≥ 80%; third letter refers to whether % mortality in the 5 ppm cotton aphid assay was ≥ 50% (LC_{50} = ≤ 5 ppm). i Quality of whole insect activity: cotton aphid LC_{50} ≤ 1ppm (A); cotton aphid LC_{50} = 1-5 ppm (B); not active (F).

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Furan Tail

Identification of a novel active tail as effective as the 2-chloropyrid-5-ylmethyl tail of IMI and combining it with known active heads was considered a good strategy for discovering new neonicotinic insecticides. Although several attempts were made at finding a novel oxacycle (Figure 7), the activity of the tetrahydrofuran-3-yl tail described in the literature (*24*) appeared to be unique. Even the activity of the structurally similar aromatic furan-3-yl ring was disappointing. The validated CoMFA models, however, suggested that the addition of a chlorine substituent to the furan ring would significantly improve potency. Although an unsubstituted furan-3-yl analog had been described in the literature (*25*), a chloro substituted analog had never been prepared. The key intermediate required several steps to synthesize (Scheme 1) and depended on a sequential blocking-deblocking strategy to obtain the desired regioisomer. The improvement in activity observed with the 5-chlorofuran-3-yl tail of **1** (Table 1) over the unsubstituted furan-3-yl tail (Figure 7) was dramatic and paralleled results observed with the pyrid-3-yl tails. On the other hand, the regioisomeric 2-chlorofuran-3-yl moiety was completely inactive. These molecules clearly demonstrated that the validated neonicotinic CoMFA models could identify better analogs to synthesize. The increased activity in this case, however, did not surpass IMI nor change the insecticidal spectrum (*26*).

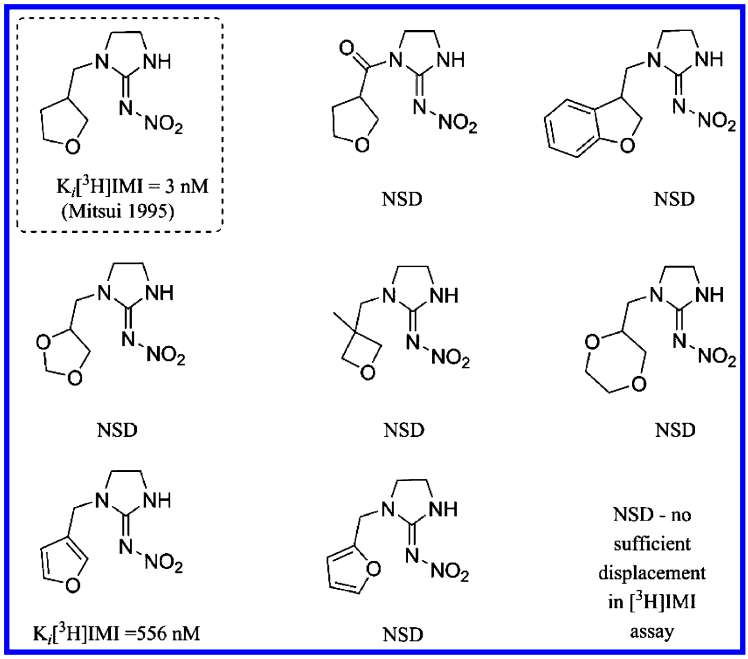

Figure 7. Comparison of oxygen heterocycles as neonicotinic tails.

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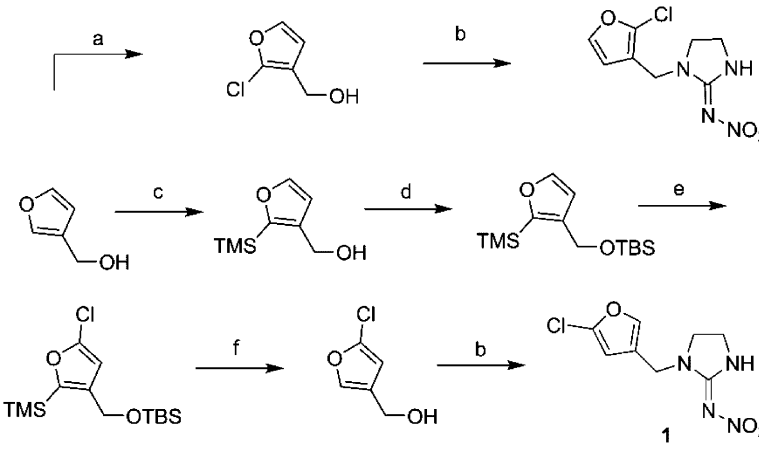

a) 3 eq n-BuLi, -30 °C, THF, C_2Cl_6 b) MsCl; NaH, DMF, 50 °C, 1hr, *N*-(imidazolidin-2-ylidene)nitramide, 36%; 48% c) 3 eq n-BuLi, -30 °C, THF, TMsCl, 78% d) TBSCl, imidazole, CH_2Cl_2, RT, 88% e) 2 eq n-BuLi, -30 °C, THF, C_2Cl_6, 92% f) TBAF, THF, 0° C, 91%

Scheme 1. Synthesis of neonicotinic targets with chlorofuranyl tails.

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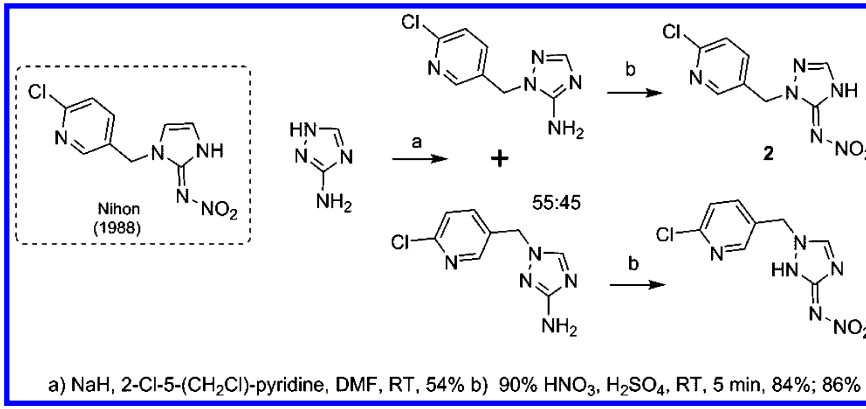
Triazole Central Scaffold

We next focused on altering the imidazoline central scaffold of IMI. The imidazole analog (*27*) was a logical extension of the known SAR and found to be highly active (Scheme 2). The corresponding triazole and tetrazole analogs were apparently unknown in the open literature. They did not differ significantly in shape from the imidazole compound and the models predicted that they would be insecticidal. Alkylation of 2-aminotriazole produced a mixture of regioisomers which were nitrated after separation. Surprisingly, compound **2** turned out to be completely inactive, as was the corresponding tetrazole analog prepared in the same fashion (the regioisomers were not active, as expected). Our first concern was that the desired compound **2** existed in an unexpected tautomeric form, not the structure that had been evaluated by the models. X-ray and ^{15}N NMR studies, however, clearly demonstrated the double bond was exocyclic.

This meant that the lack of activity was due to the additional nitrogen at a location where electronegativity was not tolerated. The prediction most likely failed because there was not enough structural diversity in that position among the molecules used to construct the pharmacophore model.



Scheme 2. Synthesis of triazole central scaffold neonicotinic compounds.

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Shortened Acyclic Central Scaffold

Our attention then turned to the bridge between the head and tail moieties of IMI. Having a shorter chain of atoms between the hydrogen bond acceptors of a neonicotinic target structure appears counterintuitive at first, but the 3-D overlay of target molecule **3** with IMI clearly showed that these critical parts could be easily superimposed (Table 1). The oxygen atoms of the nitro groups and the nitrogen atoms of the pyridyl tails lined up surprisingly well. This is because the linking atoms in the shortened molecule make a tighter turn between the two hydrogen bond acceptors, allowing their lone pairs to be in the same relative position as in IMI.

Although the shortened acyclic central scaffold compound was proposed independently, there was evidence in the literature that a nitrogen atom was not required in the linker (*28*) and that a shorter molecule (*29*) could be active. These two features, however, had never before been combined into one molecule. Standard functional group manipulations allowed us to prepare target molecule **3** from 2-chloro-5-(chloromethyl)pyridine in a straightforward manner (Scheme 3).

Testing revealed this shortened acyclic central scaffold compound and its cyanoamine analog to be highly active agonists (Table 1) and so the area was investigated extensively (*30*). The best molecules from that effort had levels of activity that were consistent with those of commercial insecticides.

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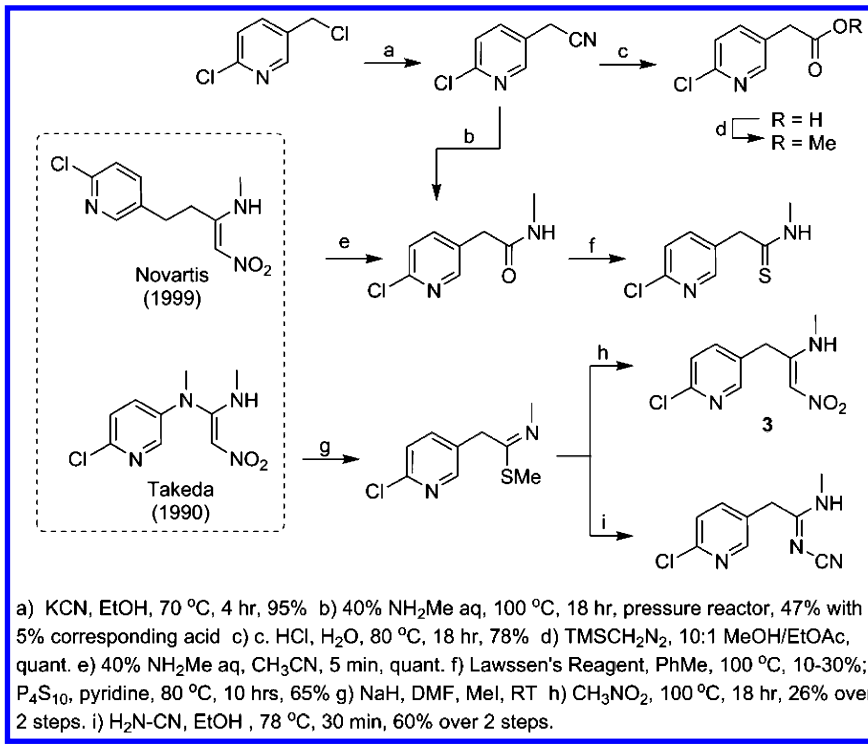

Scheme 3. Synthesis of shortened acyclic scaffold neonicotinic compounds.

Isoxazalone Head

Another structure of interest was the isoxazolone ring, which was known to be bioisosteric with the nitro group of neonicotinic insecticides (*31*). It had also been established that oxygen could be used in the linker of a neonicotinic molecule (*32*) (Scheme 4). Combining structural features from two different active molecules does not necessarily result in new efficacious materials, but in this case, the validated CoMFA models predicted there would be a beneficial effect. N-methylhydroxylamine was used to cyclize a 1,3-dicarbonyl intermediate to obtain the 2-methylisoxazalone compound **4**. It was found to be a weak agonist, but quite active (Table 1). Cyclizing with hydroxylamine, on the other hand, produced an analog in which the double bond was not in the same relative position with respect to the carbonyl. This was a substantial structural change from the one that was modeled and so it was not surprising, that this alternative motif was inactive.

The desired 2-methylisoxazol-5(2H)-one compound **4**, however, clearly demonstrates that the pair of oxygen atoms in the isoxazalone ring can interact with the nAChR target site in the same manner as a nitro group and generate an agonist response (*33*).

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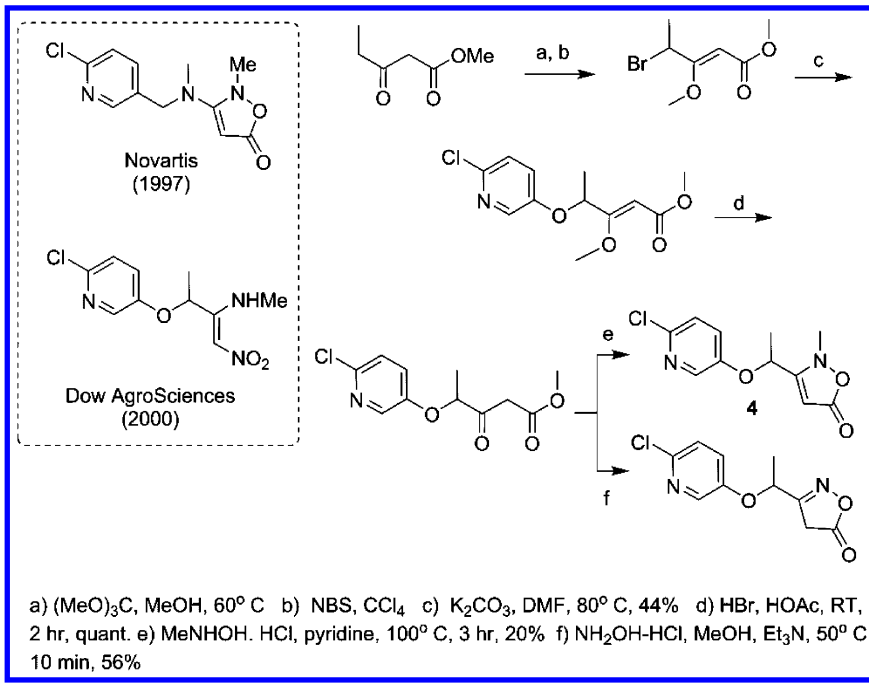

Scheme 4. Synthesis of isoxazalone neonicotinic compounds.

Tied Back Fluoropyridine Head

Within the class of neonicotinic insecticides, we hypothesized that hydrogen bond acceptor atoms can be exchanged and binding maintained if allowances are made for size and shape. Aromatic ring nitrogens and halogens should therefore be reasonable substitutes for the oxygen atoms of neonicotinic heads as suggested by target molecule **5** (Table 1). A chloropyridazine compound prepared earlier and found to be active appeared to corroborate this hypothesis (Scheme 5). The tied back 2-fluoropyridine head of target molecule **5** was constructed by the cyclization of an aminoethyl substituent back onto the heterocycle.

Compound **5** demonstrated good binding at the target site (Table 1) and was moderately active on cotton aphids. Unfortunately, it was never tested for agonist effects, but it does represent a neonicotinic molecule that has moved significantly away from the known SAR. Not only were the oxygen binding elements of the nitro group replaced by other electronegative atoms, but the central scaffold was converted into a novel rotationally restricted bicycle (*34*).

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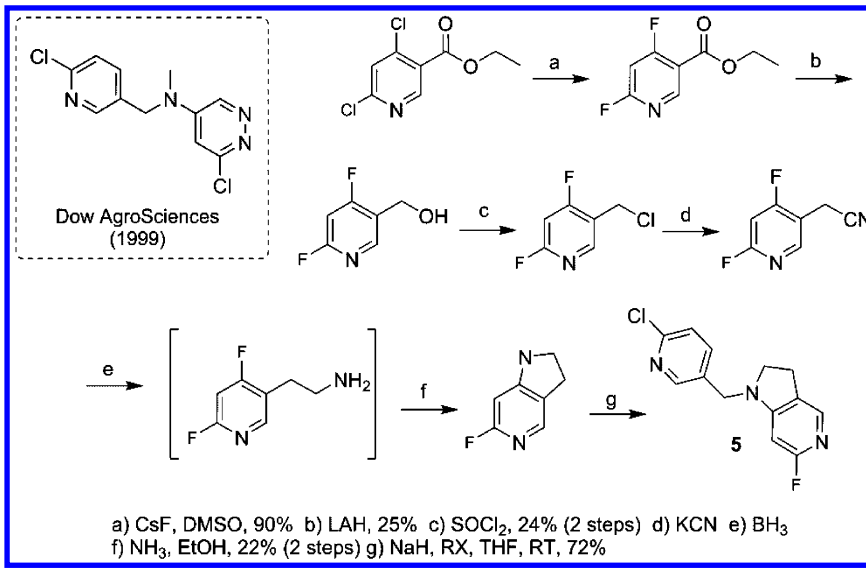

Scheme 5. Synthesis of tied-back fluoropyridine neonicotinic compound.

Evaluation of Biological Activity and Model Predictivity

Biological results for the representative five synthesized targets discussed have been summarized (Table 1). The *measured* values in the middle columns of this table should be compared directly to the *predicted* values in the first two columns. All the compounds in the table were predicted to be active against cotton aphid. Note that the cockroach injection assay was used as a surrogate for the electrophysiological tests, and a prediction of efficacy relative to nicotine of ≥ 80% corresponds to a "yes" in the tremor assay. Because our goal was to find novel neonicotinic insecticides, the last three columns evaluate each target in terms of structural diversity, model predictivity and insecticidal potency.

A qualitative assessment of structural diversity is provided by rating how large a difference there is between the target and IMI: small (S), medium (M) or large (L). Note the steady progression away from the generalized neonicotinic structure discussed earlier (Figure 2). Predictivity of the three statistical models was determined by how well the measured values matched the prediction. The options were yes (Y) and no (N), where the first letter refers to the housefly [^{3}H]IMI binding assay ± one order of magnitude (based on standard error of CoMFA binding model). The second letter refers to whether or not the % relative efficacy prediction of ≥ 80% with respect to nicotine was confirmed by tremor in the cockroach injection assay (reversible means that the insect recovered after initial tremor was observed). The third letter refers to whether or not the activity in the 5 ppm cotton aphid insecticide assay was ≥ 50%. In the last column, the quality of activity on cotton aphid was appraised: LC_{50} ≤ 1ppm (A); LC_{50} = 1-5 ppm (B); inactive (F).

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The data clearly demonstrate that the pharmacophore based statistical models worked well and led to the discovery of novel neonicotinic molecules. Although predictions may appear unnecessary for small changes in structure, seemingly minor differences can have dramatic effects, as exemplified by the inactive series of oxygen containing tails discussed earlier in comparison to compound **1** (Figure 7). Also, a pharmacophore model that lacks information in certain regions runs the risk of erroneous predictions, such as those obtained for the triazole central scaffold compound **2** in the present study. The insight gained by overlaying the shortened atom chain between hydrogen bond acceptors of analog **3** with IMI, however, allowed this molecule to be prioritized for synthesis and exploration. The resulting area was investigated extensively (*30*) and eventually led to novel compounds with levels of activity that were equivalent to commercial insecticides. Exploring greater structural diversity did challenge the validated CoMFA models, and so a lower level of potency was observed when the nature of the binding elements was changed significantly. The more modest activity observed with the isoxazalone analog **4** and the fluoropyridine analog **5**, however, were significant achievements. This is especially true because modification of molecular scaffolds changes absorption, distribution, metabolism and excretion properties as well, and these additional factors have their own unique and separate effects on potency.

Conclusions

Pharmacophore based design was used effectively to increase the probability of synthesizing biologically active neonicotinic insecticides. The graph below compares a set of 238 compounds prepared without the aid of a pharmacophore and CoMFA models to a set of 33 analogs that had passed the model threshold criteria before synthesis (Figure 8). The difference was striking. The success rate of meeting threshold criteria for the modeled structures was approximately twice that for compounds that were not evaluated in the same way. This led to a twofold increase in the number of molecules with desirable biological properties discovered over the same time period. Furthermore, this level of success was achieved with the synthesis of seven times fewer compounds.

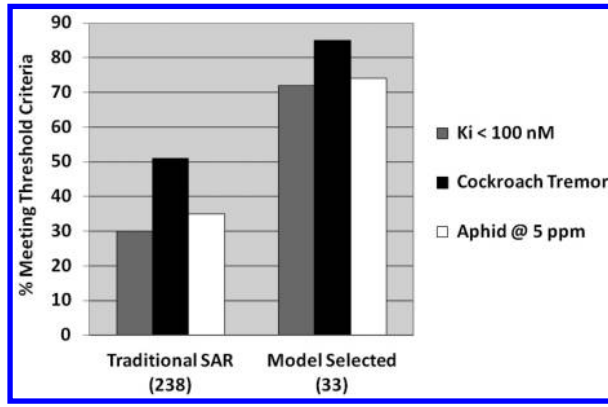

Figure 8. Comparison of success rates for traditional vs. modeled analogs.

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The pharmacophore and validated CoMFA models provided an excellent tool for prioritizing targets for synthesis. Predictions were reliable within the constraints of the model, even when diverse structures were evaluated. As a result, several classes of chemistry that interact with insect nAChRs were identified for lead generation. Pharmacophore based design proved to be an efficient and reliable method for the discovery of novel neonicotinic insecticides.

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34. Cycloxaprid is a new neonicotinic insecticide (East China University of Science and Technology, 2010) that also has a novel bicycle at its core. The key hydrogen bond acceptor, however, is still a nitro group.

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Chapter 26

Triflumezopyrim: Discovery and Optimization of a Mesoionic Insecticide for Rice

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Triflumezopyrim is an extremely effective hopper insecticide with low impact on non-target organisms including pollinators. This unique class of mesoionic chemistry targets the nicotinic acetylcholine receptor , inducing a physiological action which is distinct from that of neonicotinoids. The discovery, synthesis, optimization and mode of action will be discussed.

Introduction

Rice is the central staple food across Asia (*1*). It is grown by millions of small farmers who depend on rice for their livelihood. Because of regional preferences for particular varieties of rice the majority of rice is consumed in the country where it is grown although a growing percentage is exported. The vast majority of rice is both grown and consumed in Asia.

The brown planthopper, *Nilaparvata lugens* (Stal), is the major pest of rice in Asia (*2*). This insect causes direct damage known as "hopper burn" to the rice by feeding and also transmits viral diseases of rice. A closely related species, the whitebacked planthopper, *Sogatella frucifera* (Horvath) is also a significant pest with similar consequences to rice culture. Control of these hopper species is critical to food security in Asia and to the livlihoods of farm families across Asia.

The chemical tools for control of these hopper species are under considerable pressure because of the development of resistance and regulatory pressure (*2–4*).

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Discovery of new chemical types which can control resistant strains of these pests is an important contribution to rice culture.

DuPont™ Pyraxalt™ triflumezopyrim (Figure 1) is a novel chemical class of insecticide which controls both susceptible and resistant planthopper and leafhopper populations at low use rates. It works as an insecticide by inhibiting the nicotinic acetylcholine receptor.

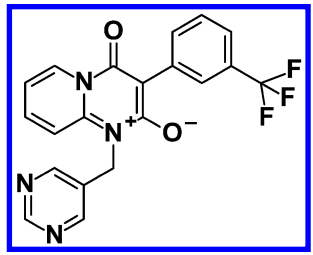

Figure 1. DuPont™ Pyraxalt™ Triflumezopyrim Insecticide

Discovery

The origins of the discovery of triflumezopyrim lie in a fungicide discovery program which led to proquinazid (*5*). The program began with pyridopyrimidinone lead **1** (Figure 2) which morphed into the quinazolinone proquinazid. The chemistry used to propare the initial lead compounds shown in Figure 2 produced the desired O-alkylated products in generally good yield but also produced in many cases a much more polar yellow compound observed as a baseline yellow spot in tlc. Some of the chemists on the program isolated these more polar compounds and characterized them as the N-alkylated mesoionic products.

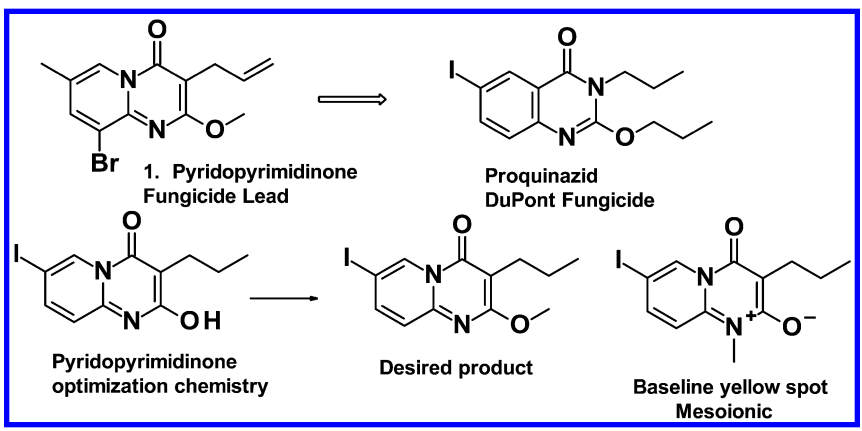

Figure 2. Origin of Mesoionics in DuPont compound collection

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Among the compounds isolated was compound **2** shown in Figure 3. None of the mesoionics isolated in this program were active as fungicides and they were not further studied at that time. However, compound 2 had activity in the insecticide screens and that activity was followed in a separate program as discussed in part in this presentation.

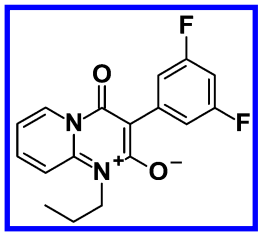

*Figure 3. Compound **2***

As mesoionic compounds are relatively rare and none have been developed for any purpose in crop protection it is appropriate to provide a definition. Mesoionics are dipolar compounds which cannot be drawn in conventional valance-bond formalism without charge separation and in which both the positive and negative charges are delocalized. They are considered by many to be non-benzenoid aromatics.

The conventional tautomers for the negative charge are shown in Figure 4 for triflumezopyrim. For each of those tautomers there are two normally drawn tautomers for the positive charge on the ring nitrogens. As can be seen in the charge distribution diagram in Figure 4 this formalism does a reasonable job of reflecting the negative charge distribution, showing nearly equal negative charge on the two oxygens and the carbon. However the positive charge distribution is quite another matter. The positive charge is primarily distributed between the two oxygen-bearing carbons and to a much lesser extent, the two nitrogens.

Synthesis

The synthesis route shown in Figure 2 for the first mesoionic compounds is clearly not a desirable one for preparing analogs. The more productive approach is to combine an amidine and a malonate in an inert solvent and heat them (*6*). In the case of most of the compounds discussed here the amidine is a 2-aminopyridine. This approach is shown in Figure 5. The malonates are generally prepared by either arylation of malonate esters (*7–9*) or by acylation of phenylacetic acid derivatives in a classic Claisen condensation as shown in Figure 6. The 2-aminopyridine derivatives are generally prepared either by reductive amination of 2-amino pyridine (*10*) or by displacement of halogen or other leaving group from a 2-halopyridine as shown in Figure 7.

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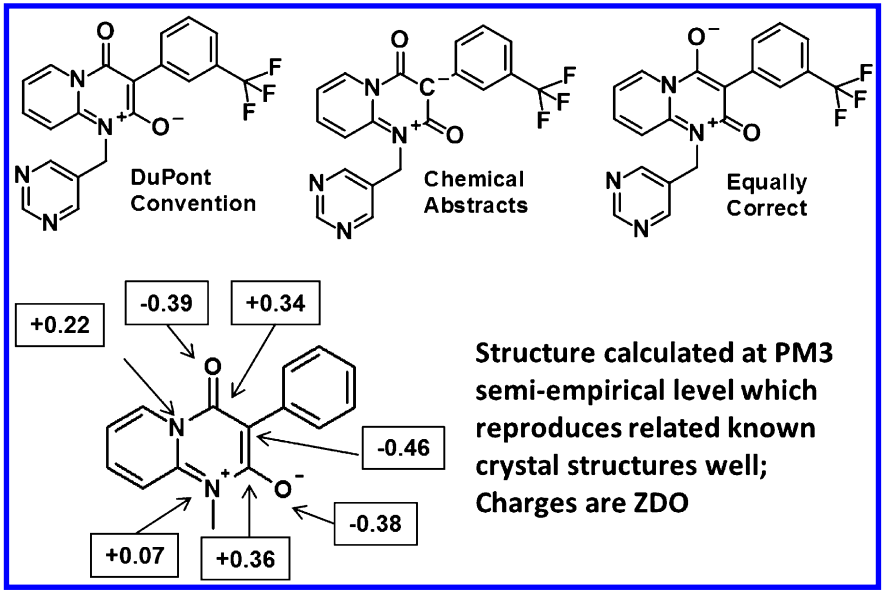

Figure 4. Negative charge tautomers of triflumezopyrim and calculated mesoionic charge distribution

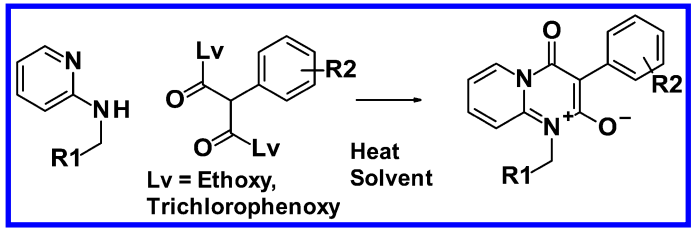

Figure 5. Cyclization step.

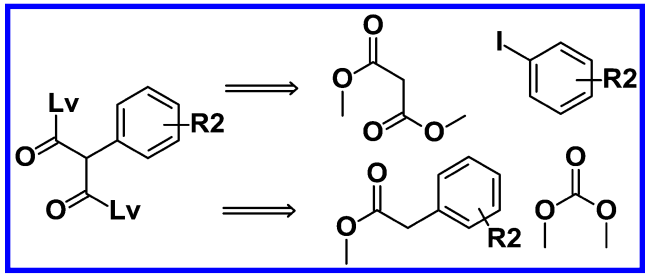

Figure 6. Malonate preparation routes

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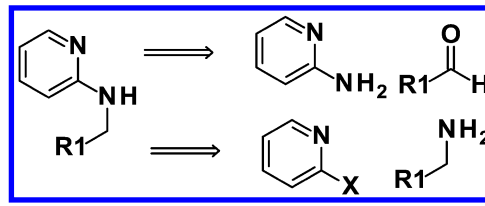

Figure 7. Aminopyridine preparation routes

Optimization

The initial compounds had modest but promising bioactivity, controlling one or two species at 250PPM or 50PPM. Compound **2** for example controlled corn planthopper at 50PPM. Smaller alkyl groups than n-propyl on nitrogen did not show insecticidal activity. Initial optimization focused on exploration of substituents on the benzene, variation of the alkyl group and in variation of the substituents on the core ring system. Initial compounds with an *N*-alkyl and a variety of substitutions on the core ring system showed decreased activity. A variety of substituents on the benzene ring showed promising activity. Longer and branched alkyl groups on nitrogen did not improve activity over the n-propyl.

The brown planthopper (BPH) and green leafhopper (GLH) tested in this program were field-harvested at the DuPont Field Research Station in Malaysia. The data present a clear picture of the development of resistance to imidacloprid over the course of this program. In the early days of the program imidacloprid controlled BPH in the tests reported in the tables with an LC_{90} of 2.0 PPM. Over the course of the next year the LC_{90} rose to >50PPM. The experimental compounds did not show significant variation in the LC_{90} over the same period clearly demonstrating efficacy against imidacloprid-resistant BPH populations.

For comparison purposes imidacloprid, used as a standard in the results reported in the Tables below, showed a typical LC_{90} against cotton/melon aphid (CMA) of 10 PPM, against green peach aphid (GPA) of 2 PPM, against corn planthopper (PLH) of 5 PPM and against potato leafhopper (PLH) of 10PPM.

The first breakthrough was found when a haloalkyl group, particularly the trifluoroethyl group replaced the *N*-n-propyl. As shown in Table 1 there was an improved spectrum and potency when compared to the n-propyl substituent on nitrogen.

While the structure-activity relationship was in early exploration phases a wide range of variations was tested. Among the less successful variations are those which are discussed in the next two paragraphs and shown in Figure 8. Variation of the "left" ring of the bicyclic core as drawn to this point did not produce improved activity. A variety of different left rings of differing sizes, both aromatic and aliphatic were made and found to have less activity than the usual core. Monocyclic cores with a variety of R3 and R4 substituents were prepared and tested. Uniformly they were less active than the core derived from 2-aminopyridine.

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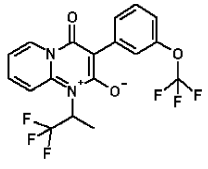
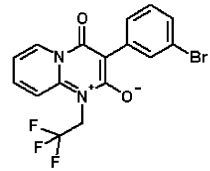
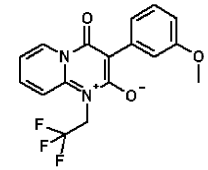
Table 1. Bioactivity (LC_{90} in PPM) of *N*-Trifluoroethyl Analogs

			
CMA	>250	>250	>250
GPA	>250	>250	>250
CPH	50	10	2
PLH	10	50	10
BPH	250	50	10
GLH	250		10

Abbreviations used in Tables: CMA, Cotton/Melon Aphid, *Aphis gossypii*; GPA, Green Peach Aphid, *Myzus persicae*; CPH, Corn Planthopper, *Peregrinus maidis*; PLH, Potato Leafhopper, *Empoasca fabae*; BPH, Brown Planthopper, *Nilaparvata lugens* (Stal); GLH, Green Leafhopper, *Nephotettix cincticeps*

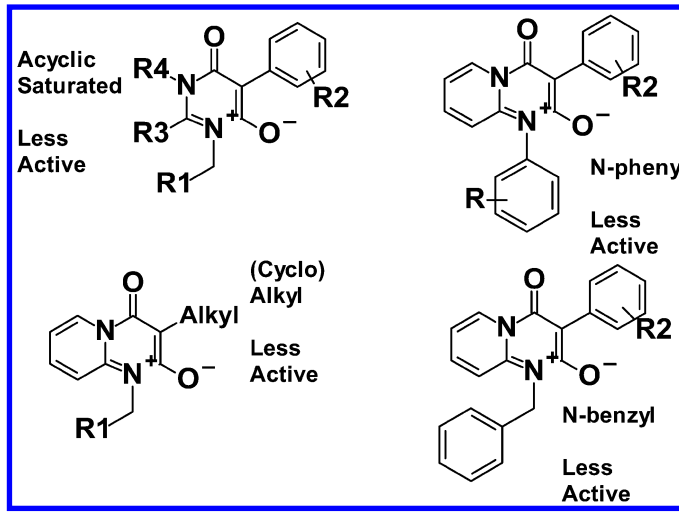


Figure 8. Less successful variations.

Replacement of the *N*-alkyl or *N*-haloalkyl substituent with substituted phenyl substantially reduced activity. Replacement of the C-phenyl group with alkyl or cycloalkyl also reduced the activity. Finally, replacement of the *N*-alkyl or *N*-haloalkyl group with benzyl substantially reduced activity.

Quite surprisingly, given the reduced activity of the benzyl analogs, the next significant improvement in activity came from the *N*-6-chloro-3-pyridylmethyl analogs shown in Table 2. There was improvement in spectrum with some control of important aphid species and in potency. More importantly, this group showed improved control of the important rice pests brown planthopper and green leafhopper.

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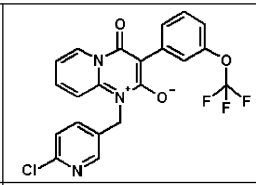
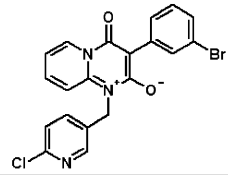
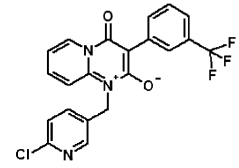
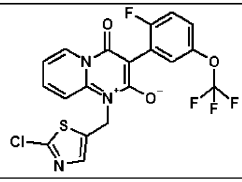
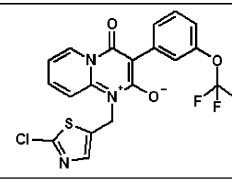
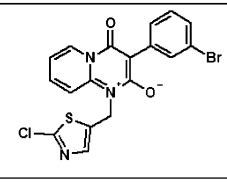
Table 2. Bioactivity (LC_{90} in PPM) of *N*-6-chloro-3-pyridylmethyl Analogs

			
CMA	>250	>250	250
GPA	>250	50	>250
CPH	50	10	10
PLH	10	10	10
BPH	10		10
GLH	50		

After the impact of the pyridylmethyl analogs was discovered we turned to the exploration of other heterocyclic groups in that position. The next one explored was the closely related 2-chloro-5-thiazolylmethyl substituent as shown in Table 3. This change provided some improvement in activity, particularly on the corn planthopper and potato leafhopper. While they continued to show good activity on the important rice pests brown planthopper and green leafhopper they did not reach a level sufficient for commercialization.

Table 3. Bioactivity (LC_{90} in PPM) of *N*-2-chloro-5-thiazolylmethyl Analogs

			
CMA	>50	250	>250
GPA	>50	250	250
CPH	2	2	10
PLH	50	2	2
BPH	10	10	50
GLH		10	50

Exploration of heterocyclylmethyl groups continued and a variety of other, less obvious, heterocycles was explored culminating in the discovery of the 5-pyrimidinylmethyl group. In the case of the pyrimidines we explored the effect of the 2-substituent on the activity. Tables 4 and 5 show the data for this comparison. The data obtained for the CMA, GPA, CPH and PLH for these analogs did not show particularly exciting activity on the aphids, however, they did show generally quite good activity on the hopper species.

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Table 4. Bioactivity (LC_{90} in PPM) of *N*-5-Pyrimidinylmethyl Analogs

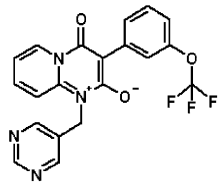
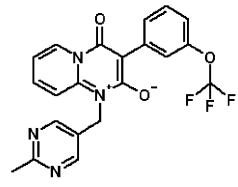
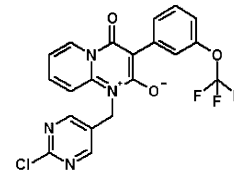
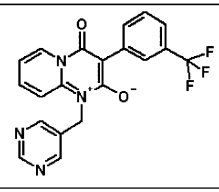
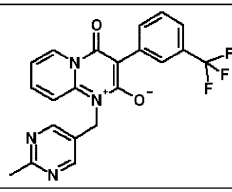
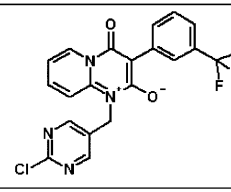
			
CMA	50	>50	250
GPA	>50	>50	>250
CPH	2	50	2
PLH	2	10	10
BPH	0.4	2	>10
GLH	2	10	

Table 5. Bioactivity (LC_{90} in PPM) of *N*-5-Pyrimidinylmethyl Analogs

			
CMA	50	50	50
GPA	50	50	50
CPH	2	2	>50
PLH	10	2	10
BPH	0.4		>10
PLH	2		

Interestingly, the unsubstituted and methyl substituented analogs showed better hopper activity than the chloro substituted analog. This was not generally the case with other heterocyclylmethyl substituents. The improved activity observed on the corn planthopper and potato leafhopper carried through to the brown planthopper and green leafhopper in laboratory tests.

Extensive field testing throughout Asia showed that the high laboratory activity on these two critical pests of rice demonstrated that the efficacy extended to the field with both direct-seeded and transplanted rice and that the efficacy was independent of rice cultivar. We were also able to demonstrate that our compounds, particularly triflumezopyrim, were extremely active against several strains of highly imidacloprid-resistant brown planthoppers both in the lab and in the field.

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Mode of Action

Injection studies conducted using the American cockroach (*Periplaneta americana*) found triflumezopyrim to have an LD_{50} values of 0.5 nmol/g body with lethargic poisoning symptoms observed within 15 minutes to several hours after injection, dependent upon dose. Even at the highest doses significant neuro-excitatory symptoms were not observed. Similar lethargic poisoning symptoms were observed with brown planthopper and green peach aphid (*Myzus persicae)* following topical application.

As the poisoning symptoms were neuronal in nature, the mesoionic insectides were evaluated against targets of commercial insecticides having a neuro-muscular mode of action. No significant effects were observed against acetylcholinesterase, GABA-gated chloride channels, voltage-gated sodium channels or ryanodine receptors, however potent activity was observed against the nicotinic acetylcholine receptor (nAChR).

Biochemical studies conducted with membranes from the green peach aphid found triflumezopyrim to potently displace ^{3}H-imidacloprid with a K_i value of 43 nM, approximately 13-fold higher than that for imidacloprid itself. Competitive ^{3}H-imidacloprid saturation studies were conducted to determine if this interaction was competitive or allosteric in nature. In the absence of triflumezopyrim, ^{3}H-imidacloprid had K_d and B_{max} values of 7.1 nM and 1265 fmol/mg, respectively. Addition of triflumezopyrim (250 and 500 nM) had no significant effect on the B_{max} value however it increased the K_d values to 11.6 and 17.6 nm, respectively. Such findings indicate a binding interaction between triflumezopyrim and imidacloprid which is competitive rather than allosteric in nature.

Given the potency for the imidacloprid binding site one would have expected excitatory rather than inhibitory poisoning symptoms. To further explore mesoionic insecticide action at the nAChR voltage clamp studies were conducted on dissociated thoracic neurons of the American cockroach. Unlike the neonicotinoids, triflumezopyrim and related mesoionic analogs exhibit extremely weak nAChR agonism activity. At triflumezopyrim's aqueous solubility limit (200 μM) very slight receptor agonism (~5% of the maximum response to acetylcholine) was observed. This was in clear contrast to neonicotinoids such as imidacloprid and dinotefuran which activate nAChR currents at concentrations below 100 nM (Figure 9).

The inhibitory action of triflumezopyrim was investigated and found to be highly potent with an IC_{50} value of 0.6 nM (Figure 10). While the mode of action of neonicotinoids is nAChR agonism, previous studies have reported inhibitory effects (*11–13*). Dose response studies on cockroach thoracic neurons show that prolonged bath application of imidacloprid, dinotefuran and cycloxaprid inhbited the acetlcholine-induced current to a much lesser degree than mesoionic insecticides. Imidacloprid and dinotefuran have IC_{50} values of 35 and >50 nM, respectively, more than 58-fold higher than that observed with triflumezopyrim (Figure 9).

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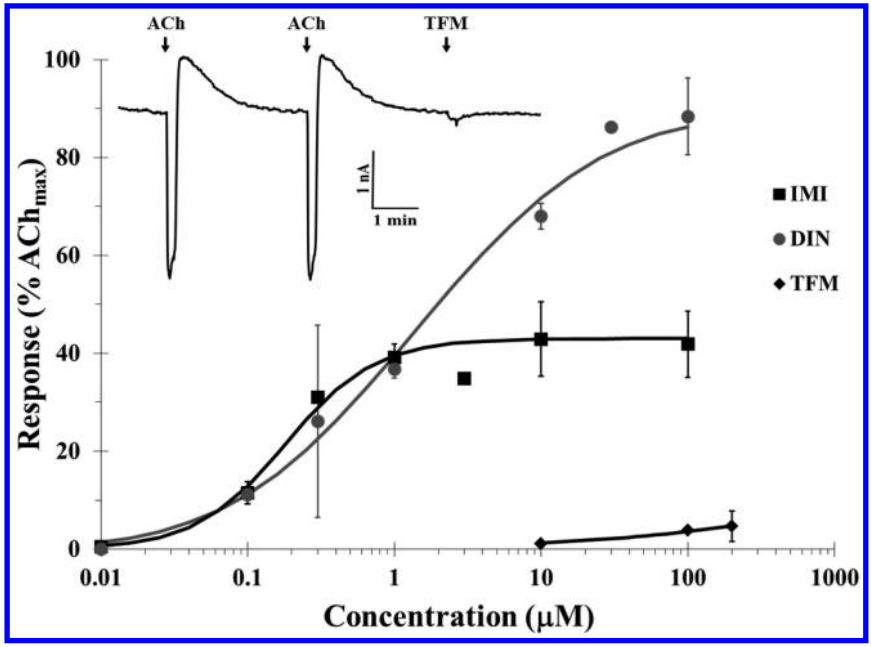

Figure 9. nAChR agonism dose response of triflumezopyrim, imidacloprid, and dinotefuran. Compounds were applied to P. americana thoracic neurons for approximately 10 sec using rapid perfusion in the immediate vicinity of neurons maintained at -60 mV via discontinuous single electrode voltage-clamp. Inset shows a typical response to 100 μM acetylcholine (ACh) and 200 μM triflumezopyrim (TFM).

Comparative kinetic studies with acetylcholine (100 μM) alone, acetylcholine + imidacloprid (1 μM) or acetylcholine + triflumezopyrim (1 μM) were conducted. Figure 11 shows that within just a few seconds of triflumezopyrim exposure the desensitizing nAChR current (nAChRD), as described by Salgado and Saar (*11*) is completely inhibited. Such an effect was not observed with imidacloprid. The inhibition induced by triflumezopyrim is consistent with the nAChR being rapidly driven to their desensitized state rather than the activated state upon binding. Such action is unique among insecticides which bind to the orthosteric site of the nAChR.

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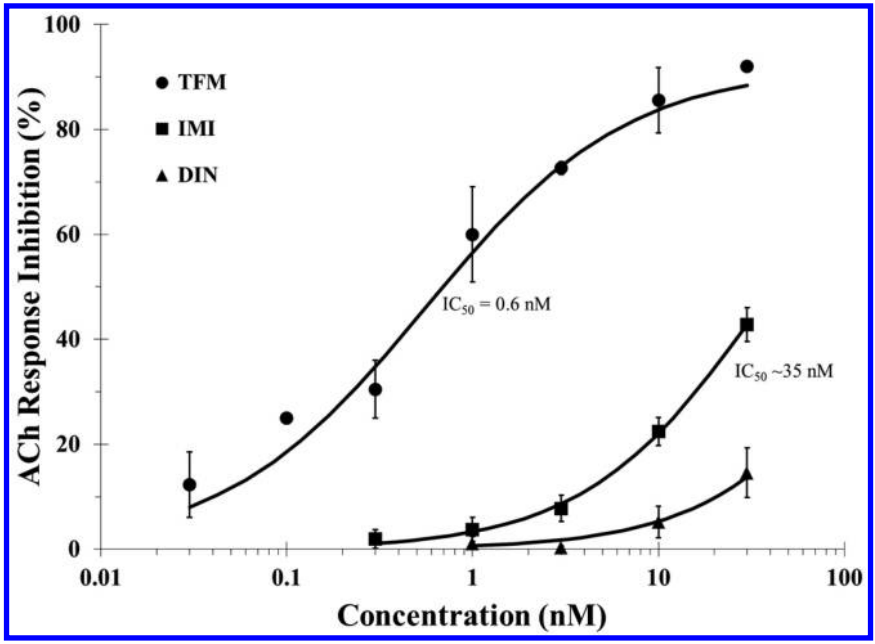

Figure 10. nAChR inhibitory dose response of triflumezopyrim (TFM), imidacloprid (IMI), and dinotefuran (DIN)). Compounds were applied to P. americana thoracic neurons for approximately 10 min using rapid perfusion in the immediate vicinity of neurons maintained at -60 mV via discontinuous single electrode voltage-clamp. Imidacloprid and dinotefuran was tested at a maximum concentration of 30 nM to avoid nAChR activation.

Environmental Profile

Triflumezopyrim has a low impact on beneficial organisms. The 96 hour LC_{50} for carp (*Cyprinus carpio*) is >100mg/L and for rainbow trout (*Ocorhyncus mykiss*) is >107mg/L. The 48 hour LC_{50} for the aquatic invertebrate *Daphnia magna* is >122mg/L. For bobwhite quail (*Colinus virginianus*) the acute oral LD_{50} is 2109 mg/kg and the dietary LC_{50} is >935 mg/kg. No adverse effects on spiders have been observed at field use rates up to and including 200g/ha. For earthworms (*Eisenia fetida*) the 14 day LC_{50} is >1000mg/kg soil. For honey bee (*Apis mellifera*) the 72 hour oral LD_{50} is 0.51 µg/bee while the 72 hour contact LD_{50} is 0.39 µg/bee. To put the bee data into perspective please see Figure 12 which shows a log-log plot of contact and oral LD_{50} data for a range of insecticides. The toxicities for commercial insecticides are European Food Safety Authority PPR conclusions and the triflumezopyrim data is GLP.

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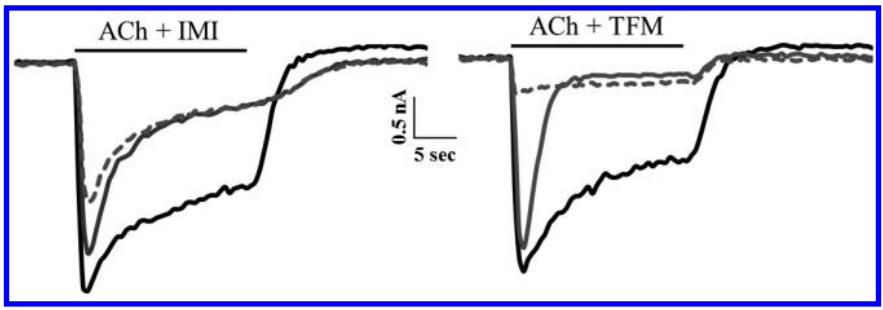

Figure 11. Effect of simultaneous acetylcholine + insecticide application to P. americana thoracic neurons. Application of 100 μM acetylcholine alone produced control responses (solid black) characterized by a rapid transient current followed by desensitization (reducing amplitude during challenge). Following saline rinse, acetylcholine + 1 μM insecticide was co-applied twice (saline perfusion between applications). Responses to the first (solid gray) and second (dashed gray) co-applications produced rapid and complete nAChRD current inhibition with triflumezopyrim. Although imidacloprid attenuated the acetylcholine response, a strong nAChRD current remained. Neurons maintained at -60 mV via discontinuous single electrode voltage-clamp.

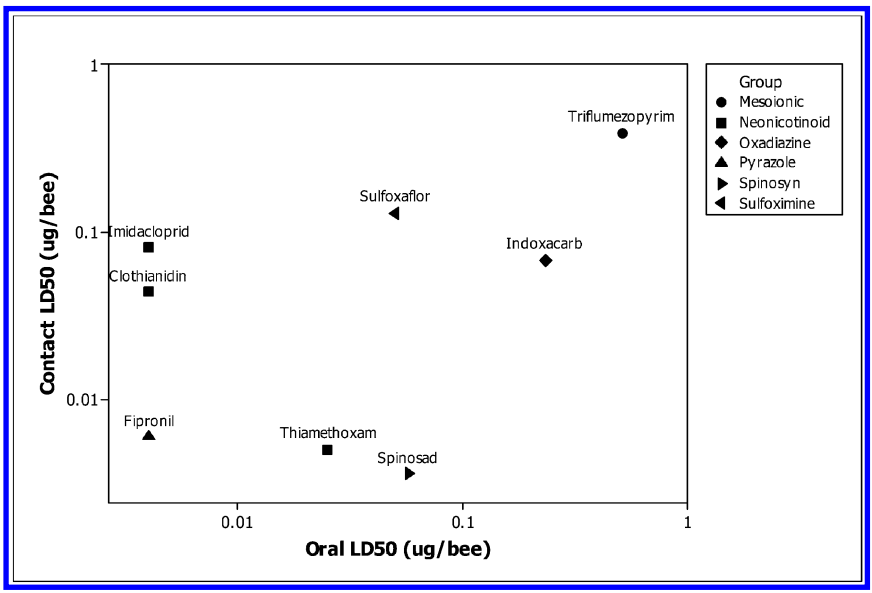

Figure 12. Honey bee hazard assessment

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Summary and Conclusion

Triflumezopyrim is the only mesoionic compound ever developed for any purpose in crop protection which makes it not simply novel but unique. It controls susceptible and resistant hopper populations at low application rates giving excellent protection of rice against a variety of planthoppers and leafhoppers. It works as an insecticide primarily be desenstitzing the nicotinic acetyl choline receptor in contrast to the neonicotinoids which act as insecticides primarily by acting as nicotinic agonists. It has a favorable environmental profile when evaluated against a variety of beneficial organisms.

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Chapter 27

Synthesis and Biological Activity of a Novel Acaricide, Pyflubumide

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Various carboxamides that inhibit succinate dehydrogenase have been created and developed. Although succinate dehydrogenase plays an important role in energy metabolism in aerobic organisms, the practical usage of the carboxamides had been primarily limited to disease control. The study to create a new carboxamide molecule revealed that introducing a fluoroalkyl group at the 4′-position on the anilino moiety remarkably enhanced the acaricidal activity. This finding prompted extensive research to ultimately identify pyflubumide, a novel complex II-inhibiting acaricide.

Introduction

Phytophagous mites are known as serious pests and some of them are notorious for their rapid development of resistance to agrochemicals. Therefore, new acaricides that are effective against the resistant population to the existing agrochemicals are always desired. Low toxicities against beneficial arthropods and natural enemies are also important for a novel acaricide in order to fit well into Integrated Pest Management (IPM) programs.

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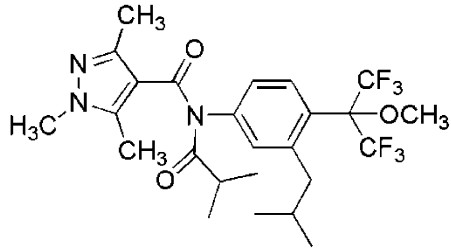
Pyflubumide (Figure 1) is a novel caboxanilide that shows remarkable activity against spider mites of the genus *Tetranychus* and *Panonychus* including resistant strains collected from the field.



Chemical name:
3′-Isobutyl-*N*-isobutyryl-1, 3, 5-trimethyl-4′-[2, 2, 2-trifluoro-1-methoxy-1-(trifluoromethyl)ethyl]pyrazole-4-carboxanilide
ISO name: Pyflubumide
Development codes: NNI-0711
Melting point: 86 ºC
Water solubility: 0.27 mg/L (20 ºC)
LogP: 5.34 (25 ºC)

Figure 1. Chemical structure, nomenclature and profile of pyflubumide

In this paper the discovery of pyflubumide are described, and the structure-activity relationships for the acaricidal activity and other biological activity are discussed.

Discovery of Acaricidal Activity

The discovery of pyflubumide originated from our interest in the various succinate dehydrogenase inhibitors (SDHI). These carboxamides such as flutolanil have been used as important tools for controlling some diseases caused by basidiomycetes. The spectrum of these carboxamides had been considered specific. However, in the late 1990's, it was reported that penthiopyrad, the carboxamide with an *ortho*-branched alkyl chain, exhibited a broader spectrum of activity (*1*).

Meanwhile, flubendiamide, a novel insecticide with unique heptafluoroisopropyl substituent was reported by our colleagues (*2*, *3*). We have synthesized various heptafluoroalkyl derivatives (*4*) but fungicide derivatives have never been synthesized.

We were very interested in introducing heptafluoroisopropyl substituent to the carboxamide and synthesized the hybrid analogue **1** (Figure 2). It only showed low fungicidal activity. This low fungicidal activity seemed to be attributed to the

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high lipophilicity of the compound, and the less lipophilic derivative **2** was then synthesized. The derivative **2** did not improve the fungicidal activity, however, it showed low larvicidal activity against spider mites (*5*).

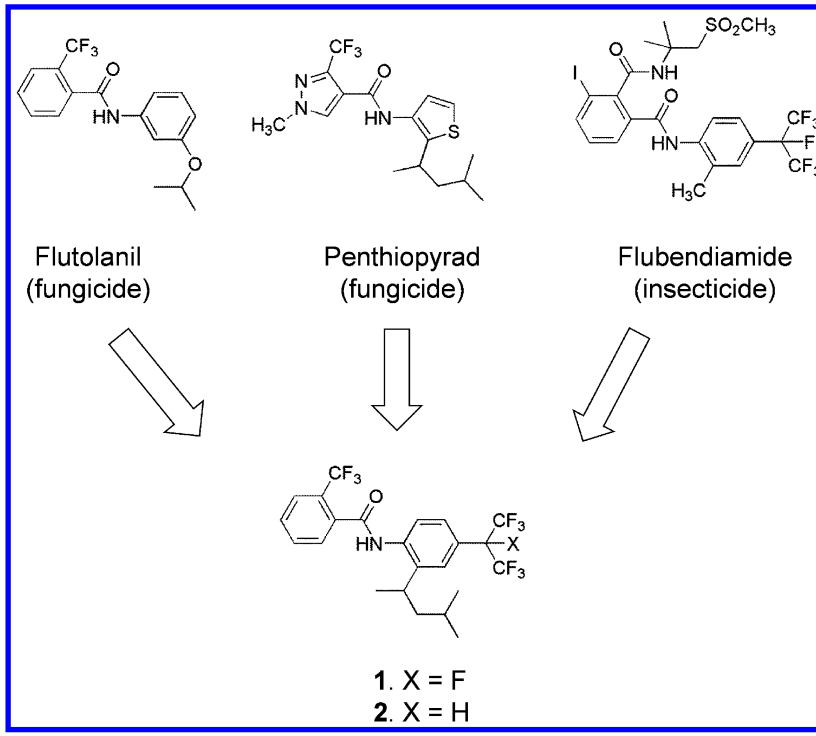

Figure 2. Discovery of acaricides

This acaricidal activity attracted our attention as so far mitochondrial complex II-inhibiting carboxamides were primarily known to deliver fungicidal activity, and acaricidal activity had never been reported for these fungcidal compounds.

Considering the structural similarity between this compound and SDHI carboxamides, we assumed that this acaricidal activity could be derived from an inhibition of mitochondrial complex II. Base on this idea, the compound **2** was modified by referring to the structures of SDHI carboxamides.

Optimization of Structures

First, the acid moiety was modified by referring to the structures of SDHI carboxamides. As a result, we found that the furametpyr derivative **3** showed higher activity. Then the substituents on the pyrazole ring were examined and the 1, 3, 5-trimethylpyrazole derivative **4** found to be best (Figure 3) (*6*).

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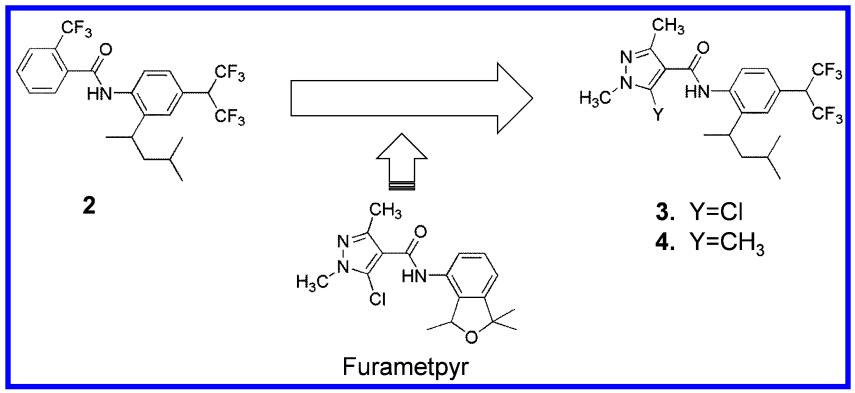

Figure 3. Modification of acid moiety

Next, the effect of the substituents on the 2′- and 3′-positon was examined.

The effect of the 2′-substituents R on the anilino moiety was shown in Table 1. The activity was strongly influenced by the number of carbon atoms and type of chain branching. Alkyl chain was required for the acaricidal activity and 1, 3-dimethylbutyl derivative **4** with the same substituent as penthiopyrad found to be best (*6*).

The effect of the 3′-substituents on the anilino moiety was shown in Table 2. By referring to the *meta*-substituted carboxamide flutolanil, isopropyloxy derivative **10** was synthesized and it showed moderate activity. From the result of 2′-substituted derivatives, substitution for alkyl chain seemed to be effective. 3′-alkyl derivatives (compound **11**-**14**) were synthesized and the derivative **13** having the same type of chain branching as flutolanil found to be best. The activity of 3′-isobutyl derivative **13** was comparable to that of 2′-(1, 3-dimethylbutyl) derivative **4**.

From the results shown in Table 1 and 2, it was concluded that an alkyl chain at the 2′- or 3′-position was required for the acaricidal activity and the favorable substituents were different between positions (2′- and 3′-) (*6*).

Finally, compounds **4** and **13** were optimized (Table 3). We examined the effect of *N*-substituent Y and $C(CF_3)_2Z$ on the 2′-(1, 3-dimethylbutyl) derivatives (type A) and the 3′-isobutyl derivatives (type B), respectively. In type A, *N*-acyl-substituted compounds retained high activity. In type B, *N*-acyl-substituted compounds retained or increased the activity. The compound 20, 23 and pyflubumide showed excellent activity. In view of their residual efficacy in the pot tests (data not shown), pyflubumide was selected as the developed compound (*6*).

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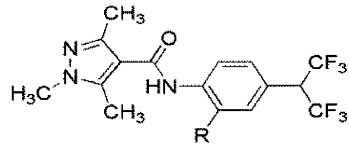
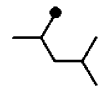
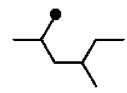
Table 1. Effects of 2′-substitutents R on *T. urticae* activity [a]



Compound	R	Activity rating [b,c]
5	H	0
6		0
7		1
8		3
4		4
9		3

[a] *Data are from reference 6.*
[b] *Activity rating, LC_{50} (mg a .i. /L);*
0: >300, 1: 100-300, 2: 30-100, 3: 10-30, 4: 3-10, 5: 1-3
[c] *Test Method: spraying method, assessment: 2 days after treatment*

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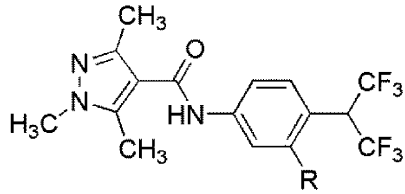
Table 2. Effects of 3′-substituents *R* on *T. urticae* activity [a]



Compound	R	Activity rating [b]
10		2
11		1
12		2
13		4
14		3

[a] *Data are from reference 6*
[b] *See Table 1*

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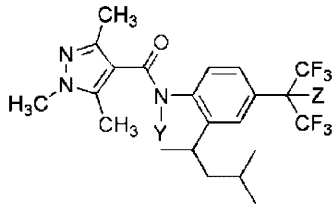
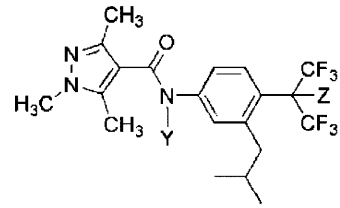
Table 3. Effect of the substituents Y and Z on the activity against *T. urticae* [a]



Type A



Type B

Compound	Type	Y	Z	Activity rating [b]
4	A	H	H	4
15	A	$COCH_3$	H	4
16	A	CO-*i*-Pr	H	4
17	A	H	OCH_3	4
18	A	$COCH_3$	OCH_3	4
19	A	CO-*i*-Pr	OCH_3	4
13	B	H	H	4
20	B	$COCH_3$	H	5
21	B	CO-*i*-Pr	H	4
22	B	H	OCH_3	3
23	B	$COCH_3$	OCH_3	5
pyflubumide	B	CO-*i*-Pr	OCH_3	5

[a] *Data are from reference 6*
[b] *See Table 1*

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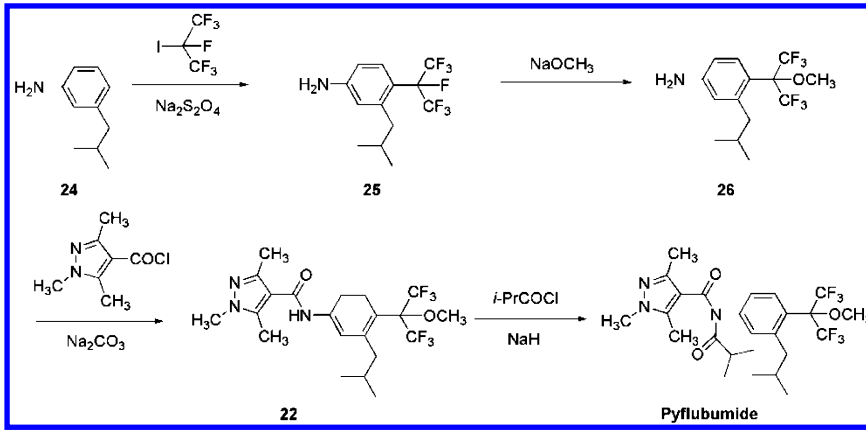
Synthesis of Pyflubumide

Synthetic pathway of pyflubumide is shown in Scheme 1 (*6–8*).



Scheme 1. Synthesis of pyflubumide

3-Isobutyl aniline **24** was heptafluoroisopropylated to give **25**. The fluorine on the benzyl position of **25** was converted to methoxy group to give **26**. The aniline **26** was reacted with 1, 3, 5-trimethylpyrazole carboxylic acid chloride to give the pyrazole carboxanilide **22**, followed by acylation to give pyflubumide.

Biological Properties of Pyflubumide

Acaricidal Activity, Resistance and Cross-Resistance

Pyflubumide showed remarkable activities against not only *Tetranychus* species but also *Panonychus* species of mites (Table 4). In addition, the acaricidal activities of pyflubumide and the conventional acaricides against the field populations of mites were examined. Pyflubumide showed excellent activity against the field populations of mites which had developed resistance to conventional acaricides (Table 4). These results indicate that this acaricide is an effective new tool for controlling spider mites which had developed resistance to existing acaricides (*6*).

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Mode of Action

Our study revealed that the metabolite of pyflubumide (compound **22**) is an inhibitor of the electron transport in mitochondrial complex II (*9*). Compound **22** shows the same inhibitory effect as described previously for *beta*-ketonitrile acaricides (*10*, *11*). However, it was reported that their binding sites on mitochondrial complex II and/or the manners of binding are not identical (*9*).

Table 4. Acaricidal activity of pyflubumide and the conventional acaricides [a]

Species	*Collected site*	LC_{50} *(mg a. i. /L)*			
		Pyflubumide	*Fenpyroximate*	*Acequinocyl*	*Etoxazol*
T. urticae	Kumamoto	0.5	> 50	> 150	> 50
	Itayanagi	0.9	> 50	> 150	> 50
	Suzaka	2.3	> 50	> 150	> 50
	Susceptible strain	1.2	0.3	3.3	0.04
P. citri	Arida	5	> 50	150	-
	Iyo	1.7	> 50	> 150	-
	Karatsu	1.5	> 50	> 150	-
	Susceptible strain	1.3	8.6	8.9	-

[a] *Data are from reference (6)*

Toxicity to Beneficial Arthropods

Table 5 shows the activity of pyflubumide on several species of beneficial arthropods and natural enemies. Pyflubumide was inactive against beneficial arthropods and natural enemies. This indicates that pyflubumide should be very safe for natural enemies, and consequently will fit well into IPM programs.

Toxicological Properties

Table 6 shows some toxicological features of pyflubumide. These results suggest that pyflubumide is safe for mammals and fishes.

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Table 5. Effects of pyflubumide on beneficial arthropods and natural enemies

Common name	*Scientific name*	*LC_{30} (mg a .i. /L)*
Silkworm	*Bombyx mori*	>100
Honey bee	*Apis mellifera*	>200
Hornfaced bee	*Osmia cornifrons*	>100
Predatory mite	*Phytoseiulus persimilis*	>200
	Amblyseius californicus	>100
	Amblyseius swirskii	>200
Lady beetle	*Harmonia axyridis*	>100
Predatory midge	*Aphidoletes aphidimyza*	>100
Parasite wasp	*Apanteles glomeratus*	>200
	Encarsia formosa	>200
Predatory bug	*Orius strigicollis*	>100
Spider	*Pardosa pseudoannulate*	>200

Table 6. Toxicological profile of pyflubumide

Acute oral :	Rat LD_{50} female	>2000 mg/kg
Mutagenicity:	Ames test	Negative
Skin irritation:	Rabbit	No irritant
Aquatic organism:	Carp LC_{50}	0.66 mg/L (96h)

Conclusions

We have found that the carboxamides with specific fluoroalkyl groups showed acaricidal activity and the modification by referring to the structures of SDHI carboxamides and introduction of substituent on amide moiety finally led to discover pyflubumide. Pyflubumide possesses excellent acaricidal activity against spider mites of the genus *Tetranychus* and *Panonychus*. Resistant strains collected from the field are controlled as well. Pyflubumide has an excellent safety profile against various beneficial arthropods and natural enemies. Our results suggest that pyflubumide is not only an effective tool for controlling spider mites which had developed resistance to existing acaricides, but is also quite suitable for inclusion into IPM programs.

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Chapter 28

Synthesis and Insecticidal Activity of New 2-Aryl-3,5-dihydro-2H-1,4-Benzoxazepine Derivatives

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As part of a retrospective screening campaign from our legacy compounds collection to identify new chemical starting points for agrochemical lead discovery programs, we identified benzoxazepine compound **1** as a new insecticidal hit with potent foliar activity against sucking insects, such as aphids, whiteflies and scales. Aspects of the synthesis, structure-activity relationships, physico-chemical properties, and biology are described herein. Our lead optimization efforts led to the discovery of compound **2**, which displayed improved potency and spectrum.

Keywords: insecticide; agrochemical discovery; privileged structure; benzoxazepine; sucking pests

Introduction

In the last 20 years, modern chemical solutions have been developed by the agrochemical industry to provide effective control of sucking pests. This is witnessed by the success of neonicotinoids, which accounted for 18.4% of global insecticides sales in 2013 (*1*) and have found broad application in the control of a wide range of sucking insects in various crops. Yet, emergence of resistance

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in sucking pest populations (*2*) threatens the long term viability of established products, and requires – beyond efficient Insecticide Resistance Management programs (*3*) – the discovery of novel insecticide classes that overcome resistance.

With this objective in mind, agrochemical companies have built effective hit discovery strategies supported by robust insecticidal screening platforms, *in vitro* and *in vivo*, fed with novel chemical input originating from diverse sources such as chemical libraries, high-speed and combinatorial synthesis, natural products, patent or literature follow-up, and rational chemistry design. These approaches often afford a large number of weakly active compounds, which makes the selection of those to actively follow-up in lead optimization programs particularly challenging. Among the various established criteria for hit selection, the concept of privileged structure has recently gained in popularity in agrochemical research (*4*). This approach, as defined by Merck medicinal chemists in the 1980's for 1,4-benzodiazepin-4-ones (*5*), refers to the ability of certain molecular frameworks to interact with multiple protein binding sites. Such scaffolds should thus be favored in lead discovery programs, especially when considering that high levels of potency and selectivity can often be achieved by subsequent optimization of their substitution pattern (*6*). Previously, we reported how the occurence of the privileged scaffold spiro[indoline-3,4′-piperidine] in a series of weak insecticidal hits led to us to start a lead optimization program, which eventually revealed a new highly potent lepidoptericide class acting through a novel mode of action (*7*, *8*). In this article, we will explain how we exploited the privileged nature of benzo-fused seven-membered ring scaffolds to the discovery of insecticidal benzoxazepines (Figure 1) of general structure **3** (Figure 2).

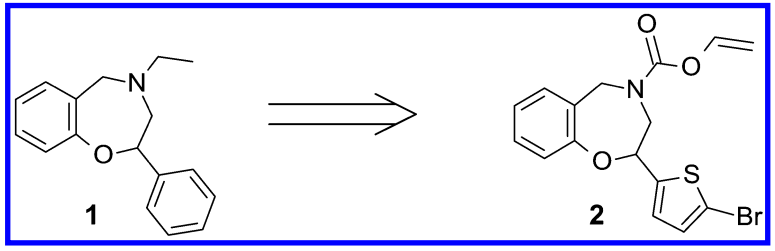

*Figure 1. From insecticidal benzoxazepine hit **1** to lead compound **2***

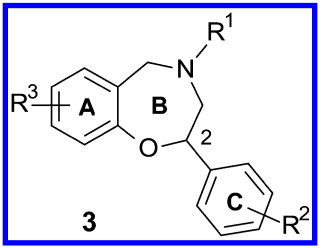

*Figure 2. General structure **3** of new insecticidal 2-aryl-3,5-dihydro-2H-1,4-benzoxazepine derivatives*

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Benzo-Fused 7-Ring Systems as Privileged Structures

Benzo-fused 7-ring bicyclic scaffolds represent popular motifs in pharmaceutical leads and drugs, as confirmed by a recent comprehensive analysis of ring occurrence in drugs (*9*). In contrast to most benzo-fused 5-ring and 6-ring bicyclic compounds, their structures are usually non-planar, an attractive feature to improve solubility (*10*) and reduce unspecific binding (*11*). Despite the potential flexibility of the 7-membered ring, their structure often possess a well-defined preferred conformation simply dependent on the level of saturation of the 7-ring, which can be predicted by quantum mechanics (*12*) or analysis of small molecule crystal databases (*13*). Not only does this unique topology allow the introduction of substituents in controlled geometry to fit into protein binding pockets, but some benzo-fused 7-ring bicyclics such as benzodiazepines have also been proposed as protein secondary structure (β-turn) mimicking scaffolds (*14*, *15*).

Although such bicyclic ring systems have found repeated success in the pharmaceutical industry (Figure 3), their use as agrochemical leads has so far remained very limited.

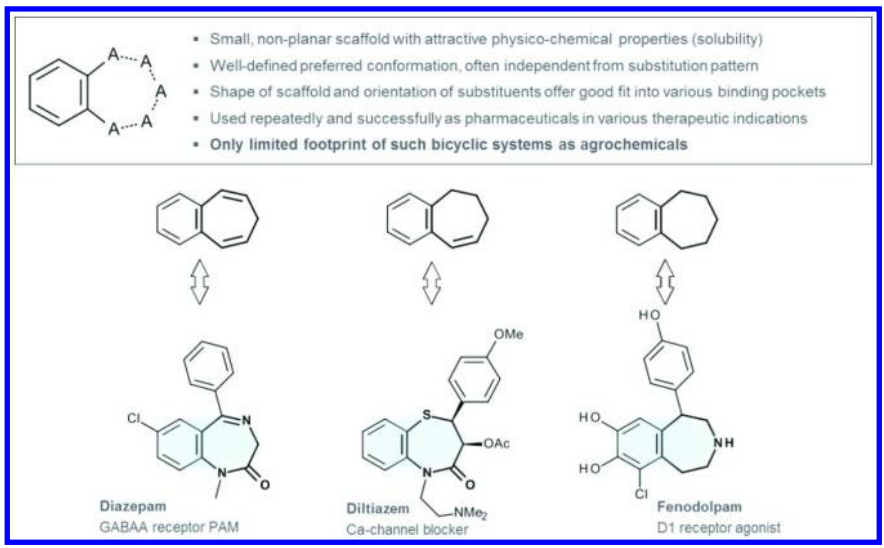

Figure 3. Properties and selected pharmaceutical examples of benzo-fused 7-ring bicyclic structures

Insecticidal Benzoxazepine: Hit Discovery

As part of a retrospective screening campaign from a Syngenta legacy companies compound collection, we identified **1** as a new insecticidal hit with moderate aphicidal activity against *Myzus persicae* in feeding/contact and systemic (hydroponic test) assays. The synthesis of a series of close analogues (Figure 4) confirmed the aphicidal activity and revealed some preliminary structure-activity relationships (Table 1): The N-dealkylated compound **4** retained

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the feeding/contact aphicidal activity of **1** but not the systemic activity (Figure 5), which could suggest an N-dealkylation bioactivation pathway as has been shown in other pesticidal classes (*16*). It was also found that only one of the two enantiomers ***(R)*-1** contributes to the biological activity, which indicated good enantiomeric stability *in vivo* and was an encouraging sign of genuine insecticidal effect through selective interaction with an important protein target in aphids. Finally, following a classical strategy in agrochemical design (*17*), a chlorine was introduced in **5** on the aromatic substituent in order to improve metabolic stability, which significantly increased the potency. However, systemic activity was no longer observed for this compound.

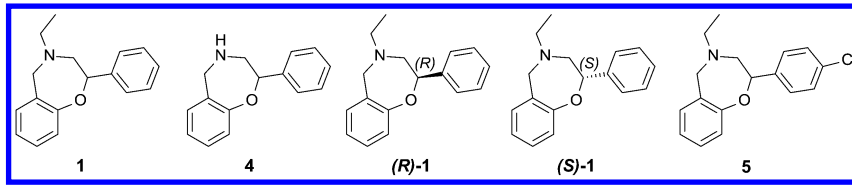

*Figure 4. Hit compound **1** and hit validation series*

Table 1. Biological activity (BP_{80} values in ppm ie effective concentration to control 80% of insect population)

Compound	*1*	*4*	*(R)-1*	*(S)-1*	*5*
Myzus persicae feeding/contact assay[a]	**200**	**200**	**< 200**	> 200	**12.5**
Myzus persicae Systemic assay[b]	**24**	> 24	**6**	> 24	> 24

[a] Sunflower, preventative application mixed population, 6 days incubation. [b] Pea seedlings, into water application; mixed population, 6 days incubation.

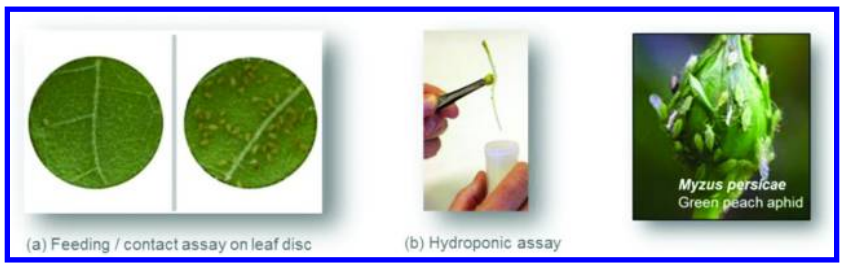

Figure 5. Biological assays on Myzus persicae (green peach aphid)

With the lead compound **5** in hand, preliminary biochemical and physiological studies suggested a novel mode of action and a favorable preliminary product safety profile. A literature review revealed some similar or related compounds in the pharmaceutical field, but no agrochemical prior art. Selected examples of

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bioactive benzoxazepines are presented in Figure 6, including some more recent examples, which appeared after our own research effort. These preliminary findings encouraged us to start a lead optimization campaign.

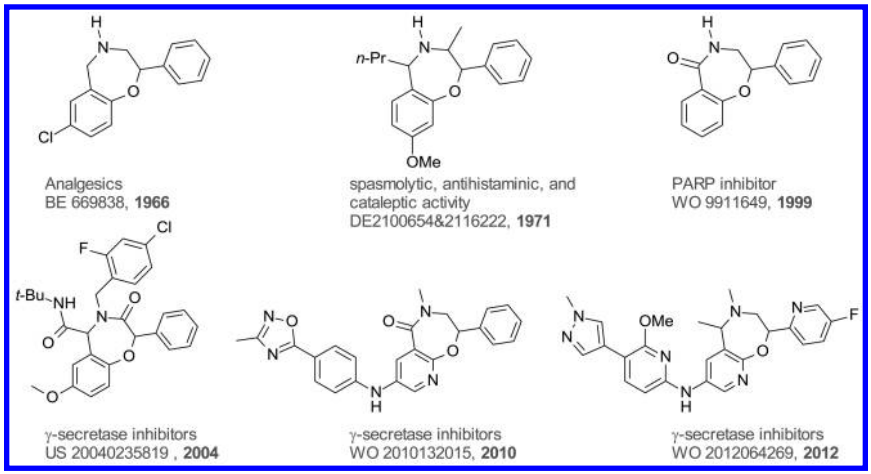

Figure 6. Selected examples of bioactive 2-aryl-3,5-dihydro-2H-1,4-benzoxazepine compounds

Cycloheptene is known to adopt three major conformations with a preference for the Chair form over the Twist-boat and Boat conformations, as computed by Boggs et al. (*12*) DFT calculations on our benzoxazepine lead compound **5** confirmed this conformational preference for the chair conformation with 2.5-4.5 kcal/mol lower energy than the twist and boat conformations (Figure 7).

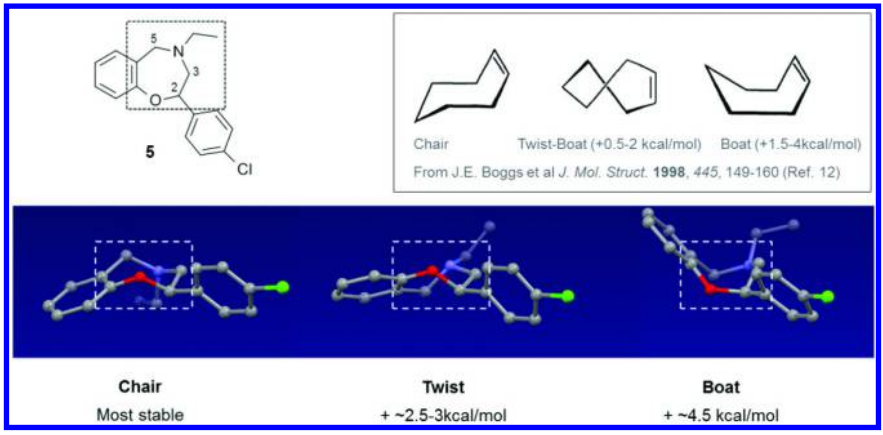

*Figure 7. Conformational analysis of 2-aryl-3,5-dihydro-2H-1,4-benzoxazepine by DFT method: DFT-M06-2X/6-311++G***

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General Synthesis

Our initial approach to the synthesis of benzoxazepine derivatives involved a Schmidt rearrangement of the flavanone **7** as illustrated in Figure 8 for the synthesis of the fluoro benzoxazepine derivative **10**. Flavanone **7** was obtained in two unoptimized steps from 2-hydroxyacetophenone (*18*, *19*). Upon treatment with sodium azide in trifluoracetic acid (*20*) **7** converted into the rearranged product **8** in good yield. Interestingly, treatment of **7** with sodium azide in a mixture of sulfuric acid and acetic acid afforded exclusively the oxazoline **9**, probably as the result of an intramolecular trapping of a transient benzylic cation by the amide carbonyl. **8** was finally reduced to the benzoxazepine **10**.

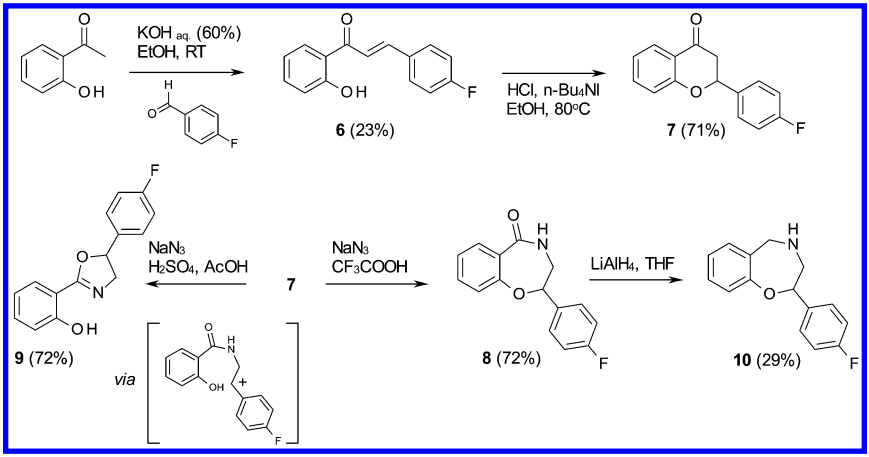

Figure 8. First synthetic approach to benzoxazepine compounds

In order to overcome the limitations of this initial approach, we developed a convergent and scalable synthesis of benzoxazepine compounds illustrated on Figure 9 with the synthesis of the lead compound **5** from readily available starting material salicaldehyde **11** and mandelic acid **12**. Reductive amination of **11** with ethylamine afforded the amino alcohol **13** which was coupled with the acid chloride **14** to afford the intermediate **15**. Intramolecular cyclisation under basic conditions produced the benzoxazepin-3-one derivative **16** which was finally reduced to the lead compound **5**. It is noteworthy that the corresponding secondary amide (N-deethylated) did not undergo cyclisation under these conditions; we presumed that the *s*-cis amide conformation required for the cyclisation was more populated for the tertiary amide when the *s*-trans conformer predominates for secondary amides. Dealkylation of **5** to **17** was achieved by reaction with vinyl chloroformate followed by acidic cleavage.

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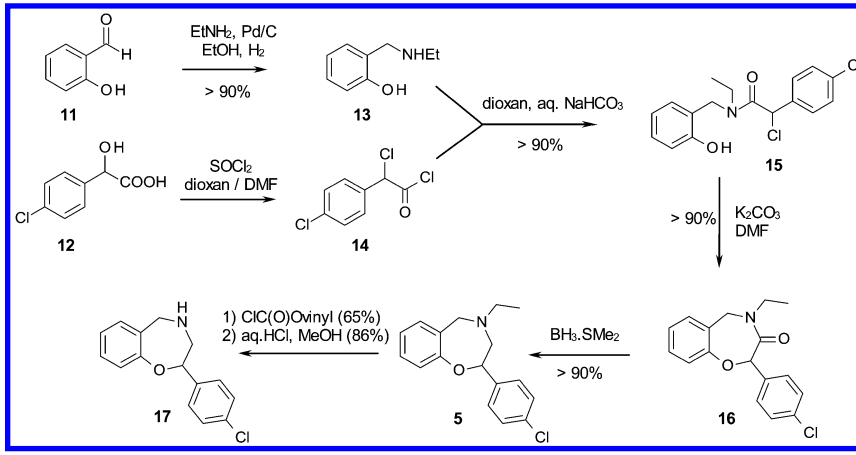

*Figure 9. Scalable synthesis of **5** and N-dealkylation*

This synthetic route allowed the preparation of enantiomerically pure isomers ***(R)*-1** and ***(S)*-1** from *(*chloromandelic acid chlorides ***(S)*-14** and ***(R)*-14** respectively. Since final compounds were found enantiomerically pure, we indeed assumed a clean inversion of stereochemistry during the intramolecular SN_2 reaction (**15→16**). ***(R)*-5** and ***(S)*-5** were obtained by chiral separation of racemic compound **5**; their absolute stereochemistry was assumed from comparison of optical rotation and biological activity with isomers ***(R)*-1** and ***(S)*-1** (Figure 10).

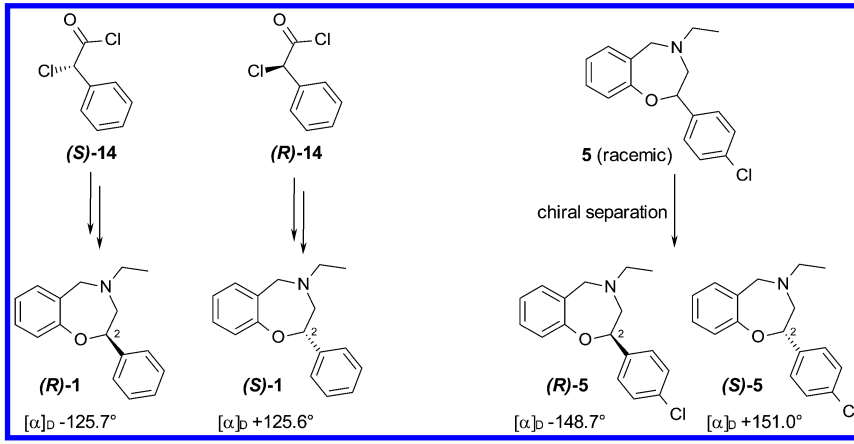

*Figure 10. Enantiomers of **1** and **5***

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An alternative synthesis was developed to allow more flexible substitution of the aromatic part of the benzoxazepine ring system (Figure 11). 2-Bromo-1-(4-chlorophenyl)ethanone **18** was converted into amino alcohol **19a**, which underwent an intramolecular SNAr reaction to afford **5** quantitatively. A complementary Cu-catalysed cyclisation from **19b** was also developed, which later proved useful for the preparation of benzoxazepines with electron-rich substituents on the aromatic ring. This synthetic route also allowed the direct preparation of the dealkylated compound **17** in only 3 steps.

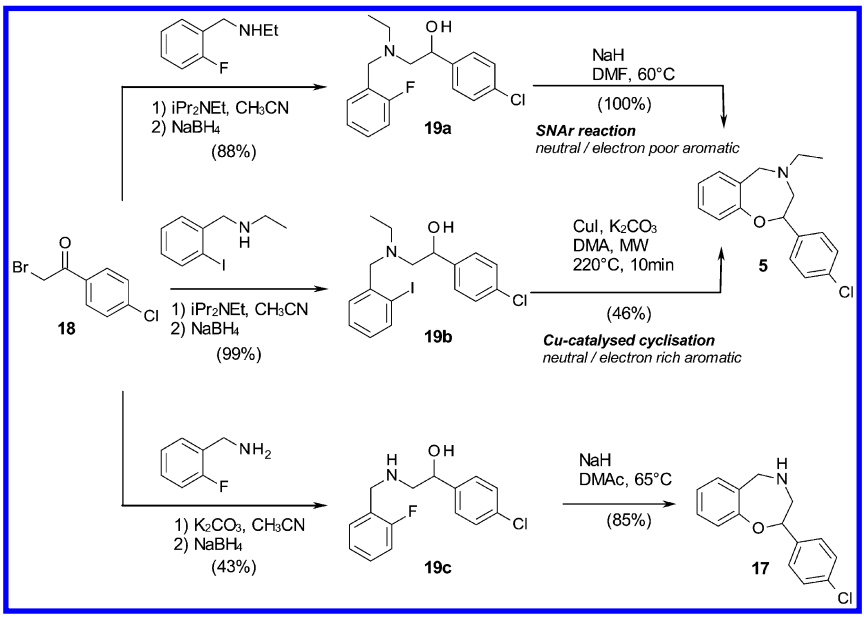

*Figure 11. Alternative synthesis of benzoxazepines **5** and **17** from phenacyl bromide derivative **18***

Structure-Activity Relationships and Synthetic Variations on the Benzoxazepine Aromatic Ring A

In the absence of structural information on ligand-protein interactions, a substituent scan remains a powerful option to explore Structure-Activity Relationships (SAR). We explored all positions of the benzoxazepine aromatic ring A by systematic substitution with fluorine and methoxy groups. In addition all four pyridine derivatives (aza-variations) were prepared. A very steep SAR picture was observed as outlined in Figure 12 with only position C-6 tolerating a fluorine substituent or an aza variation. This position was explored in more details but no other substituent introduced retained the level of activity of the initial lead compound **5**. The aromatic ring was also replaced by a thiophene in **20**, which retained some insecticidal activity, whereas deletion of the aromatic ring in **21c** led to an inactive derivative.

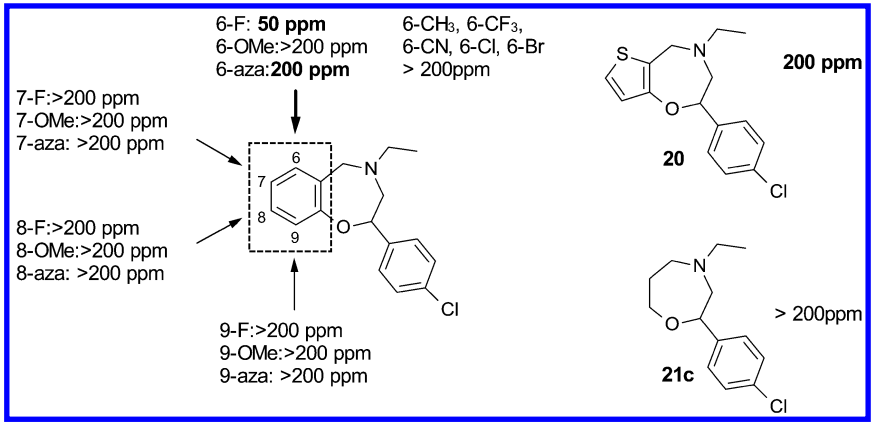

*Figure 12. Structure-Activity relationships on ring A Biological activity given on Myzus persicae feeding/contact assay, BP80 (ppm) (Reference compound **5** 12.5 ppm)*

Most of the 6-, 7-, 8-, and 9-substituted benzoxazepine derivatives were obtained using the general methods described in Figures 9 and 11. 6-Substituted salicaldehydes were obtained by *ortho*-lithiation of the 1,3-dimethylimidazoline protected derivative **22** as described in Figure 13 (*21*).

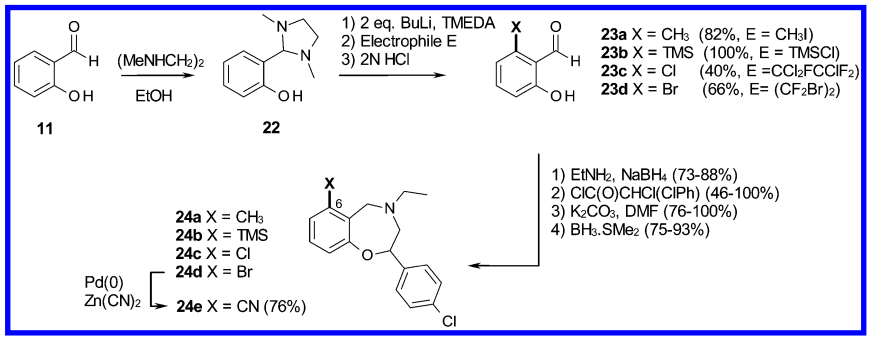

Figure 13. Synthesis of 6-substituted derivatives

Heteroaromatic benzoxazepines were obtained by adapting the general synthetic routes as described in Figure 14. While cyclisation to form the pyridine derivatives **28a-d** occurred smoothly under typical intramolecular SNAr conditions, the formation of the thiophene compound **20** required Cu-catalysed conditions to perform the cyclisation. Although none of these compounds displayed interesting insecticidal activity, the straightforward access to these unusual heterocycles may offer further opportunities in lead discovery.

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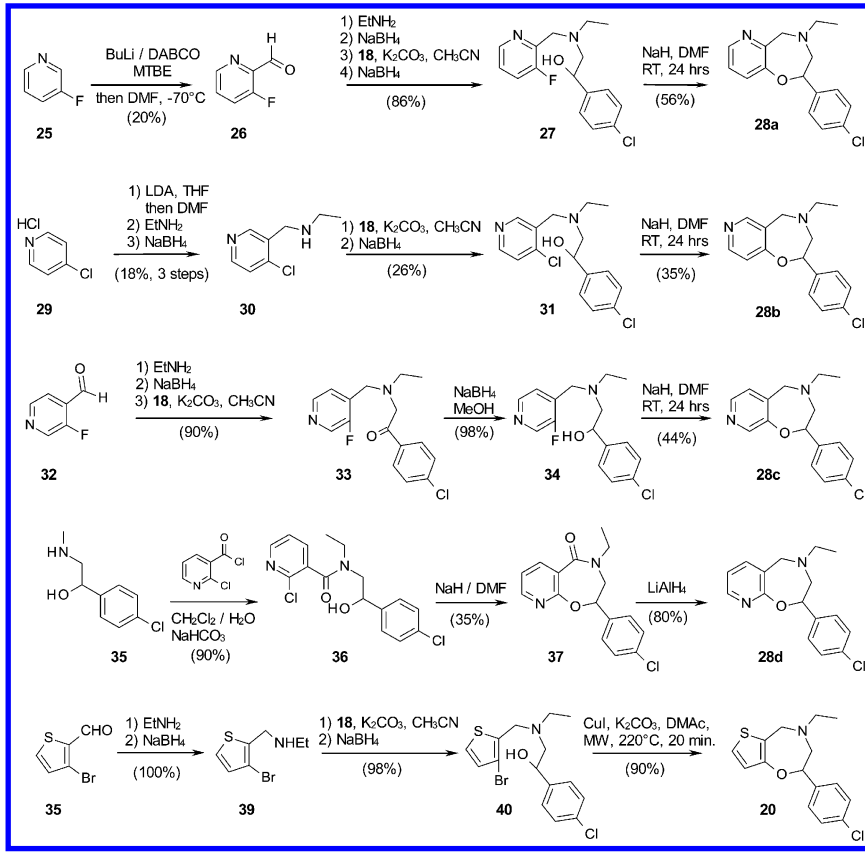

Figure 14. Synthesis of heteroaromatic benzoxazepines

The synthesis of compounds lacking the A-ring was designed using Ring-Closure Metathesis (RCM) as a key step, which offered a general and elegant entry to 6-, 7-, and 8-membered analogues from a single precursor **41a** as described in Figure 15. **41a** underwent RCM with Grubbs II catalyst to afford the oxazocinone compound **42a** (*22*)**.** On the other hand, isomerization of **41a** with Grubbs I catalyst or the ruthenium catalyst [$RuClH(CO)(PPh_3)_3$] (*23*) afforded intermediates **41b** and **41c** respectively. The latter compounds were then cyclized under RCM conditions to afford the oxazinone and oxazepinone (under high dilution conditions (*24*)) compounds **42b** and **42c**. Hydrogenation followed by reduction of **42a-c** afforded the corresponding compounds **21a-c.**

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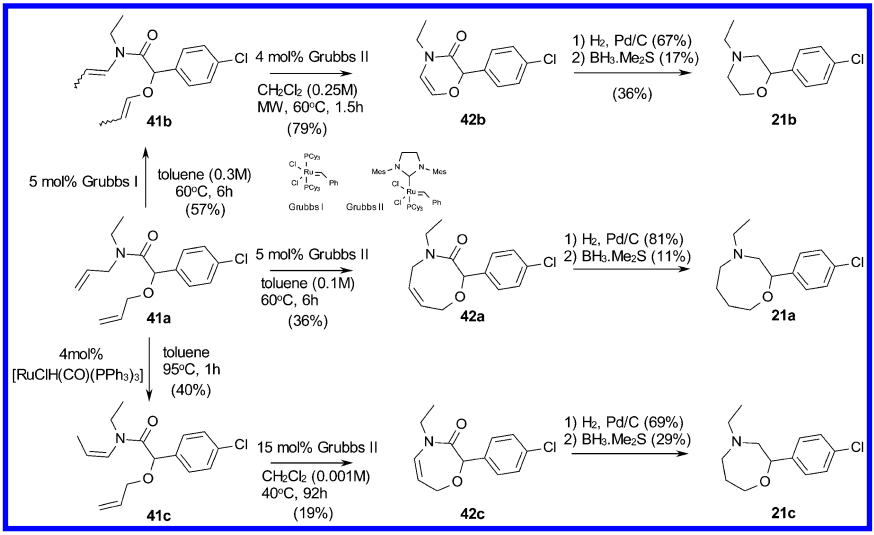

*Figure 15. RCM strategy for the synthesis of oxazocane, oxazepine and morpholine derivatives **21a-c***

Structure-Activity Relationships and Synthetic Variations on the Benzoxazepine Aromatic Ring C

A substituent scan was also extensively investigated on the aromatic ring C as outlined on Figure 16. Substituents at the *ortho* position were not tolerated, possibly as a result of the change of conformation induced. Both *meta* and *para* positions tolerated a range of substituents, including an additional aromatic ring, but most active compounds included halogen atoms at either or both *para* and *meta* positions. A range of heteroaromatic rings were introduced as replacement for the aromatic ring, but most of them were found to be biologically inactive, with the notable exception of the 2-thienyl ring **43a-b**, which stood out as a very interesting compound series with improved potency compared to the chloro-phenyl compound.

The synthesis of 2-(2-thienyl)-benzoxazepine derivatives is illustrated in Figure 17 with the straightforward preparation of the lead candidate **43a**, as well as the unusual bis-thienyl analogue **49**.

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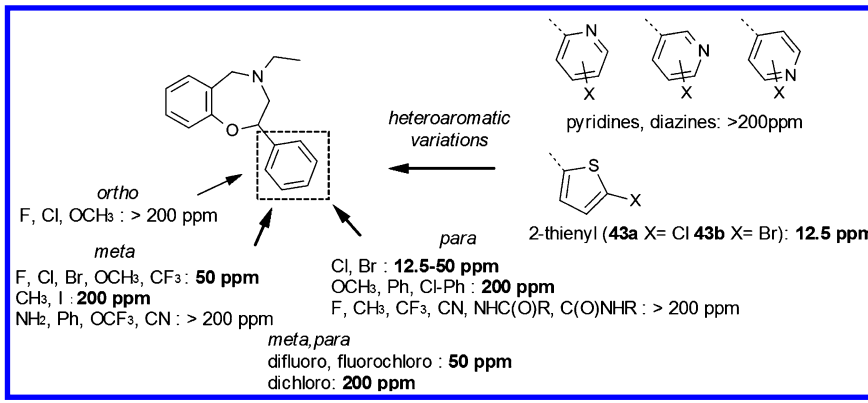

*Figure 16. Structure-Activity relationships on ring C Biological activity given on Myzus persicae feeding/contact assay, BP80 (ppm) (Reference compound **5** 12.5 ppm)*

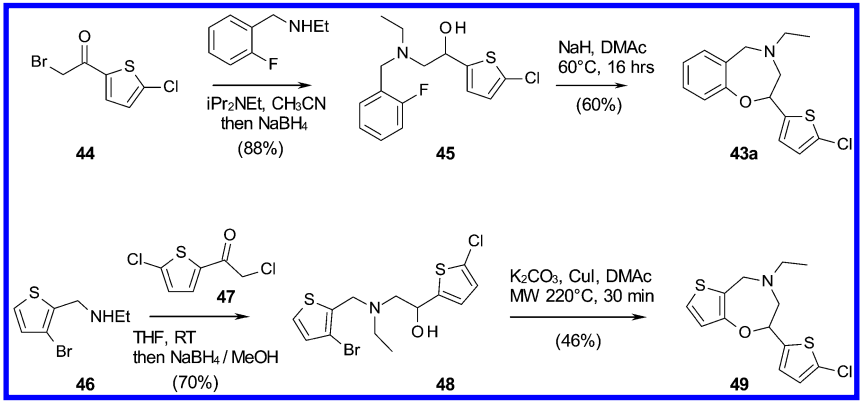

Figure 17. Synthesis of 2-(2-thienyl) benzoxazepine derivatives

Structure-Activity Relationships and Synthetic Variations on the Core Oxazepine Ring B

The nitrogen substitution on the core oxazepine ring was investigated extensively; a selection of representative substituents is outlined in Figure 18. A wide range of alkyl groups retained some biological activity but ethyl and cyclopropyl gave the best results. As mentioned previously, N-dealkylated analogues retained some biological activity, albeit lower than the N-alkylated compounds. The hypothesis of a cleavage of the alkyl side-chain *in vivo* can not be excluded, although no evidence of such a metabolic activation could be obtained by biokinetics studies. Most of the N-acyl, N-sulfonyl, and N-carbamoyl derivatives were found to be inactive, with the notable exception of cleavable carbamates such as the vinyl carbamate derivative, which displayed better activity than the N-ethyl lead compound. We postulated that such derivatives would

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improve bioavailability, especially distribution in plant tissues, and could deliver the insecticidal effect after conversion to the secondary benzoxazepine amine in insects.

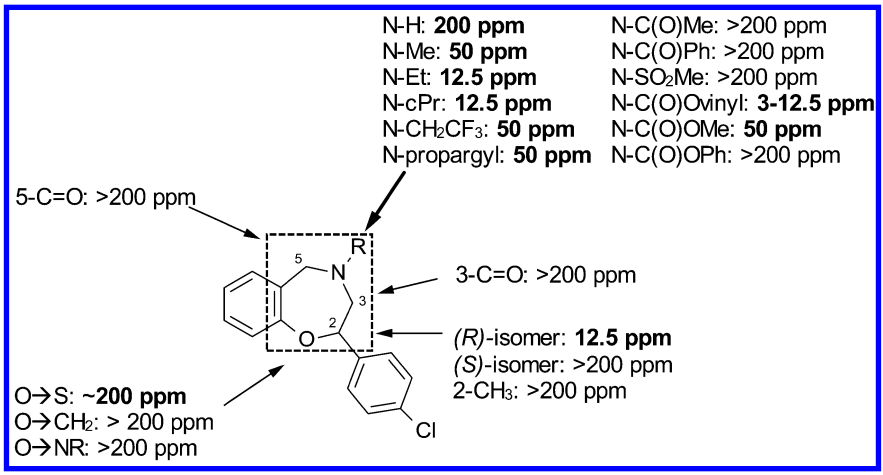

Figure 18. Structure-Activity relationships on the oxazepine ring Biological activity given on Myzus persicae feeding/contact assay, BP80 (ppm)

Other variations on the oxazepine ring were investigated, in particular the replacement of the ring oxygen by carbon and other heteroatoms. Only the benzothiazepine derivative retained some weak insecticidal activity, but significantly reduced compared to the parent benzoxazepine. The synthesis of benzothiazepine **53a**, benzodiazepine **53b** and benzazepine **53c** (*25*) are outlined in Figure 19.

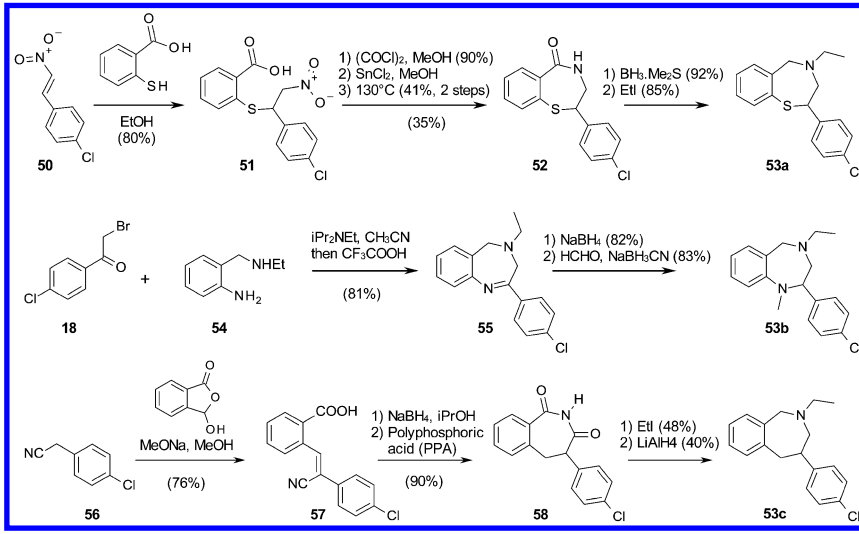

Figure 19. Synthetic variations at the benzoxazepine oxygen

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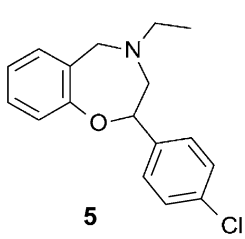
Physico-Chemical Properties and Relationships to Biological Activity and Spectrum

The combination of optimal 2-thienyl substituent at C-2 and vinyl carbamate substituent at benzoxazepine nitrogen led to compound **2**, which was selected as new lead candidate. Physico-chemical properties of compounds **5** and **2** were measured and compared as shown in Figure 20.



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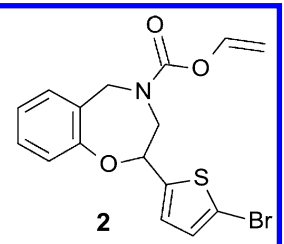

2

5	**Properties**	**2**
4.36	Log P	4.43
7.18	pKa	-
1.5 ppm	Aq. Sol. (@pH 9)	0.1 ppm
44 min	Photostability (T50)	> 24 hrs
66 min	Volatility (T50)	12 hrs

*Figure 20. Physico-Chemical properties of lead compounds **5** and **2***

Compound **5** displays medium lipophilicity, low water solubility, moderate photostability and volatility. More importantly, due to its basic pKa, we suspected that **5** might be trapped as protonated form in acidic plant vacuoles.

As demonstrated in other chemical classes (*26*, *27*), then rationalized and quantified by Buchholz and Trapp (*28*, *29*), vacuole trapping of basic compounds results from the lack of membrane permeation of protonated species and the differential pH between plant vacuoles and other compartments in plant cells (Figure 21). Since sucking insects exhibit different feeding behavior, vacuole trapping often results in spectrum limitation (for example, whereas aphids can feed to some extent in all plant compartments, whiteflies tend to feed selectively in phloem and xylem and may not be exposed to chemicals trapped in plant vacuoles, except through curative contact activity).

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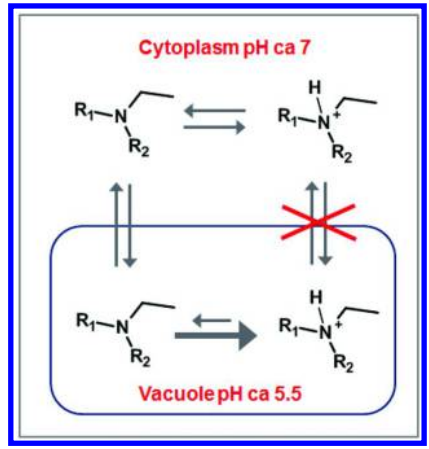

*Figure 21. Schematic representation of vacuole trapping mechanism of basic compound such as **5** in plant vacuoles (26–29)*

In contrast, lead compound **2** possesses more favorable physico-chemical properties, in particular higher photostability and lower volatility, but more importantly **2** is a non basic compound, possibly acting as a procide. We hypothesized that if bioactivation of the carbamate would only occur in insects and not simply in plants, vacuole trapping could be avoided and broader spectrum revealed. Indeed, we were delighted to observe that **2** not only showed higher potency than **5** against aphids but also displayed broader spectrum and in particular good activity against whiteflies (Table 2). Besides aphids and whiteflies, benzoxazepines display good activity against scales, but only marginal activity against thrips (data not shown) and mites.

Table 2. Biological spectrum of benzoxazepines 5 and 2 (BP_{80} in ppm)

	Aphis craccivora[a]	*Aphis gossypii*[a]	*Myzus persicae*[a]	*Bemisia tabaci*[b]	*Aonidiella aurantii*[c]	*Tetranychus urticae*[d]
5	**50 ppm**	**200 ppm**	**50 ppm**	~100 ppm	**3 ppm**	> 50 ppm
2	**50 ppm**	**50 ppm**	**25 ppm**	**3 ppm**	**3 ppm**	> 50 ppm

[a] Pea seedlings, contact/feeding activity, aphid mixed population, curative test. [b] Bean plants, contact/feeding activity, larvicide test, curative. [c] Potato tubers, contact/feeding activity, larvicide test, preventative. [d] Bean plants, contact/feeding activity, mixed population, curative.

This example highlights the importance of understanding the distribution of of chemicals in plant tissues combined with the knowledge of insect feeding behavior, and serves as a good illustration of the powerful Buchholz-Trapp model in modern agrochemical design.

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Field Biology

We selected compound **2** for field studies considering the good activity observed in the greenhouse and its favorable physico-chemical properties, in particular good photostability and low volatility. Selected field trials data are shown in Figures 22-24 against aphids and whiteflies. A good activity was observed against both aphids *Aphis gossypii* and *Myzus persicae*, but at higher rates than commercial standards such as thiamethoxam. Control of whiteflies was also validated in the field, but a relatively slow effect and only partial control was achieved.

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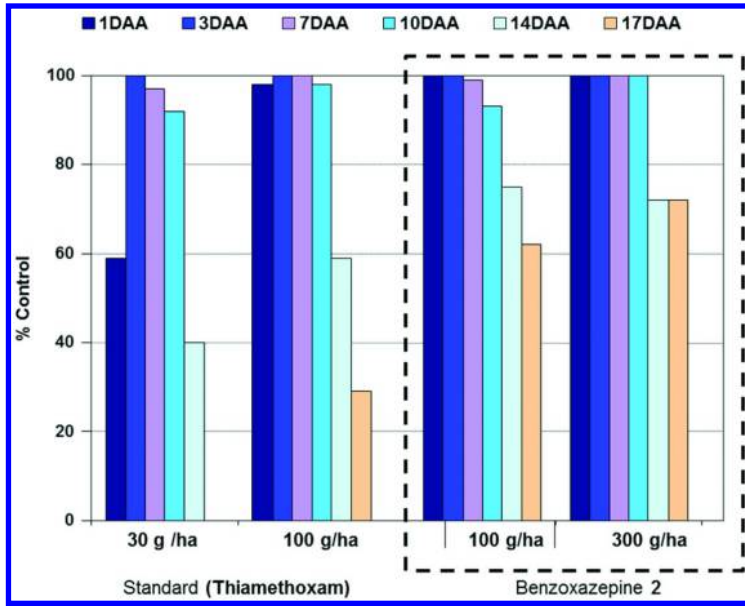

*Figure 22. Field studies of compound **2** (EC formulation) against Aphis gossypii on cotton (DAA: Days After Application)*

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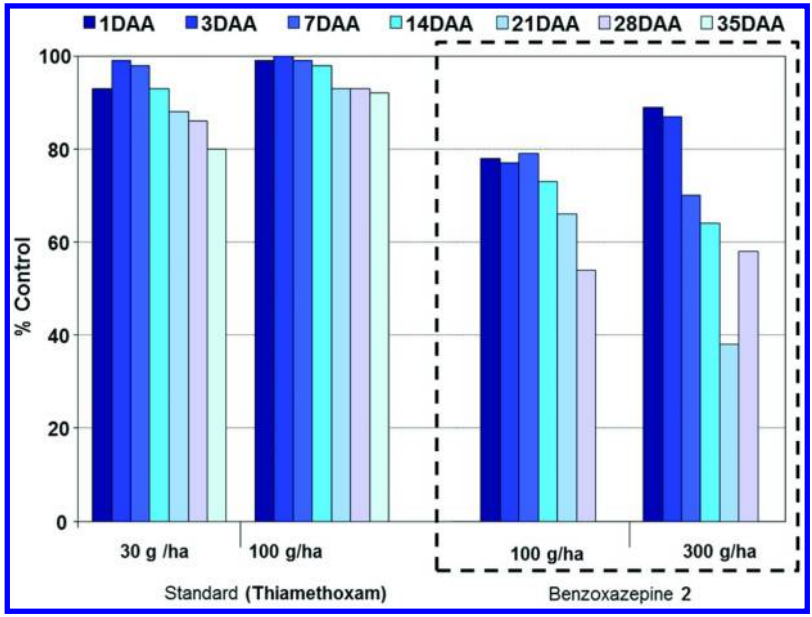

*Figure 23. Field studies of compound **2** (EC formulation) against Myzus persicae on cauliflower (DAA: Days After Application)*

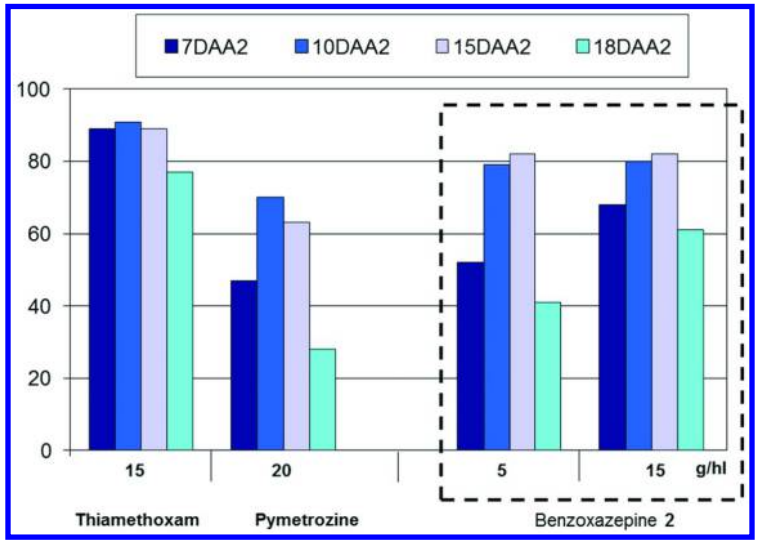

*Figure 24. Field studies of compound **2** (EC formulation) against Bemisia tabaci on eggplant (DAA2: Days After 2nd Application)*

Conclusion

A new insecticidal class was discovered based on a benzoxazepine core scaffold. Efficient synthetic routes were established and used to explore structure-activity relationships. Improvement of both activity and spectrum was achieved after systematic exploration of the different parts of the molecule. Highly active non-basic analogues such as **2**, likely to act as procides, were identified. Good control of important sucking pests such as aphids, whiteflies and scales could was validated both in the greenhouse and in the field. Specific aspects of the mode of insecticidal activity of benzoxazepines remain to be studied, in particular the identification of the molecular target in insects to determine the mode of action, but also the confirmation of the metabolic activation in insects to understand the exact nature of the active ingredient.

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Chapter 29

Synthesis and Insecticidal Activity of New Benzyl- and Indanyl-Oxazolines, Thiazolines and Alkoxy-Alkyl-Imidazolines

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Compounds **1**, **2** and **3** are examples of a new chemical class with potent activity against sucking insects, comparable to or greater than commercialised standards. The discovery, synthesis, biology and structure-activity relationships are described herein. Modelling of their distribution in plants encouraged us to invoke "vacuole trapping" to explain the weak field performance of this class of insecticides.

Introduction

This paper deals with the insecticidal activity of compounds of the structural types **1**, **2** and **3** (Figure 1). During a retrospective screening of compounds from the pharmaceutical departments of the Syngenta legacy companies, compounds **4** and **5** (Figure 2) were found to show an interesting activity against aphids (Figure 3). The oxazoline **4**, which had been prepared by chemists in Sandoz (*1*) is related to **6**, which was claimed by Bayer in 1971 to have insecticidal activity (*2*), and which was later shown by American Cyanamide scientists to be an octopamine agonist (*3*). Urea **5** had been prepared by chemists in Ciba-Geigy, or in one of the Ciba-Geigy legacy companies. It was thought that **5** would readily cyclise to the amino-oxazoline **7**, which turned out to be the case as **5** and **7** showed an almost identical spectrum and potency of insecticide action. A ready interconversion of **5** and **7** is inferred by their behavior in solution. The hydrochloride salt of **7** and the chloroethyl urea **5** give identical ^{1}H-NMR spectra in D_6-DMSO indicating a rapid interconversion. As **7** is similar to compounds **16** (see below) which are known to be insecticidal with an octopaminergic mode of action (*3*), we regarded the two compounds **6** and **7** as sub-types of the same insecticidal class.

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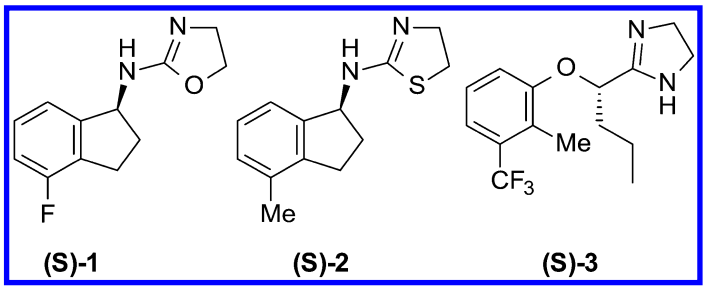

Figure 1. Examples of oxazoline, thiazoline and imidazoline chemical classes described in this chapter.

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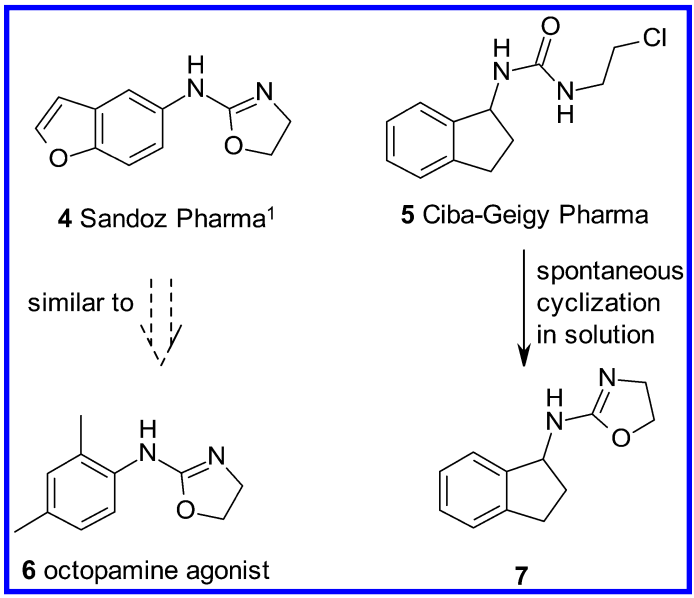
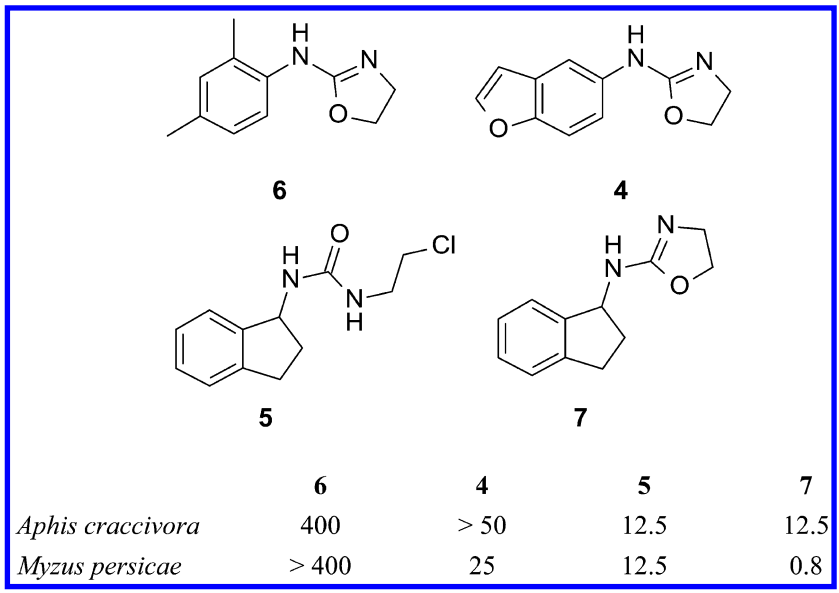


*Figure 2. Origin of the lead compounds (**4** and **5**) for this series*



	6	4	5	7
Aphis craccivora	400	> 50	12.5	12.5
Myzus persicae	> 400	25	12.5	0.8

Figure 3. Aphicidal activity of the lead compounds (BP80 in ppm)

The oxazolines **4** and **7** showed a promising aphicidal activity against both Aphis and Myzus species as indicated in Figure 3. The data show the BP80 (breakpoint concentration in ppm where where 80% mortality is observed) using a mixed population of insects on bean or pea plants. Although **4** is more potent than the Bayer/American Cyanamide compound **6**, the much more potent activity of the indanyl oxazoline **7**, led us to concentrate on this structural type.

The two sub-types differ from each other in having either one or two atoms linking the phenyl ring with the oxazoline ring. These compounds were overlayed successfully together with octopamine using the basic nitrogen and the aromatic ring as pharmacophoric units (Figure 4). These two moieties are typical for the pharmacophore of octopaminergic compounds (*4*) and indeed for compounds binding to biogenic amine receptors in general (*5*).

The oxazoline **6** with one atom bridging the phenyl and oxazoline rings, is a member of the formamidine class of insecticides (*6*). Chlordimeform **9**, introduced by Ciba in 1966 and Amitraz **10**, introduced by Boots in 1971 are both procides, which are cleaved *in vivo* to the active metabolite **11** shown in Figure 5. Chlordimeform **9** was since withdrawn from the market, but Amitraz **10** is still in use. The formamidine insecticides were shown in 1980 to be octopamine agonists (*7*, *8*).

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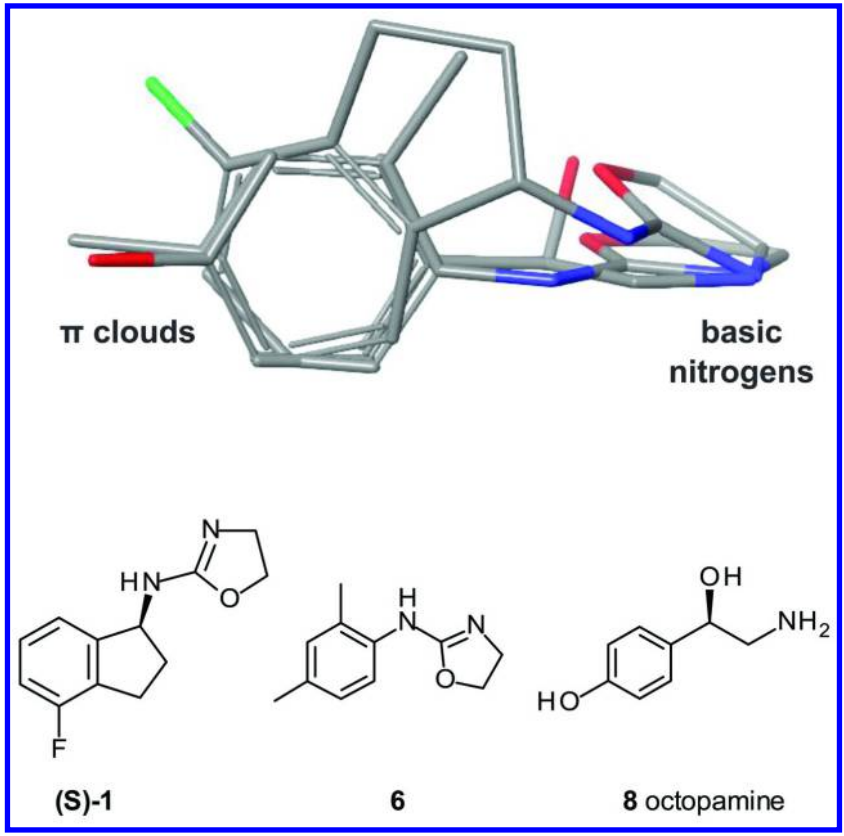

Figure 4. Common Pharmacophore (typical for biogenic amine receptors) (see color insert)

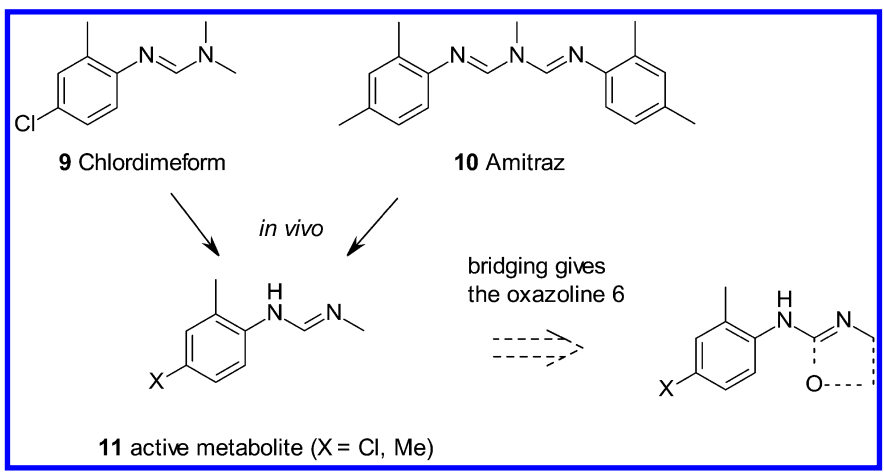

Figure 5. Formamidine insecticides (octopamine agonists) are procides.

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Compound **7** bearing a two atom bridge between the phenyl and oxazoline rings is precedented by a number of different sub-types shown in Figure 6. In each case we have attempted to identify the pioneering patent (**12** (*9*), **13-15** (*10*), **16** (*3*), **17** (*11*), **18** (*12*), **19** (*13*), **20** (*14*), **21** (*15*), **22** (*16*)). In some cases the compound types are protected by only a few patents, but there was considerable work invested in some structural types, such as **13** and **14**, which are claimed in dozens of patents. Demiditraz **23** was recently introduced to the market for the treatment of ectoparasites on dogs (*17*). The indanyl compound **7** is related to the benzyl analog **16** by formation of an additional ring. They share a similar spectrum of activity, but **7** is much more potent. A similar benzyl- to indane-type formal ring formation is precedented by the series **12**, which was formally formed by cyclisation of the benzyl type compound **13**. The indanyl amino oxazolines **7** were claimed independently by both BASF (*18*) and Bayer (*19*) within a year of the Syngenta (*20*) claims. Some of the work described here was published in lecture (*21*) or poster (*22*) form.

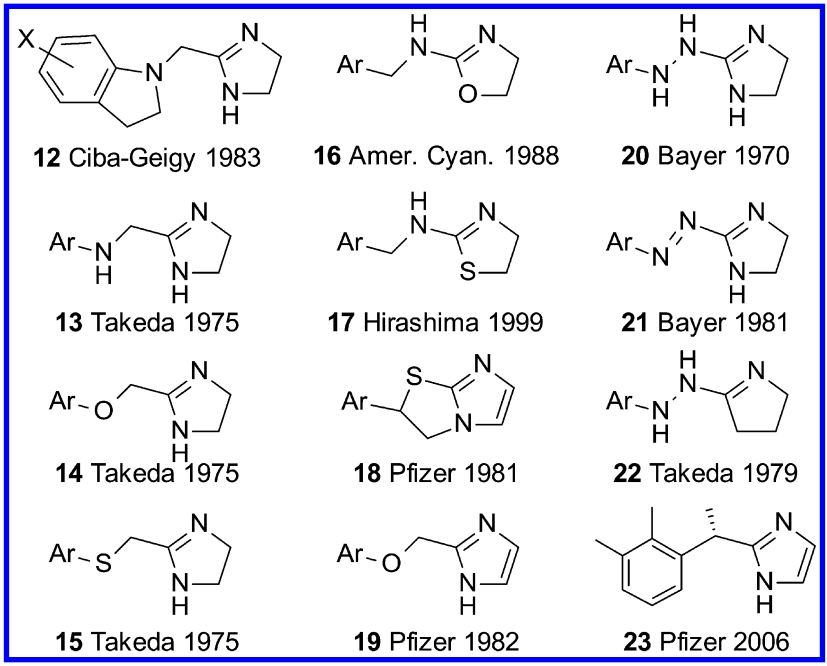

Figure 6. Prior art. Compounds of similar structure, which were known to have insecticidal activity.

Indanaminooxazolines

The oxazolines were mostly prepared by cyclisation of the chloroethyl ureas, which were easily prepared by addition of chloroethyl isocyanate to the indanamines (*23*). The base used for the cyclisation is of great importance. Three modes of cyclisation were observed (Figure 7). The stronger bases methoxide

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(*24*) and NaH led to cyclisation on N to the imidazolidinone **26** or aziridine **27** respectively, but cyclisation without base, or with a weak base to absorb HCl, led to the desired oxazolines (*25*).

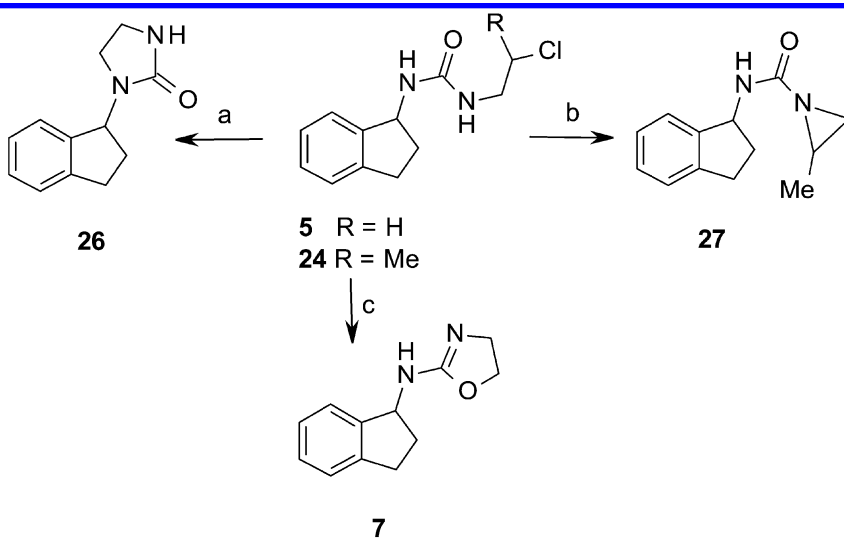

Figure 7. Synthesis of indanamino-oxazolines - three different modes of cyclisation

Formal addition of substituents at various positions on the molecule usually resulted in compounds with less potent insecticidal activity, but formal addition of certain small groups at C(4) resulted in compounds with greatly increased activity. The insecticidal activity of the 4-fluoro compound (rac-**1**) is compared with that of thiamethoxam **28**, a leading potent commercial insecticide (*26*, *27*) (Figure 8). The activity against aphids and whitefly is excellent, superior to thiamethoxam, and a highly potent activity was seen against the spider mite. Unfortunately the compound showed unacceptable acute toxicity to rodents, and was Ames positive. The mutagenicity of aminooxazolines was described by the Sandoz scientists responsible for our original lead (*28*). Convincing evidence was presented to show that the mutagenicity is caused by alkylation of DNA at the position shown by the arrow in Figure 8. In the same paper it was further shown that the corresponding thiazolines and imidazolines are not mutagenic. The SAR for mutagenicity described by the Sandoz authors, makes it clear that replacement of the indanyl group with other alkyl or aryl groups would most likely lead to mutagenic compounds. Compounds with methyl groups on either C-atom of the oxazoline ring show reduced mutagenicity, but showed no insecticidal activity in our screens. Consequently other sub-classes were regarded as more promising.

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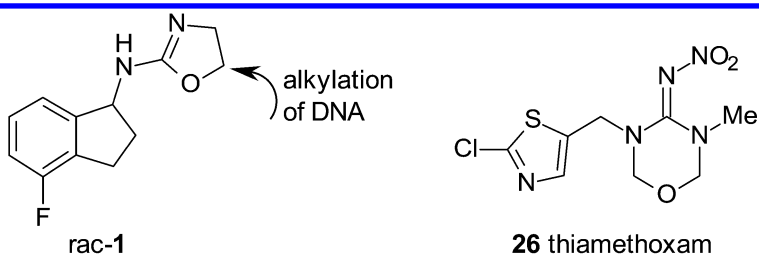

	rac-**1**	**28**
Aphis craccivora	1.6	6.3
Bemisia tabaci	3.1	25
Tetranychus urticae	3.1	/

*Figure 8. Insecticidal activity of the oxazoline rac-1 compared to that of thiamethoxam **28**. (BP80 in ppm on bean plants)*

As the thiazolines and imidazolines were reported to be non-mutagenic, we examined their insecticidal activity. Figure 9 shows activity against *Myzus persicae* (mixed population) on sunflower leaf discs. The imidazoline **30** was inactive in our insecticide screens, but was highly potent as an octopamine agonist (see below for assay). We suspect the higher basicity leads to a problematic biokinetic/biodynamic behavior. However the insecticidal activity of the thiazoline **29** is similar in spectrum, but somewhat weaker in potency than the corresponding oxazoline **7**. The SAR of this sub-class was then studied carefully (*29*). As was the case for the oxazolines, BASF (*18*) and Bayer (*19*) filed patent applications within a year of the Syngenta filing.

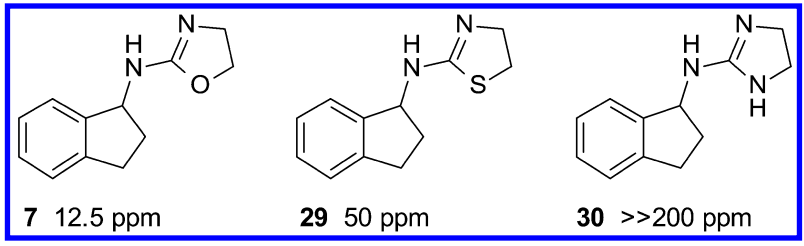

Figure 9. Aphicidal activity: oxazoline > thiazoline >> imidazoline (BP80 in ppm)

Indanaminothiazolines

The thiazolines were mostly prepared in one step from the indanamines **31** (Figure 10). Reaction with chloroethyl isothiocyanate **32** was a very mild procedure, but led to the thiazolyl-thiazoline by-product **33** (*30*). Reaction with methylthiothiazoline **34** led to the desired product, but required long reaction times at >100 °C (*31*). We found the methoxy compound **35** to be much more reactive, reacting slowly even at RT, but requiring expensive **32** (*32*) for its

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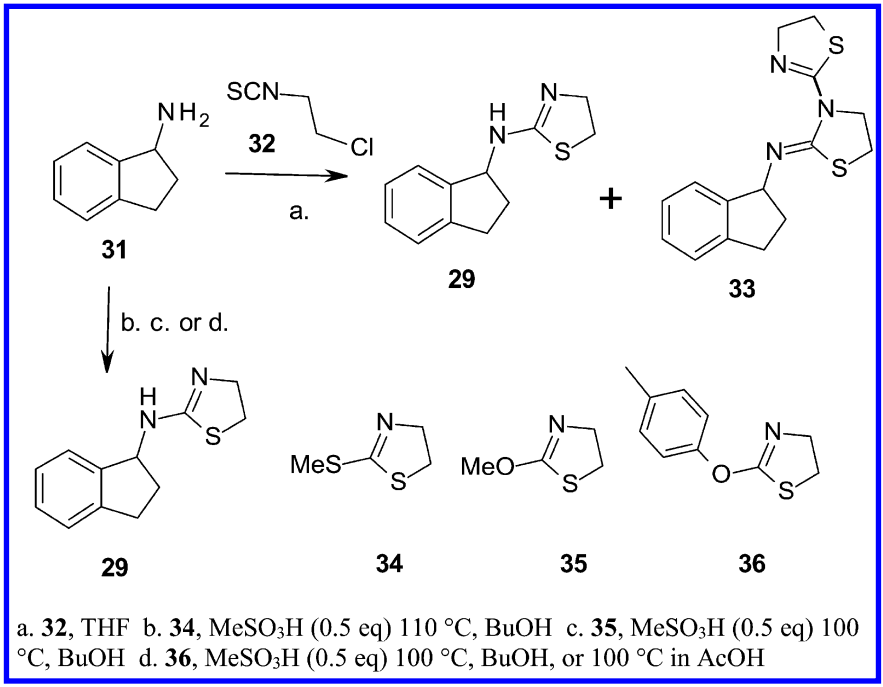
synthesis. We thus preferred to use the tolyloxy analog **36**, which was prepared from tolylchlorothionoformate. The coupling was greatly improved by acid catalysis, using either less than one equivalent of strong acid, or acetic acid as solvent.



a. **32**, THF b. **34**, $MeSO_3H$ (0.5 eq) 110 °C, BuOH c. **35**, $MeSO_3H$ (0.5 eq) 100 °C, BuOH d. **36**, $MeSO_3H$ (0.5 eq) 100 °C, BuOH, or 100 °C in AcOH

Figure 10. Novel and robust one step synthesis of alkylaminothiazolines.

A number of indanyl thiazoline compounds were prepared with substituents at various positions. While this work was underway, a publication appeared recommending placing a methyl group exhaustively at each position of a bioactive molecule as a basis for understanding the SAR (*33*). As most of the methylated compounds had already been made at that time, the rest were prepared and screened for their insecticidal activity. The activity of this series is shown in Figure 11 with the values showing the BP80 in ppm against *Myzus persicae* on leaf discs. In all the SAR can be regarded as steep. Methylation at most positions resulted in inactive compounds, which was also typical for substituents other than methyl. Usually the octopamine agonist activity mirrored the *in vivo* activity, but compounds with trans substituents at C(2) were often good octopamine agonists, even with quite large groups. The stereochemistry at C(1) was important. The S-isomer was active and the R-isomer inactive in our screens. Introduction of a methyl group at C(4) led to a compound **2** with very good insecticidal activity. Compounds with ethyl or any of the larger alkyl groups at C(4) showed very poor activity, but halides at C(4) were tolerated. In particular the fluoro compound **37** showed good insecticidal acitivity.

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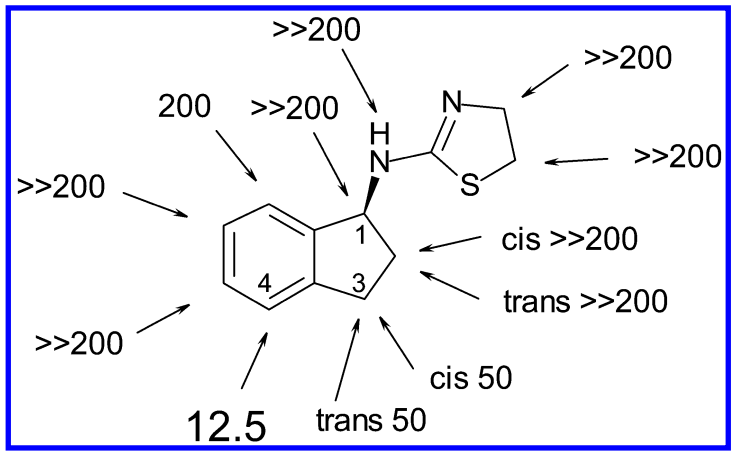
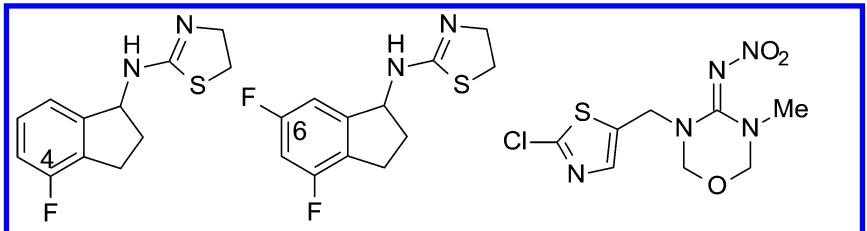


Figure 11. Methyl scan pinpoints postion 4 as having best aphicidal activity.

The insecticidal activity of two of the best thiazolines **37** and **38** were compared with that of thiamethoxam **28** on whole plants (Figure 12). The activity of **37** and **38** against aphids and whitefly was a little weaker than that of thiamethoxam, but they showed good spider mite activity. They showed good activity when applied to a hydroponic solution, indicating good systemicity through xylem mobility. However, when applied as a soil drench no activity was seen, reflecting the tight binding of basic compounds to soil. The thiazolines have an attractive fast speed of kill, and control neonicotinoid resistant aphids well. **37** showed an acute rodent LD50 of >200 mg/kg, and is Ames negative. However despite these favourable results **37** and **38** performed badly in field trials, which led us to disfavor this sub-class.



37 **38** **28** thiamethoxam

	Aphis craccivora	*Aphis gossypii*	*Myzus persicae*	*Bemisia tabaci*	*Aonidiella aurantii*	*Tetranychus urticae*
37	12.5	12.5	12.5	> 50	25	12.5
38	12.5	12.5	12.5	25	12.5	50
28	3	25	3	3	25	/

Figure 12. Insecticidal activity (BP 80 in ppm) of best thiazolines compared to that of thiamethoxam

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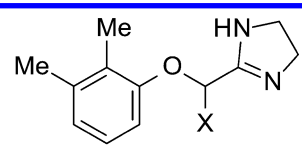
Aryloxyalkyl-imidazoles 14

On screening the various sub-types shown in Figure 6, the aryloxyalkyl-imidazoles **14** were found to show high potency. While compound **39** showed good aphid activity (Figure 13), its ethyl analog **40** showed even better aphicidal activity and in addition good spider mite activity (*34*). The values in the table are BP80 in ppm on leaf discs. **40** showed more potent activity against whitefly, mites and scales than the best thiazolines. Compounds where X comprises simple alkyl groups were available from the Ciba-Geigy work in the 1970s and 1980s. When X is a large alkyl group the compounds are poor insecticides, but they retained potent octopamine agonist activity. Although **40** shows unacceptable acute rodent toxicity, the compound is Ames negative as expected. With the possibility of an additional substituent X to modify, we hoped to break out of the steep SAR found with the oxazolines and thiazolines.



	X	Myzus persicae	Tetranychus urticae
39	H	12.5	>> 200
40	Et	3	12.5

Figure 13. Insecticidal activity (BP 80 in ppm) of lead imidazolines

Imidazolines were easily prepared from reaction of the corresponding nitriles with ethylene diamine (*34*) (Figure 14). A method for the late stage introduction of substituents via deprotonation of the methylene group of **41** was developed. Treatment with LDA or LiTMP gave no reaction, and reaction with nBuLi resulted in nucleophilic attack on the imidazoline ring. However, mesityl lithium deprotonated the compound in the desired way and a large number of substituents were introduced by these means using the appropriate electrophile, thus enabling a rapid scoping of these substituents, despite the moderate yields.

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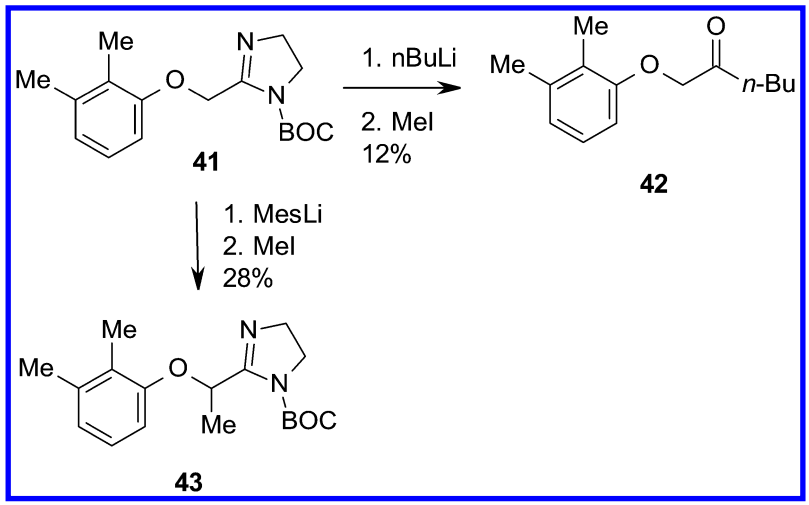

Figure 14. One step alkylation of aryloxymethylimidazolines

Most aspects of the SAR are shown in Figure 15. Listed are the substituents on the compounds we considered for field trials (*35–40*). On the aryl ring we found good activity when Z is CF_3, CHF_2, NO_2, Me, or Cl. In particular compounds with CF_3, CHF_2, and NO_2 substituents show good mite activity in addition to the aphid and whitefly acitivity seen with other compounds. Compounds wherein Y is Me, Cl, or F show good activity against aphids, whitefly, and mites. The compounds we prepared with substituents at other positions in the aryl ring did not show increased insecticidal activity. Compounds with alkyl groups at the imidazoline ring were inactive, but compounds with procidal groups on the nitrogen were of course active. Substituents on the bridging alkyl group did not show such a rapid fall off of insecticidal activity when the size of the group was increased. When X is CH_2OMe, allyl, nPr, cPr, Et, or iPr high activity was seen. *In vitro* results show larger groups to be accepted here. Compounds with stereochemistry shown were much more active than their stereoisomers.

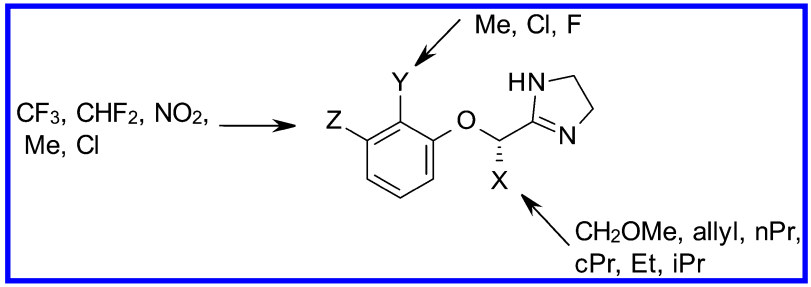

Figure 15. Highly active X,Y,Z-substituted aryloxymethylimidazolines

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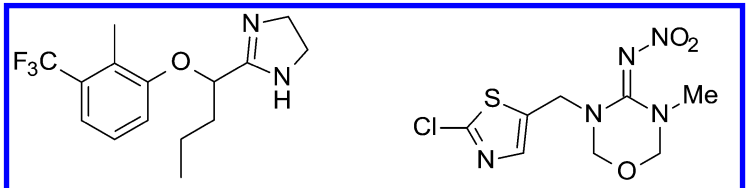
The insecticidal activity of **3** is compared with that of thiamethoxam **28** (Figure 16). It shows very potent aphid and whitefly activity, excellent activity against the crawling stages of scales, and excellent activity against spider mites. There is a fast speed of kill, and the compounds fully control neonicotinoid resistant *Myzus persicae* at the same rate used for susceptible aphids. The compounds are xylem mobile, showing good activity when applied to the root solution of hydroponically grown plants. Again there is no activity on soil drench, in keeping with the strong binding of basic compounds to soil. Several field candidates showed a rodent acute toxicity alert and were dropped from further testing. Compounds were preferentially applied in the field as salts, because the free bases racemised (*41*) and their imidazoline rings were slowly hydrolysed (*42*) in solution. Disappointingly, despite this excellent greenhouse activity, the performance of the HCl salt of **3** in most of the field trials against aphids and whiteflies was poor, while good tetranychus control was observed.



3 **28**

	Aphis craccivora	*Bemisia tabaci*	*Aonidiella aurantii*	*Tetranychus urticae*
3	3	0.8	< 0.8	3
28	3	3	25	/

*Figure 16. Insecticidal activity of the imidazoline **3** compared to that of thiamethoxam **28.***

Biochemistry

Three *in vitro* assays were developed. A functional cell-based assay using CHO cells expressing a *Heliothis virescens* octopamine receptor (*43*) with aequorin reporting was generated. With this assay both agonist and antagonist potency were determined. Two radioligand binding assays using whole *Myzus persicae* (ground-up and suspended in buffer) were also developed. This assay only measures affinity at the receptor. The tritium labelled ligands were prepared according to Figure 17.

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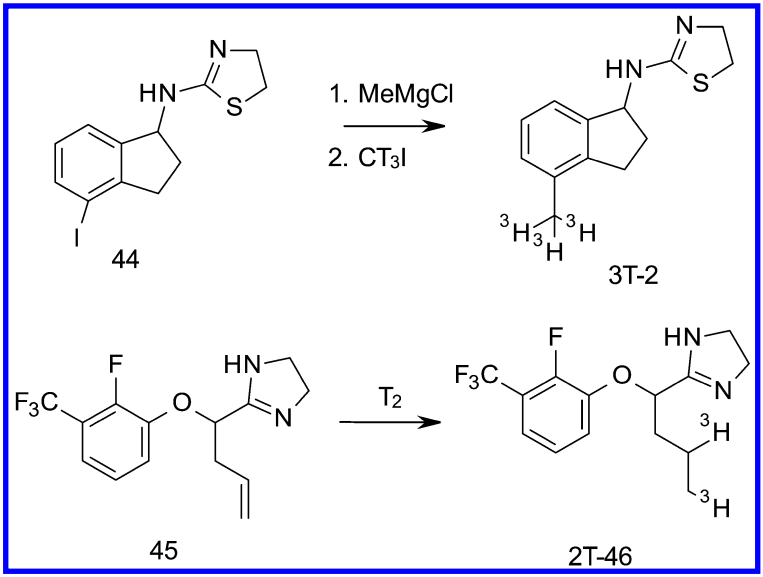

Figure 17. Synthesis of tritium labelled compounds

It seems that the azole insecticides described herein express their activity at more than two target sites in aphids. The three compounds shown in Figure 18 are all highly active against Myzus. The thiazoline (S)-**29** displaces 3T-**2** several hundred times more effectively than 2T-**46** from aphid membranes. The imidazoline **45** displaces 2T-**46** ca 40x more effectively than 3T-**2**, and the BASF compound **47** (*44*) displaces neither effectively, and presumably exerts its insecticidal activity by means of another receptor or binding site. The imidazolines and thiazolines do not bind mutually exclusively. Some imidazolines have a higher affinity for thiazoline 3T-**2** displacement and vice versa. There are five octopamine receptors in the pea aphid (*45*). It is possible that three or more of these, or in fact any of the 18 biogenic amine receptors annotated in the pea aphid, may play a role as effective insecticide targets.

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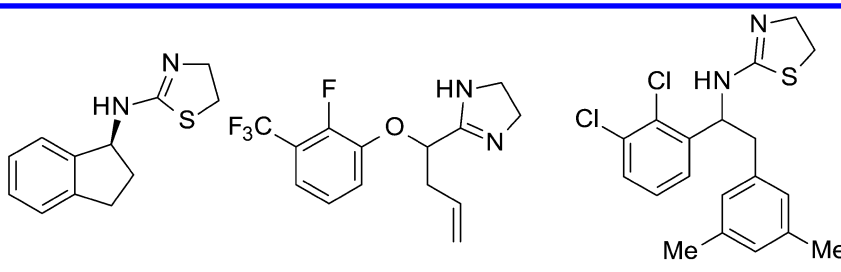

(S)-29 **45** **47**

	Myzus BP80 ppm	**3T-2** affinity IC50 (nM)	**2T-46** affinity IC50 (nM)
(S)-**29**	50	10	3982
45	12.5	856	20
47	12.5	1241	694

Figure 18. Binding affinities and aphicidal acitivity of three insecticides of this class

Vacuole Trapping (*46*)

All of the sub-classes of azole insecticides described herein showed excellent greenhouse activity but were disappointing in the field, so on bean plants their translaminar activity were compared with the contact and sachet results (Figure 19). In a translaminar screen the insects are held on the side of the leaf opposite to the one sprayed. Curative application is on the side of the leaf the arthropods are on. With the sachet set-up there is no plant material and the insects suck solutions containing insecticide through Parafilm™. Mites are unable to feed through Parafilm™ like this. The BP80 is given in ppm. **1** and **3** show greatly reduced translaminar activity against aphids and particularly against whitefly. In the field compounds with such solely contact activity would clear the sprayed plant from these insects, but not control any insects flying to the treated plant, nor any feeding on the undersides of leaves, which are not directly sprayed. **1** and **3** suffer no drop-off in spider mite activity, which correlates with their good field activity seen against this pest.

Although the lack of field activity can be explained by the weak translaminar activity, there was still a surprising discrepancy between the three arthropods. Of these, the mites have the shortest feeding stylets reaching in to the cells nearest the surface of the leaf, and yet they are controlled well translaminarally. Clearly the compounds diffuse well through the cuticle (*46*) and unobstructedly right through the leaf, but they appear to circumvent the vascular bundle, where the aphids and whitefly feed. An explanation for this behaviour was found by considering the basicity of these compounds. In acidic compartments they exist largely in their protonated form, which do not diffuse across membranes, resulting in an accumulation in acidic compartments (Figure 20), which in plants are the vacuoles. The accumulation of basic compounds in the vacuoles can be compared with the behaviour of weak acids in the basic phloem (phloem trapping), which is a similar

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phenomenon of exactly opposite polarity (*47*). A model was developed to quantify vacuole trapping, and more generally to predict the distribution of these and indeed any compound through all plant compartments (*48*).

		oxazoline rac-**1**	imidazoline **3**	thiamethoxam **28**
Aphis craccivora	*sachet*	0.06	0.06	0.2
mp	*curative*	1.6	3.13	6.2
	translaminar	12.5	200	6.2
Bemisia tabaci	*sachet*	0.8	0.2	0.2
adult	*curative*	3.1	12.5	25
	translaminar	1200	300	12.5
Tetranychus	*curative*	3.1	3.1	inactive
urticae mp	*translaminar*	3.9	3.8	inactive

Figure 19. Translaminar activity drops off with certain insects.

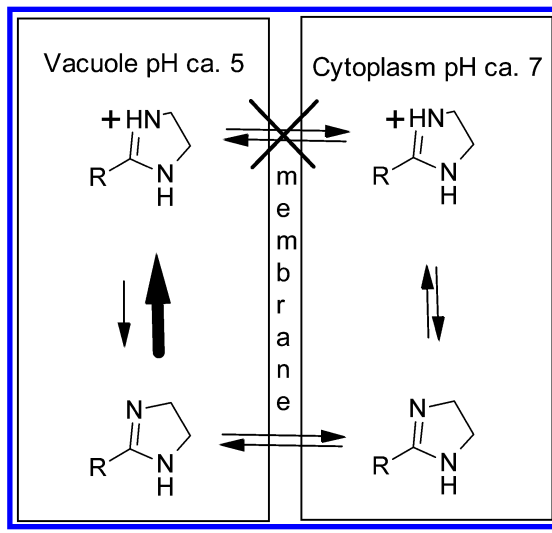

Figure 20. Vacuole Trapping. Basic compounds concentrate in the acidic vacuoles

The observed insecticidal activity can now be explained by the feeding behaviour of the insects (Figure 21). Spider mites insert their stylet into cells, and suck up the whole contents including the vacuoles. Aphids puncture cells with their stylets before spending most of their feeding time sucking the contents of the vascular bundle (phloem and xylem), and whiteflies avoid cells completely

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while inserting their stylets through the apoplast to reach their preferred feeding source in the vascular system. The mathematical model thus not only predicts the distribution of compounds in a plant, but, in consideration of feeding behaviour, allows the prediction of the systemic activity of any insecticidally active compound with measurable or calculatable physical properties.

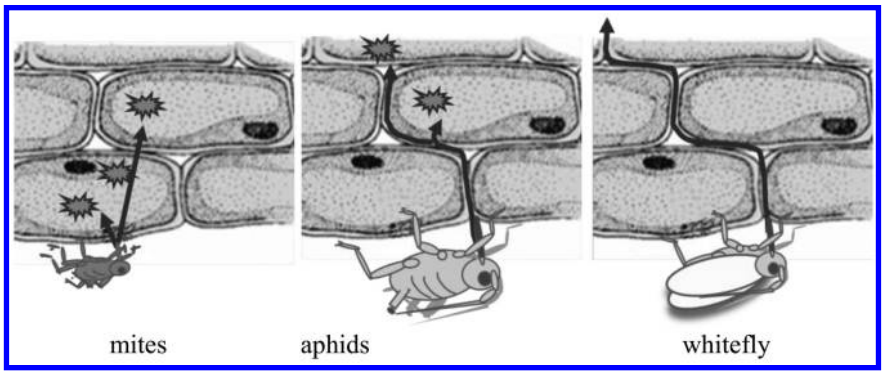

Figure 21. Feeding sites of arthropods explain the translaminar activity

Summary

Herein is described a novel insecticide class with excellent activity against sucking pests, and a mode of action not yet exploited for sucking pest control. The mutagenicity of oxazolines was understood and avoided by turning to thiazolines and imidazolines. Novel expedient syntheses were developed for all of these classes. The poor field performance was explained after experimental (translaminar bioassays) and theoretical (modelling) studies, by postulating the accumulation of basic compounds in vacuoles (vacuole trapping), which is a new agronomic concept. Modelling the distribution of compounds in plants, together with knowledge of the feeding behaviour of insects, allows the prediction of systemic activity of insectidally active compounds.

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Chapter 30

Multiple Sites of Insecticidal Action in Ionotropic GABA Receptors

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Pentameric ligand-gated ion channels (LGICs) of insects are among the targets of existing insecticides. Given the presence of multiple ligand-binding sites and the allostery in LGICs, more studies could provide further opportunities for insecticide discovery. Ionotropic γ-aminobutyric acid (GABA) receptors (GABARs) fall into the family of LGICs, and insect GABARs are insecticidal targets of fipronil, a conventional noncompetitive antagonist (cNCA), and of isoxazolines and benzamides, which are a novel type of NCAs and have a different site(s) of action from that of cNCAs. The mechanism of interaction of competitive antagonists with the orthosteric site merits further investigation for developing novel insecticidal compounds.

γ-Aminobutyric acid (GABA) is a principal inhibitory neurotransmitter in both vertebrates and invertebrates. GABA released by the presynaptic neuron binds to ionotropic GABA receptors (GABARs) on the postsynaptic membrane, causing chloride ion influx into the postsynaptic neuron or muscle cell. The resulting hyperpolarizing potentials counteract the depolarizing potentials to inhibit nerve or muscle excitability (*1*). GABARs fall into the family of

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pentameric ligand-gated ion channels (LGICs), the subunits of which include a large extracellular domain and four α-helical transmembrane domains (TMs). LGICs have multiple allosteric ligand-binding sites in addition to an orthosteric agonist-binding site (*2*).

Insect LGICs have multiple sites of action for insecticidal compounds, including sites for neonicotinoids, spinosad, and nereistoxin analogs in nicotinic acetylcholine receptors; those for macrolides in inhibitory glutamate receptors (iGluRs); and those for chlorinated hydrocarbons, phenylpyrazoles, isoxazolines, and benzamides (also known as meta-diamides) in GABARs (*3*–*5*). Given the existence of similar multiple sites in the LGIC family, LGICs remain potential targets for the further development of novel insecticides. In this chapter, we mainly review our work on competitive and noncompetitive antagonists of GABARs as insecticidal ligands.

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Conventional Noncompetitive Antagonists

Picrotoxinin, a sesquiterpene of plant origin, is among the best known noncompetitive antagonists (NCAs) or channel blockers of GABARs. This compound is classified as a conventional NCA (cNCA) in this review. This compound does not simply plug the pore as a channel blocker but stabilizes the closed conformation of the channels to antagonize the action of GABA (*6*, *7*); therefore, it may also be classified as a negative allosteric modulator in this respect. cNCAs include structurally diverse compounds, ranging from naturally occurring compounds to synthetic chemicals (*3*, *5*). Phenylpyrazoles such as fipronil, which are currently used as insecticides and ectoparasiticides, and 4′-ethynyl-4-*n*-propylbicycloorthobenzoate (EBOB), which is an important probe for studying GABARs (*8*), have the same mode of action as picrotoxinin. Lindane (γ-BHC), cyclodienes such as dieldrin, and *tert*-butylbicyclophosphorothionate (TBPS) also fall into the same category. The structural diversity (i.e., lack of specificity) of cNCAs implies that the binding mechanism conforms to the induced-fit model rather than the lock-and-key model.

To identify the EBOB-binding site, we expressed wild-type and mutant human β3 GABARs in *Drosophila* S2 cells (*9*). Ala274 (-1′), Ala277 (2′), and Thr281 (6′), which line the cytoplasmic side of the ion channel pore, on the second TM were mutated to Ser or Val (Figure 1a). Homomeric β3 GABARs were used because the cNCA pharmacology resembles that of insect GABARs (*10*). The β3 homo-oligomers are not endogenously expressed in the human brain. The mutation of Thr281 to Val resulted in a loss of [^{3}H]EBOB binding, whereas the mutations of Ala274 and Ala277 to Ser caused decreases in [^{3}H]EBOB binding compared with the wild type (Figure 1b). Semi-quantitative RT-PCR analysis revealed that the decreases in [^{3}H]EBOB binding in the A274S and A277S mutants are attributable to their low expression levels in S2 cells (*9*). However, Scatchard analysis indicated that the decreased binding in the A277S mutant is due to not only its low expression (shown by the decrease in the ordinate intercept) but also the decrease in the affinity for EBOB (shown by the decrease in the line slope) (Figure 1c). Ligand-docking simulation using a β3 GABAR homology model predicted

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that EBOB forms a hydrogen bond with Thr281 and has a hydrophobic interaction with Ala277 (Figure 1d,e). These findings indicate that the 2′ and 6′ amino acids on the cytoplasmic side within the channel are important for interacting with EBOB.

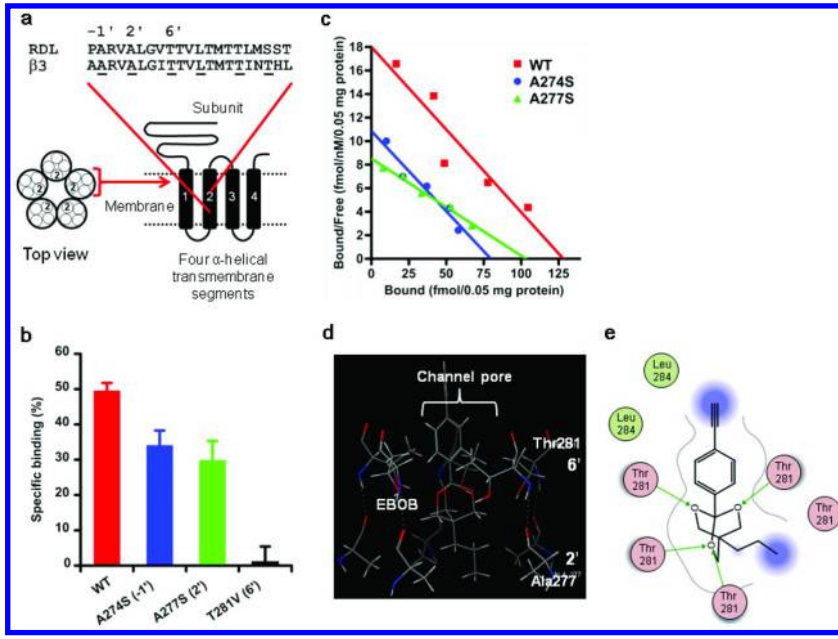

Figure 1. Binding site of EBOB in GABARs. (a) Sequences of the second TM and the location of binding site. The index number in the second TM increases toward the C-terminus, with the conserved Arg numbered 0. (b) [³H]EBOB binding to wild-type and mutant β3 GABARs expressed in Drosophila S2 cells. The concentration of [³H]EBOB was 1 nM. Data are means ± SD (n = 3). (c) Scatchard plots of EBOB binding data. (d) EBOB docking into a β3 GABAR homology model. The template used was the Torpedo marmorata nicotinic acetylcholine receptor (PDB ID 1OED). (e) Interaction map in the EBOB-binding site. (Reproduced with permission from reference (9). Copyright 2007 Springer.)

Numerous efforts have been implemented to identify the binding sites of individual cNCAs. Chemical and molecular biological approaches provided important information about the binding sites of fipronil and TBPS. Chemically reactive fipronil analogs labeled the 2′ and 17′ amino acid residues of rat α1β1γ2 GABARs (*11*). The 2′ and 3′ amino acid residues were identified as key residues interacting with TBPS by amino acid replacements of the second TM of α1β3 GABARs (*12*). Most of other studies consistently indicate that the binding site of cNCAs is located on the cytoplasmic side within the channel pore (*5*), although a TBPS site separate from the site for more elongated cNCAs such as EBOB has recently been proposed (*13*). With the understanding of the location of the cNCA site, we demonstrated that cNCAs with certain levels of the receptor selectivity of insect versus mammalian GABARs can be designed (*14*).

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Novel Type of NCAs

Two different chemotypes of novel GABAR antagonists, isoxazolines and benzamides (also known as meta-diamides), have recently been reported (*15*, *16*). These antagonists may be classified as a novel type of NCAs (nNCA) because the binding site(s) evidently differ(s) from that of cNCAs. The future of GABARs as an insecticidal target looked bleak because insect pests with resistance to cNCA insecticides have predominated in certain regions. However, the ability of nNCAs to circumvent this serious situation makes further development of novel NCA insecticides feasible.

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Isoxazolines

Insecticidal isoxazolines have recently been highlighted as nNCAs (*15*, *17–21*). Fluralaner (A1443; Figure 2) is an isoxazoline class ectoparasiticide used for flea and tick control in dogs. For the mode-of-action studies, we used native and cloned housefly (*Musca domestica*) GABARs. GABARs composed of RDL subunits are highly expressed in housefly heads (*22*). Fluralaner potently inhibited specific [^{3}H]EBOB binding to housefly head membranes; however, the inhibition was partial, unlike the full inhibition by fipronil (Figure 2) (*15*). The partial inhibition might be a sign of negative heterotropic cooperativity (*23*); the fluralaner-binding site allosterically interacts but is not directly associated with the EBOB site. Fluralaner most likely holds the GABAR channel in a closed conformation that hinders EBOB from approaching the cNCA site.

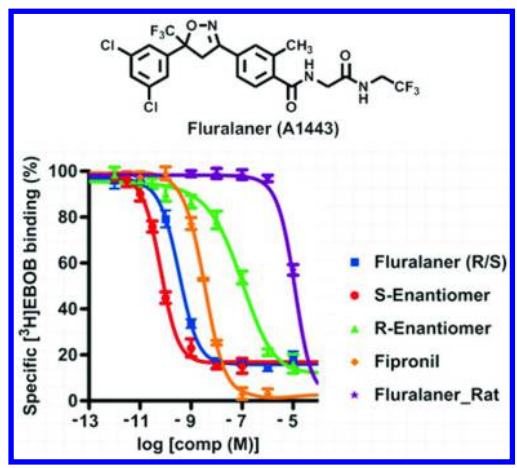

Figure 2. Inhibition of specific [^{3}H]EBOB binding to housefly head membranes and rat brain membranes by fluralaner, its enantiomers, and fipronil. The concentration of [^{3}H]EBOB was 0.5 nM. Data are means ± SE (n = 3). (Reproduced with permission from reference (15). Copyright 2010 Elsevier.)

Two-electrode voltage clamp (TEVC) analyses of fluralaner functions were also performed using housefly GABARs (RDL variant bd, RDL_{bd}) and iGluRs (GluCl variant A, $GluCl_A$) expressed in *Xenopus* oocytes. Fluralaner inhibited both GABA-induced currents in GABARs and L-glutamate-induced currents in iGluRs; however, the GABARs were more sensitive than the iGluRs (*15*). The *S*-stereochemistry at the chiral carbon on the isoxazoline ring was strictly required for high potency. More importantly, GABARs in the rat brain were much less sensitive to fluralaner compared to housefly GABARs (Figure 2).

The binding site of cNCAs is located within the channel as described above. In particular, the 2′ amino acid, Ala299 in the case of the housefly GABAR, on the second TM plays an important role in binding cNCAs. To examine whether fluralaner shares a common binding site with fipronil or binds to a distinct site, we mutated Ala299 of housefly GABARs (RDL variant ac, RDL_{ac}) and Thr348 on the third TM (Figure 3a), the equivalent amino acids of which were reported to affect fipronil binding in other insects (*24–28*). We expressed the wild type and several mutants in *Xenopus* oocytes and examined the effects of the mutations on fluralaner antagonism using a TEVC method. Antagonism of housefly GABARs by fluralaner was not affected by these mutations, whereas the ability of fipronil to antagonize GABARs was undermined by these mutations (Figure 3b-d, Table 1). Notably, a marked reduction in sensitivity to fipronil was observed in the A299N mutation, which is equivalent to the mutations identified in RDLs of highly fipronil-resistant whitebacked planthoppers and small brown planthoppers (*25*, *26*). By contrast, fluralaner remains effective against this mutant. Given that the A2′S and A2′G mutations were originally identified as the mutations associated with dieldrin resistance (*29*), it is plausible that antagonism by fipronil was less affected by these mutations than by the A2′N mutation. These findings indicate that fluralaner is an antagonist that binds to a site distinct from the site of cNCAs. The binding site of fluralaner has yet to be definitively determined.

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3-Benzamido-N-phenylbenzamides

The insecticide broflanilide is an example of 3-benzamido-*N*-phenylbenzamides (BPBs) (Figure 4a). Assays using a cell line transfected with an RDL cDNA became an opportunity to discover a novel "*meta*-diamide" chemistry, which differs from the "*ortho*-diamide" chemistry of ryanodine receptor activators (*30*). It was postulated that insecticidal BPBs enter at a transmembrane interface between adjacent subunits, which differs from the cNCA site, in the *Drosophila* GABAR to exert antagonistic effects (*16*). Bulky amino acid residues at a position in the third TM play a critical role in conferring BPB insensitivity to mammalian LGICs such as $GABA_A$ receptors (*31*).

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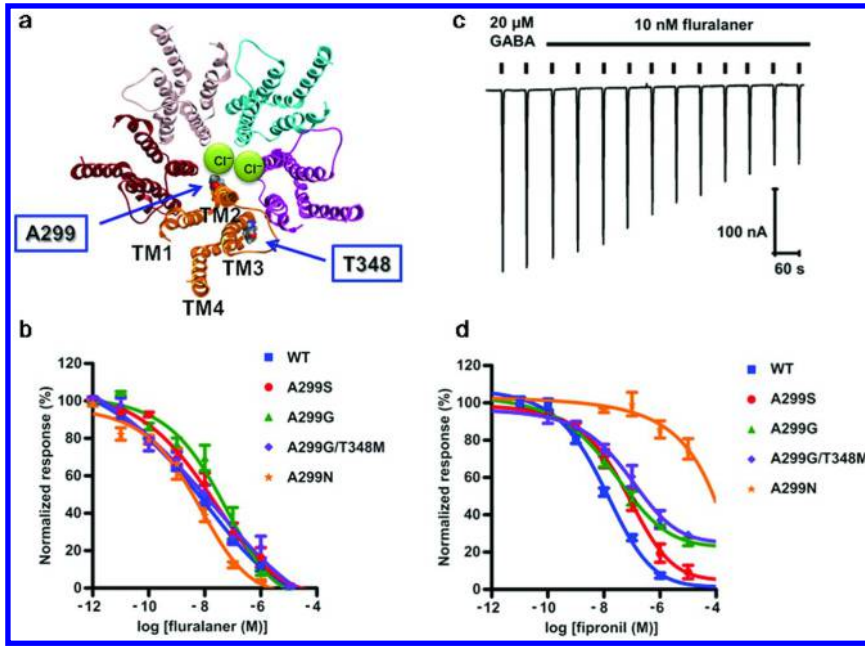

Figure 3. Effects of 2' amino acid mutations on fluralaner and fipronil antagonism of housefly GABARs. (a) Sites of mutation in the channel domain. Top view. The Caenorhabditis elegans iGluR (PDB ID 3RIF) was used as a template. (b) Concentration-response curves of the effects of fluralaner on GABA-induced response in wild-type and mutant GABARs. The EC_{50} of GABA for each receptor type was used to activate GABARs. (c) Trace of GABA-induced currents in the presence of 10 nM fluralaner in the A299N mutant. (d) Concentration-response curves of the effects of fipronil on GABA-induced currents in wild-type and mutant GABARs. The EC_{50} of GABA for each receptor type was used to activate GABARs. Data are means ± SE (n = 4-6).

We have shown that BPBs 1 and 2 potently inhibited specific [^{3}H]EBOB binding to housefly head membranes (Figure 4b) (*32*). The BPBs were partial inhibitors, as was fluralaner, whereas phenylpyrazoles were full inhibitors (Figure 4b). BPB 3, the *N*-methyl analog of BPB 1, was a very weak inhibitor, although it has high insecticidal activity (*32*), indicating that BPB 3 is a prodrug that becomes an antagonist upon *N*-demethylation. It is important to note that cNCAs such as fipronil, ethiprole, EBOB, and dieldrin enhanced specific [^{3}H]BPB 1 binding to housefly head membranes, whereas BPB 1 and GABA inhibited the binding (Figure 4c). The pyrethroid etofenprox, which has a different mode of action from that of GABAR NCAs, had no effect. The cNCA enhancement of [^{3}H]BPB 1 binding clearly indicates that cNCAs and BPBs do not compete for a binding site but that they allosterically interact with one another via distinct sites in the housefly GABARs (*23*). BPB 1 showed similar potencies in head membranes from wild-type and A299S mutant houseflies, supporting the view that the BPB site differs from the cNCA site (Figure 4d).

Table 1. IC_{50} values of fluralaner and fipronil in housefly wild-type and mutant GABARs

RDL_{ac}	*IC_{50} ± S.E. (nM)*	*n*
Fluralaner		
WT	5.76 ± 1.22	6
A299S	9.43 ± 1.22	4
A299G	26.4 ± 11.1	4
A299G/T348M	11.3 ± 4.4	4
A299N	2.73 ± 0.82	6
Fipronil		
WT	12.3 ± 1.2	6
A299S	85.0 ± 33.1[a]	4
A299G	196 ± 71[a]	4
A299G/T348M	634 ± 312[a]	4
A299N	>10000	8

[a] Significantly different from WT, $P < 0.05$ (unpaired *t*-test). The EC_{50} of GABA for each receptor type was used to activate GABARs.

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To delineate the mechanisms underlying the cNCA enhancement and the GABA inhibition of [^{3}H]BPB 1 binding, we examined the binding isotherms in the presence and absence of fipronil or GABA. The BPB 1 binding curve shifted upward in the presence of 100 nM fipronil (Figure 5a). The Scatchard transformation of the data indicates that fipronil increases the affinity ($1/K_d$) of the binding site for BPB 1 without a significant change in the maximal number (B_{max}) of binding sites (Figure 5b). This result implies the positive heterotropic cooperativity of cNCA binding relative to BPB 1 binding (*23*). In contrast to the upward shift induced by fipronil, the BPB 1 binding curve shifted downward in the presence of 30 μM GABA (Figure 5c), and the Scatchard plot indicates that GABA noncompetitively inhibits BPB 1 binding by reducing the B_{max} value without a significant change in the K_d of BPB 1 for the binding site (Figure 5d). This finding indicates that GABA, upon binding to the orthosteric site, might allosterically destabilize the channel conformation favorable for binding BPB 1.

We also tested whether ivermectin B_{1a} and milbemycin A_4 affect specific [^{3}H]BPB 1 binding to housefly head membranes. They were potent inhibitors of specific [^{3}H]BPB 1 binding (Figure 6a). Antiparasitic macrolides such as ivermectin B_{1a} were previously shown to induce chloride currents by acting at iGluRs (*33*, *34*). However, L-glutamate only slightly inhibited [^{3}H]BPB 1 binding even at 10 mM. To understand whether [^{3}H]BPB 1 binding occurs at iGluRs, GABARs, or both in housefly heads, we more directly examined the effects of BPB 1 on housefly RDL_{ac} GABARs and $GluCl_A$ iGluRs expressed in *Xenopus* oocyte using a TEVC technique. BPB 1 at nanomolar concentrations inhibited

GABA-induced currents in GABARs but not L-glutamate-induced currents in iGluRs (Figure 6b-d). Thus, BPB 1 is an antagonist of GABARs but not iGluRs. Our findings indicate that the tested macrolides inhibited [^{3}H]BPB 1 binding at GABARs. The topological relationship between the BPB site and the macrolide site in GABARs has yet to be clarified.

Competitive Antagonists

Bicuculline and gabazine (SR95531) are generally considered to be competitive antagonists (CAs), which bind to the orthosteric site in mammalian GABARs. However, these compounds are not simple CAs that preclude agonist binding to the orthosteric site. These compounds produce conformational changes in GABARs, thereby blocking the channels (*35*). Therefore, CAs could also serve as insecticides like NCAs.

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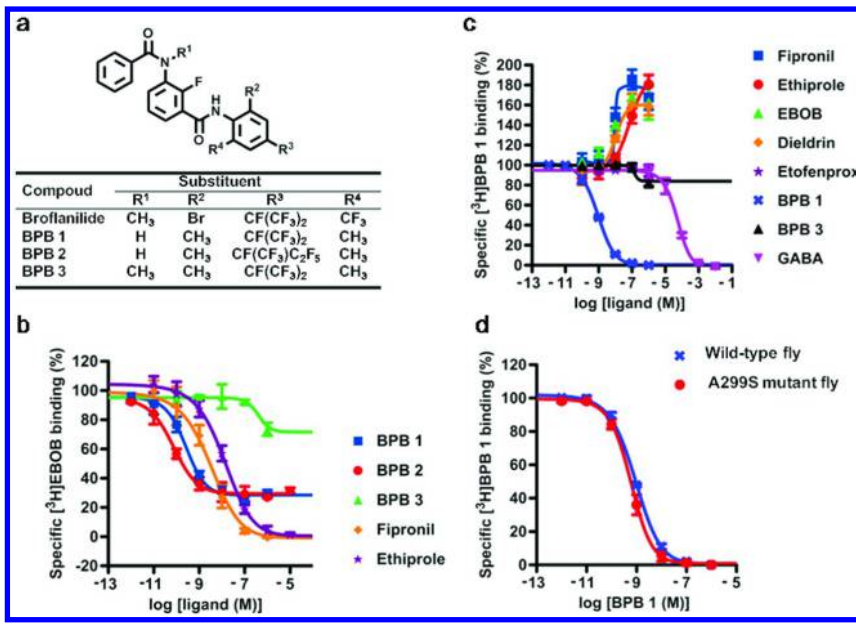

Figure 4. Effects of BPBs and related ligands on specific [^{3}H]EBOB and [^{3}H]BPB 1 binding to housefly head membranes. (a) Structures of BPBs. (b) Inhibition of [^{3}H]EBOB binding by BPBs and phenylpyrazoles. The concentration of [^{3}H]EBOB was 0.5 nM. (c) Effects of ligands on [^{3}H]BPB 1 binding. The concentration of [^{3}H]BPB 1 was 0.5 nM. (d) Inhibition of [^{3}H]BPB 1 binding by BPB 1 in head membranes from wild-type and A299S mutant houseflies. The concentration of [^{3}H]BPB 1 was 0.5 nM. Data are means ± SD (n = 3). (Reproduced with permission from reference (32). Copyright 2013 Elsevier.)

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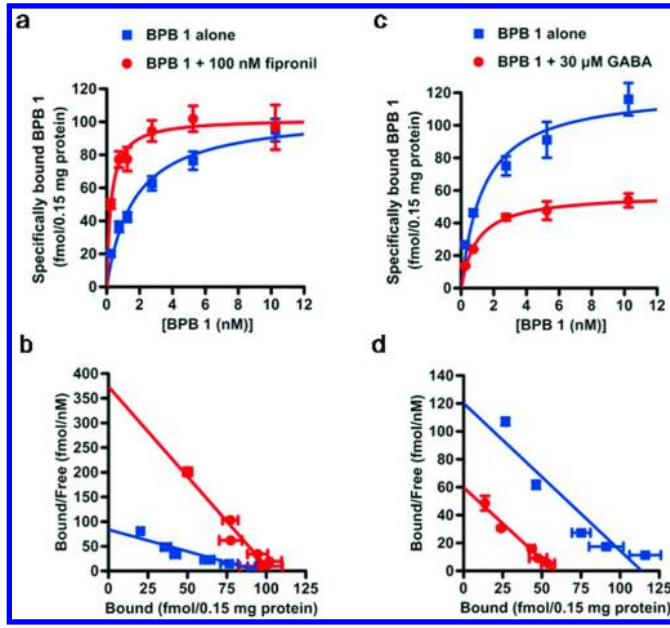

Figure 5. Effects of fipronil and GABA on specific [³H]BPB binding. (a) BPB 1 binding isotherm in the presence and absence of 100 nM fipronil. (b) Scatchard transformation of the data in a. (c) BPB 1 binding isotherm in the presence and absence of 30 µM GABA. (d) Scatchard transformation of the data in c. Data are means ± SD (n = 3). (Reproduced with permission from reference (32). Copyright 2013 Elsevier.)

Bicuculline has no effect on insect GABARs, whereas gabazine shows weak or moderate antagonism of insect GABARs (*5*). There is little information about the action of CAs on insect GABARs. No effective CA for insect GABARs is available. Therefore, we sought to examine whether modifying gabazine or known GABAR agonists could produce insecticides with selectivity.

1,6-Dihydro-6-iminopyridazines

The 1,6-dihydro-6-iminopyridazine gabazine showed only a marginal antagonistic activity in the case of housefly RDL_{ac} GABARs (*36*). We first aimed at enhancing the potency of 1,6-dihydro-6-iminopyridazines against insect GABARs by modifying the structure of gabazine (Figure 7a). The antagonism of cloned housefly, common cutworm, and small brown planthopper GABARs by synthesized compounds were investigated by a TEVC or fluorescence membrane potential assay (*36*).

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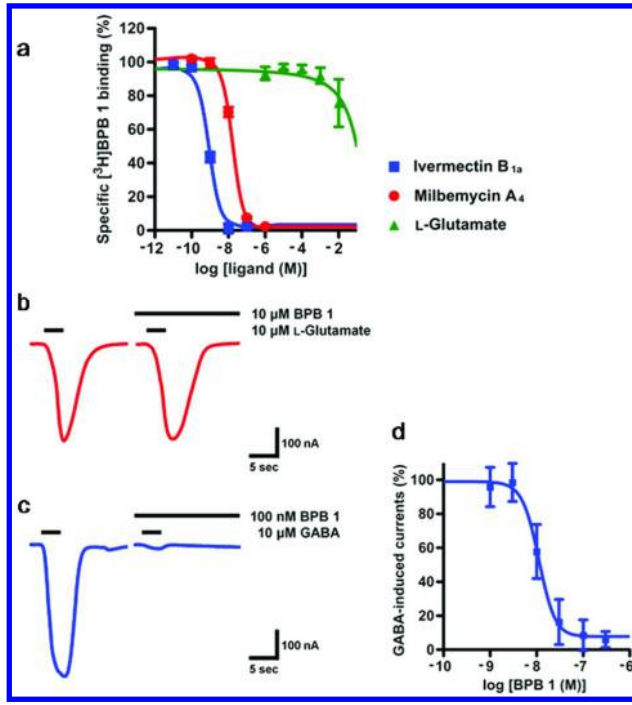

Figure 6. Effects of macrolides on GABARs and iGluRs. (a) Inhibition of specific [³H]PBP 1 binding to housefly head membranes. The concentration of [³H]BPB 1 was 0.5 nM. Data are means ± SD (n = 3). (b) Effects of BPB 1 on L-glutamate-induced currents in housefly iGluRs expressed in Xenopus oocytes. (c) BPB 1 inhibition of GABA-induced currents in housefly GABARs. (d) Concentration-dependent inhibition of GABA-induced currents by BPB 1 in housefly GABARs expressed in Xenopus oocytes. Data are means ± SD (n = 6). (Reproduced with permission from reference (32). Copyright 2013 Elsevier.)

1,6-Dihydro-6-iminopyridazine **7d**, with 1-(3-cyanopropyl) and 3-(4-biphenylyl) substitution, at 100 µM inhibited GABA-induced currents in housefly RDL_{ac} GABARs expressed in *Xenopus* oocytes (Figure 7b). Of the synthesized compounds, analogs with large aromatic substituents at the 3-position showed over 50% inhibition of GABA-induced currents at 100 µM. The IC_{50} values of **7d**, **7e**, and **7f** were approximately 38, 42, and 76 µM, respectively (Figure 7c). Substituting the cyano group of **7d** with an ethyl phosphono group, affording **8**, resulted in a 2-fold increase in potency.

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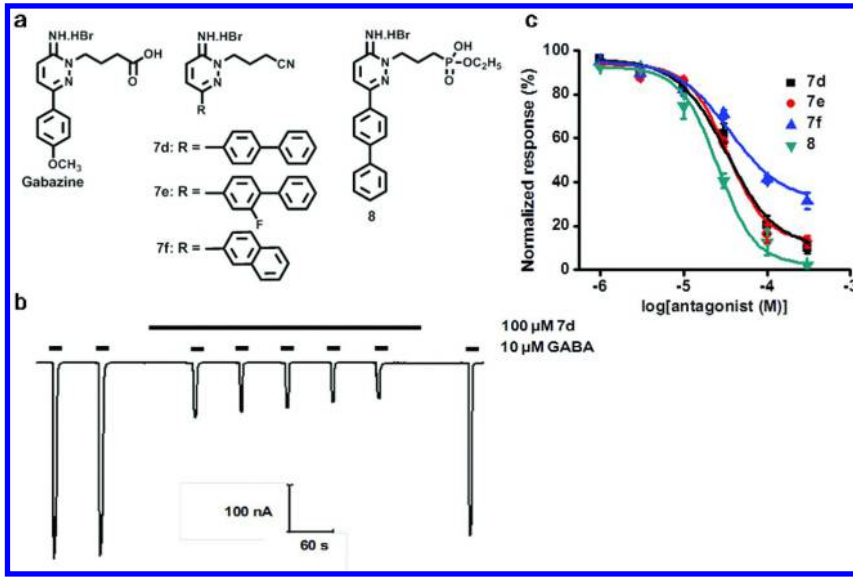

*Figure 7. Iminopyridazine inhibition of GABA-induced currents in housefly GABARs expressed in Xenopus oocytes. (a) Chemical structures of 1,6-dihydro-6-iminopyridazines. (b) Current trace when inhibited by 100 µM **7d**. (c) Concentration-response curves in the presence of **7d**, **7e**, **7f**, and **8**. The EC_{50} of GABA was used. Data are means ± SE (n = 4-6). (Reproduced with permission from reference (36). Copyright 2014 Pesticide Science Society of Japan.)*

Compounds **7d** and **8** caused a parallel rightward shift of the GABA dose-response curve, indicating competitive antagonism by these compounds (*36*). In ligand-docking simulation using a housefly GABAR homology model, GABA bound to the orthosteric site in the extracellular domain where the ammonio group of GABA was predicted to interact with Glu202 and Ser203 and the carboxylate group was predicted to interact with Arg109 and Ser174. When **7e** was docked into the orthosteric site, the cyano group interacted with Arg109 as a bioisostere of the GABA carboxylate and the biphenylyl group was well accommodated in the cavity of the orthosteric site (*36*). Information from this molecular interaction should help design CAs for potency enhancement.

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5-(4-Piperidyl)-3-isothiazolols

Muscimol [5-(aminomethyl)-3-isoxazolol] is a naturally occurring GABAR agonist (Figure 8a). This compound has served as a scaffold for the development of many GABAR agonists and antagonists for mammalian GABARs (*37*), but little is known about the activity of the derivatives against insect GABARs. Therefore, we synthesized 4-substituted 5-(4-piperidyl)-3-isothiazolols and examined their antagonism of housefly, common cutworm, and small brown planthopper GABARs (*38*).

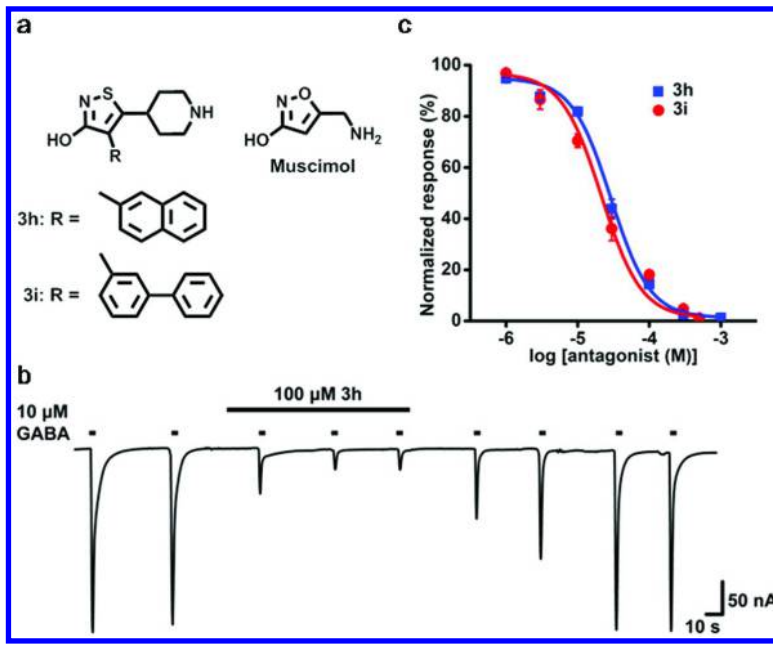

*Figure 8. Isothiazolol inhibition of GABA-induced currents in housefly GABARs. (a) Chemical structures of muscimol and isothiazolols. (b) Current trace when inhibited by 100 µM **3h**. (c) Concentration-response curves in the presence of **3h** and **3i**. The EC_{50} of GABA was used to activate GABARs. Data are means ± SE (n = 4-6). (Reproduced with permission from reference (38). Copyright 2014 Elsevier.)*

Applying **3h**, an isothiazolol with a 2-naphthyl group at the 4-position, at 100 µM reduced GABA-induced currents in housefly RDL_{ac} GABARs expressed in *Xenopus* oocytes (Figure 8b). Of the synthesized compounds, **3h** and a 3-biphenylyl analog (**3i**) showed the highest inhibition, with IC_{50} values of approximately 29 and 20 µM, respectively (Figure 8c), and insecticidal activity (*38*).

Similar to GABA, isothiazolols bound to the orthosteric site of a housefly GABAR homology model (Figure 9). The piperidyl nitrogen atom and the isothiazolol moiety were predicted to interact with Glu202 and Arg109,

respectively. The biphenylyl and the naphthyl rings were well accommodated in the cavity of the orthosteric site. Creating a strong interaction between CAs and amino acid residues in the cavity remains a challenge for enhancing potency.

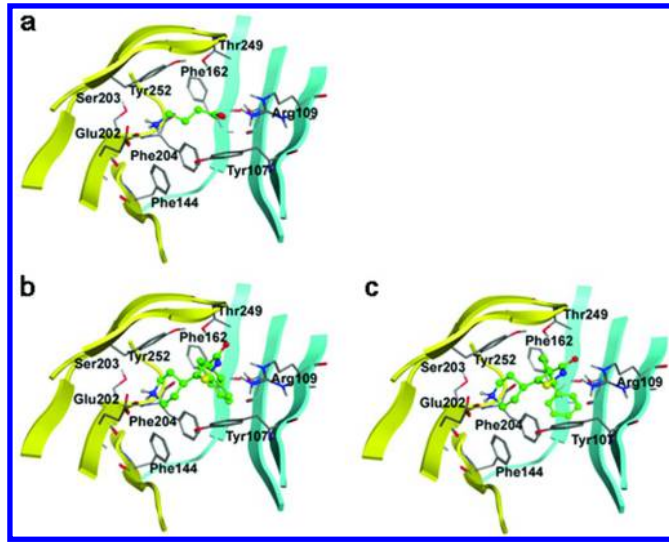

Figure 9. Docking of GABA and 3-isothiazolols into the orthosteric site of a housefly GABAR homology model. (a) GABA. (b) ***3h****. (c)* ***3i****. The adjacent subunits are represented using different colors. Ligands are shown in green. The Caenorhabditis elegans iGluR (PDB ID 3RIF) was used as a template. (Reproduced with permission from reference (38). Copyright 2014 Elsevier.)*

Conclusion

To conclude, insect GABARs are an established target of insecticides that has multiple sites of action for insecticidal compounds. Therefore, further efforts may provide opportunities to discover novel chemistry for crop protection.

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We thank Dr. Kunimitsu Nakahira and Ms. Miho Asahi (Nissan Chemical Industries) and Dr. Toshifumi Nakao and Dr. Kangetsu Hirase (Mitsui Chemicals Agro) for long-lasting research collaborations. This work was supported in part by JSPS KAKENHI Grant Number 26292031.

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Chapter 31

Insect Voltage-Gated Chloride Channels as a Possible Insecticide Target Site

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Voltage-gated chloride channels are involved in a number of physiological functions, including chloride ion secretion and resorption, cell volume regulation, maintanence of electrical excitability, and intravesicular acidification. With the various physiological functions these channels regulate, chloride channels are important potential target sites for insecticides with novel modes of action. This chapter details the recent developments in the search for novel insecticides targeting the chloride channel and examines the factors that influence strategies for insecticide discovery and development.

Insect Chloride Channels and Their Physiology

At present, three families of chloride channel proteins have been confirmed in insects, including chloride transporters (ClC type), anoctamin calcium-activated chloride channels, and ligand-gated chloride channels (*1*, *2*). Insect ligand-gated chloride channels (LGCC) are channels activated by several neurotransmitters including histamine, γ-aminobutyric acid (GABA), and glutamate (*1*). They have a pentameric formation of subunits that span the membrane with an intrinsic chloride

In Discovery and Synthesis of Crop Protection Products; Maienfisch, et al.;
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channel that conducts the flow of Cl^- ions to regulate membrane excitability (*3*). These channels have been the subject of numerous studies and reviews (*4–7*). Accordingly, we will not consider them further in this review, but will instead focus on other chloride channel subtypes as novel insecticide target sites.

The chloride channel (ClC) protein family can be organized as two functional groups; voltage-gated chloride channels (VGCC), which are found in plasma and intracellular organelle membranes (*1*), and $Cl^-:H^+$ exchangers (*8*). Both ClC groups mediate chloride flux and are involved in many cell functions including volume regulation, stabilizing membrane potentials of excitable tissues, and acidification of intracellular compartments (*1*, *8*). For example, the ClC-1 channel is the dominant chloride channel in skeletal muscle (*9*), and ClC-2 is thought to be involved in both electrical excitability and volume regulation (*10*). Both ClC-1 and ClC-2 are thought to be "double-barreled" (i.e. the channel is homodimeric with two pores in one channel complex). The structure of the chloride channel makes independent or simultaneous gating possible (*11*). The gating of some ClC channels is linked to the Cl^- concentration gradient, which can lead to changes in membrane voltage characteristics. For example, a 10-fold change in the concentration gradient of external Cl^- shifts the voltage needed to open the fast gating chloride channel of *Torpedo* electric organ by 60 mV (*11*). Therefore, by Cl^- binding to the chloride channel pore binding site, it acts as the gating charge for the channel (*11*).

The anoctamin family of transmembrane proteins, which encode for calcium-activated chloride channels (CACC), have more recently been found in nematodes and insects (*2*). Chloride movement through CACC is dictated by three factors: chloride concentration gradient, the cell membrane potential, and the calcium concentration (*12*). An increase in calcium is required for the activation of CACCs, which can come from either Ca^{2+} transmembrane flux or release from intracellular stores (*12*). In general, the resting membrane potential of most cells is more negative than the equilibrium potential of Cl^- (E_{Cl}), and when intracellular free Ca^{2+} increases, it causes Cl^- to exit and the cell membrane to depolarize. Moreover, this membrane depolarization can stimulate voltage-gated Ca^{2+} channels to open, additional Ca^{2+} influx, and further depolarization (*12*). Two CACC have been found in *Drosophila melanogaster* (ANO1 and ANO2) and in *Caenorhabditis elegans* (ANOH-1 and ANOH-2) that parallel mammalian CACC functions such as epithelial transport, membrane excitability, and signal transduction in insects (*2*). Further results have shown that these channels aid in detecting environmental osmotic changes in *C. elegans* (*2*). Both CACC and VGCC subtypes of chloride channels were previously identified in Sua1B cell cultures derived from embryonic *Anopheles gambiae* (*13*). Outwardly rectifying voltage-sensitive currents observed in native Sua1B cells seem to be mediated either primarily by CACCs or by a combination of CACCs and volume-sensitive outwardly rectifying anion channels (*13*). Further characterization of these chloride channels will be critical if they are to be exploited as insecticide target sites.

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Insecticide Discovery and Chloride Channels

Difficulties in insecticide discovery include the increased cost of chemical synthesis, and the high costs of developing the complex, novel compounds required for the market (*14*). Another difficulty is finding chemical compounds with high toxicity and high selectivity for target organisms (*15*). However, the need for novel chemical classes is imperative for the management of relevant agricultural, medical, and veterinary pest species.

As mentioned above, chloride channels are important target sites for established insecticides, with the potential to exploit novel modes of action. In the development process, identifying structural and functional differences in vertebrate and invertebrate ion channels will further aid the development of insecticides with acceptable selectivity. The remaining sections of this article detail recent developments in the search for novel insecticides targeting the chloride channel and examine the factors that influence their potential for insecticide discovery and development.

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DIDS and Related VGCC Blockers

An example of chemistry with insecticidal activity via chloride channels is the established VGCC blocker, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS). This compound has documented insecticidal and nematicidal properties, where it is an efficacious stomach poison in lepidopteran larvae and paralytic in nematodes (*16*, *17*). DIDS toxicity develops in nematodes over 7 d, and it was toxic at low ppm levels in both caterpillars and worms (*16*, *17*). Effects similar to DIDS were shown by the VGCC blocking compounds anthracene-9-carboxylic acid, NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid, and IAA-94 (indanyloxyacetic acid) (*16*, *17*) (Fig. 1).

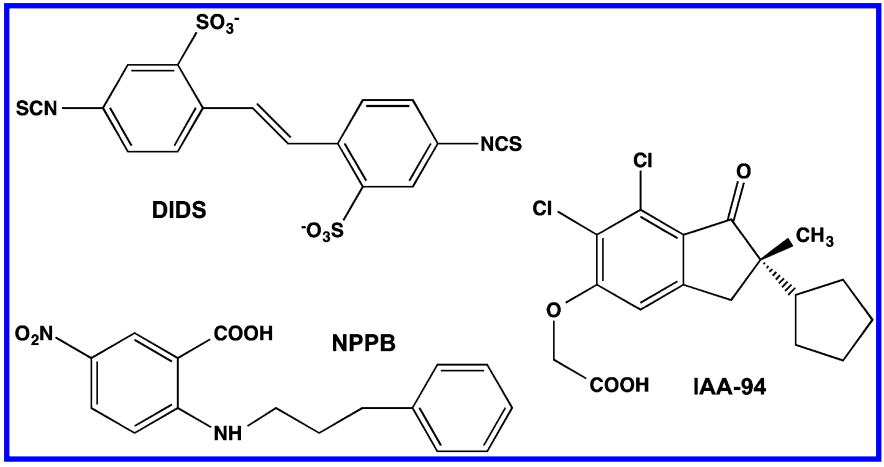

Figure 1. Chemical structures of established chloride channel blockers.

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Recent evidence suggests that DIDS may serve as an alternative acaricide for *tau*-fluvalinate- and coumaphos-resistant *Varroa destructor* (varroa mite) in honey bee hives (*18*). Vu et al. (*18*) report that DIDS has *ca*. 25% and *ca*. 22% higher efficacy than *tau*-fluvalinate and coumaphos, respectively, to acaricide-resistant varroa mites and, thereby, may reduce the infestation of this parasite in honey bee hives. Moreover, DIDS, NPPB, and IAA-94 showed no cross resistance in the *rdl* strain of *Drosophila melanogaster* expressing an altered GABA receptor (*3*). At the cellular level, the VGCC blockers had a dose-dependent effect in *Ostrinia nubilalis* (European corn borer) on both the larval midgut chloride ion transport (*ca.* IC_{50} = 10 μM) and the midgut alkalinity (*ca*. IC_{50}= 25 μM) (*17*). These effects explained their toxicity via dietary exposure. In terms of mammalian safety, oral treatment of mice at a dose of 2000 mg/kg resulted in no toxicity or poisoning signs.

Further, DIDS inhibited cell growth (*19*) and blocked outward VSCC currents expressed in Sua1B cells (*8*), which suggests these cells would be useful for chloride channel screening. Using the planar patch clamp technique, the most potent inhibitory effect of DIDS on insect cells clamped at +60 mV was IC_{50}= 5.1 μM and Hill coefficient = 0.5 (*19*). Dietrich and Lindau (*20*) reported a similar result for DIDS in rat peritoneal mast cells patch clamped at +70 mV (IC_{50} = 2.3 μM and a Hill coefficient of 0.7). The double-barreled structure of some chloride channels, together with Hill coefficients <1 found with the IC_{50} values of DIDS, suggests that more than one molecule of DIDS binds to these chloride channels in order to block ion flux.

Notably, some chloride currents do not show inhibition by DIDS. A recent study by Jenson (*19*) found that a small population of cells within the Sua1B cell line displayed chloride currents that were insensitive to DIDS. Similarly, where phenylalanine (residue 508) is deleted, DIDS insensitivity (0.1-0.5 mM) occurred in a mutated chloride channel protein of the cystic fibrosis transmembrane conductance regulator (ΔF508-CFTR) (*21*). The mutated, DIDS-insensitive chloride channels discovered by Pasyk and Foskett (*21*) displayed outwardly rectifying chloride channel (ORCC) currents that are activated by cyclic AMP and showed reduced current amplitudes when bathed in low chloride solutions (*21*).

Previously, Diykov *et al.* (*13*) described the presence of CACC in Sua1B cells, and reduced current amplitudes were present when calcium was replaced in the external media. Jenson (*19*) reported the majority of channels were VSCCs when characterizing the chloride channels present in Sua1B cells, whereas Diykov *et al.* (*13*) identified predominantly the CACC channel subtype. Both chloride channel subtypes were found in each study, but each subtype is activated under different physiological circumstances and occur independently of one another. The reason for the variation in chloride channel expression could be related to different aliquots/passages of Sua1B cells. Alternatively, Diykov et al. (*13*) conducted manual patch clamp experiments, while Jenson (*19*) focused on planar patch clamp of cell suspensions. Thus, there might be sampling bias between the two studies. However, both studies demonstrated a reduced current amplitude in the presence of DIDS (69%) and, thereby, demonstrate that DIDS-sensitive chloride channels existed in both groups of cells. The contribution of CACC blockage to the toxicity

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of DIDS and other compounds is unknown, and the issue of consistent expression would impact use of these cells for CACC screening purposes.

Pyrethroid Toxicity to VGCC

The neurotoxic effects of pyrethroids have been accredited primarily to their action on mammalian and insect voltage gated sodium channels. It has been reported that pyrethroids extend the period of time that the sodium channel is in the "open" state following an action potential, which ultimately produces signs of intoxication in vertebrates and invertebrates (*22*). The signs of intoxication of pyrethroids differ between the two categories (type 1 and 2) in mammals where the compounds described in the type 1 category (e.g., allethrin) displays a tremor syndrome involving hyperexcitation, ataxia, and convulsions followed by flaccid paralysis. In contrast, the compounds described in the type 2 category (e.g., fenvalerate) present choreoathetosis (sinuous writhing) and extreme salivation, followed by paralysis (*22*)(Fig 2).

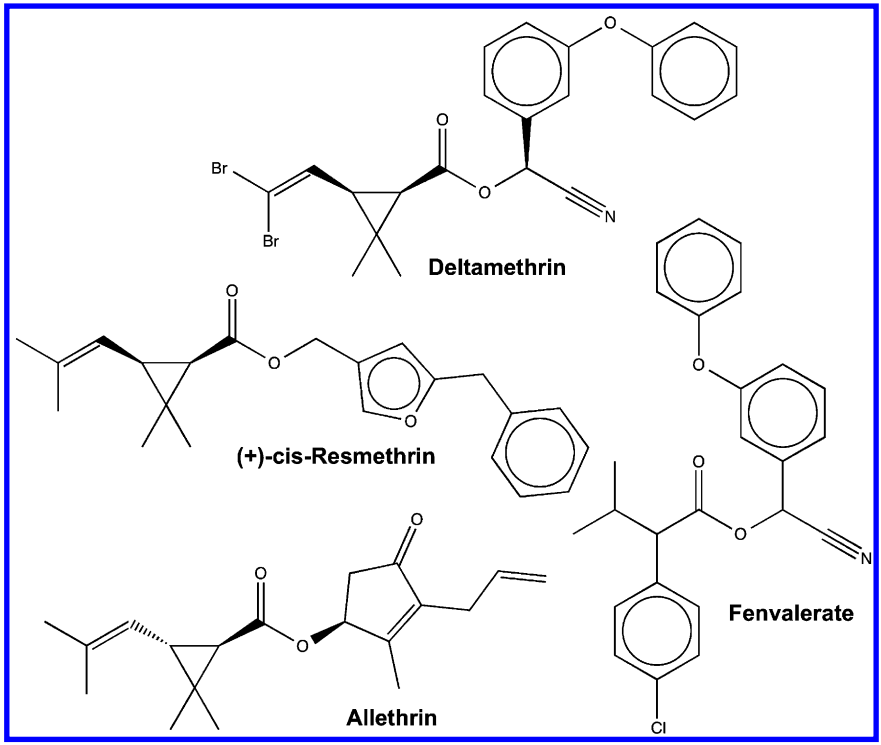

Figure 2. Chemical structures of standard use pyrethroids from both type 1 and type 2 categories.

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More recently, it has been proposed that the toxicity of pyrethroids may be the result of interactions with the γ-aminobutyric acid (GABA) receptor-ionophore complex (*23*), or on VGCC (*24*, *25*). Forshaw et al. (*24*) reported that a type 2 pyrethroid (deltamethrin) reduced chloride conductance concomitantly with an increase in membrane resistance in mammalian skeletal muscle and non-myelinated nerve fibers. Notably, these same results were not found with cismethrin, a type 1 pyrethroid (*24*). Additional studies confirmed the effects of deltamethrin on chloride channels in mouse neuroblastoma cells (*25*). Jenson (*19*) reported an inhibition of chloride current amplitude after the application of 10 μM fenvalerate (type 2 pyrethroid), while no significant inhibition on the chloride current was observed in the presence of 10 μM allethrin (type 1 pyrethroid). This study documents the first observation of type 2 pyrethroid effects on chloride channels in invertebrates. The extent to which chloride channel blockage might contribute to the toxicity of type 2 pyrethroids in insects, typically toxic via sodium channel modulation, is yet to be determined.

Conclusions

Studies over the last decade demonstrated the toxic action of compounds affecting additional classes of VGCC, but the activity is not yet at commercially viable levels for industrial development (*13*, *16–19*). Accordingly, additional development efforts such as structural optimization in novel chloride channel blockers is needed to increase target site binding affinity. The use of insect cell lines for screening native ion channels in high-throughput formats is advantageous, but the issue of consistent expression of CACC or other possible insecticide targets may limit their usefulness. Nonetheless, information provided in this review serves as a foundation to improve the discovery and development pipeline for alternative active ingredients against pests via invertebrate VGCCs.

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