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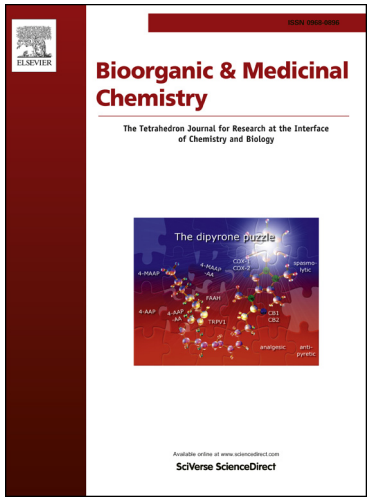

Discovery of oxathiapiprolin, a new oomycete fungicide that targets an oxysterol binding protein

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Discovery of oxathiapiprolin, a new oomycete fungicide that targets an oxysterol binding protein

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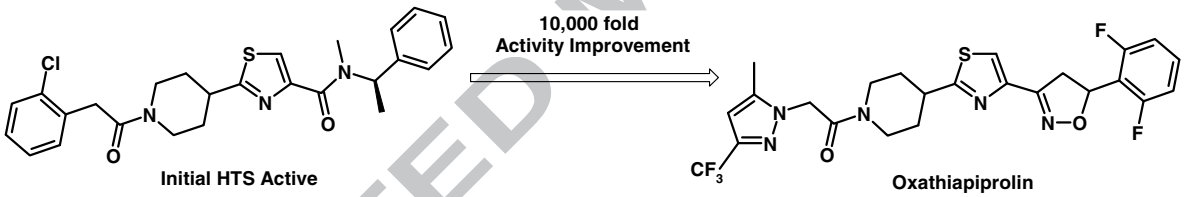
Abstract

Oxathiapiprolin is the first member of a new class of piperidinyl thiazole isoxazoline fungicides with exceptional activity against plant diseases caused by oomycete pathogens. It acts via inhibition of 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, synthesis, optimization and biological efficacy are presented.



1. Introduction

Enhancing world food production represents a significant challenge as the global population increases by 2.4 billion people over the next 35 years.[1] A key step in meeting this challenge is the development of better tools to prevent the estimated 20% – 40% global crop losses caused by competition with weeds, insects and plant diseases, despite the use of modern crop protection products.[2] Plant diseases caused by oomycete pathogens are particularly devastating for potato, grape and vegetable crops, with the global crop losses due to *Phytophthora infestans* on potatoes (potato late blight; PLB) alone estimated at $6.7 billion annually.[3] The ability of plant pathogens to develop resistance to current control technologies necessitates the search for novel chemical classes that can control diseases by acting against new biochemical targets. Accordingly, one goal of our laboratory is to seek out new chemical classes whose biological properties have not yet been well explored. In this paper, we will discuss the discovery, synthesis, mode of action and biological efficacy of a new class of oomycete fungicides based on a novel piperidinyl thiazole isoxazoline core and its optimization to oxathiapiprolin, a new tool to address key grower disease control needs.

2. Results and discussion

2.1 Hit Identification and activity

The discovery of oxathiapiprolin began with the purchase of a diverse bis-amide 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 (Table 1). This hint of curative activity, an important but rarely seen attribute for oomycete fungicides, caught our attention and an optimization program was initiated.

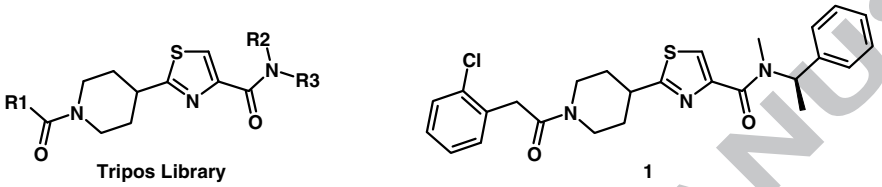
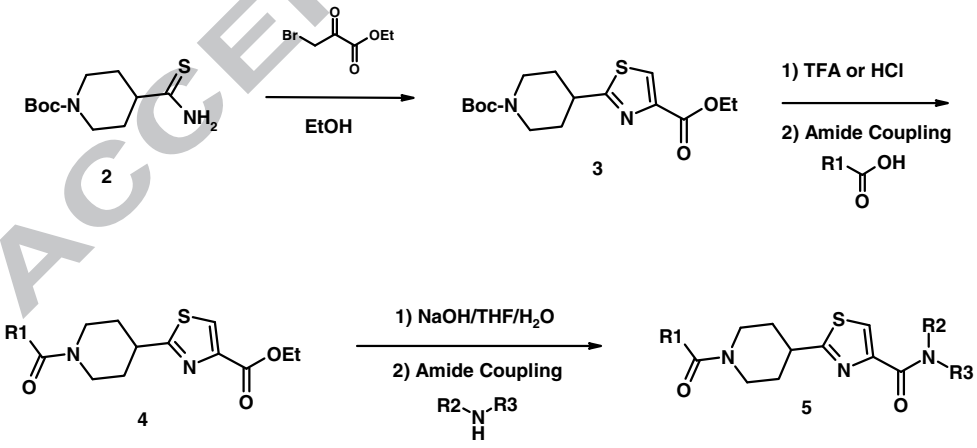


Figure 1. Tripos bis-amide library details: R1 and R3 are aryl, heteroaryl and benzyl; R2 is hydrogen or methyl. Compound **1** is a library member.

2.2 Chemistry and Initial SAR

The synthesis of compounds such as **1** is outlined in Scheme 1 and begins with condensation of the commercially available Boc-protected piperidine thioamide **2** with ethyl bromopyruvate to give the orthogonally protected piperidinyl thiazole **3**. Amine deprotection, followed by acylation affords ester **4**, which upon base hydrolysis and amide coupling provides the bis-amides **5**. These steps can be reversed where ester hydrolysis and amide coupling precede amine deprotection and acylation.[4]



Scheme 1. The synthesis of bis-amides **5**.

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. Numerous amine variations were explored, but did not lead to significant improvement over the original *N*,α-dimethyl benzyl amine moiety. Both the *N*-methyl and the α-substituent on the benzyl moiety were found to be required for activity, with the (*R*)-enantiomer being preferred. An α-ethyl was slightly better than α-methyl, but longer alkyl, alkenyl or halo alkyl groups reduced activity. Removing the α-substituent caused loss of activity. Adding substituents to the phenyl ring did not appear to enhance activity, nor did replacing the phenyl ring with pyridine or thiophene rings. Removal of the benzylic carbon or replacing it with a nitrogen or oxygen atom eliminated activity. Simple anilides or alkyl amides were not active.

Variation of the acid moiety, holding the (*R*)-*N*,α-dimethyl benzyl amine moiety constant, was more productive. 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 phenyl analog **6** (Figure 2), showed a 5-fold increase in activity vs. the 2-chlorophenyl analog **1**, with > 90% control of preventative and curative TLB and curative GDM observed at an application rate of 40 ppm (Table 1).

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. The dimethyl phenyl ring was replaced with various heterocycles in an effort to lower the logP. Appropriately substituted pyridines, thiophenes, imidazoles, triazoles and thiazoles had similar activity as the phenyl, but a 3,5-disubstituted *N*-linked pyrazole gave a significant boost in activity and systemicity. Thus, pyrazole acetamide **7** (Figure 2) showed a 20-fold increase in activity vs. phenyl acetamide **1**, with > 90% control of preventative and curative TLB and curative GDM observed at an application rate of 10 ppm (Table 1).

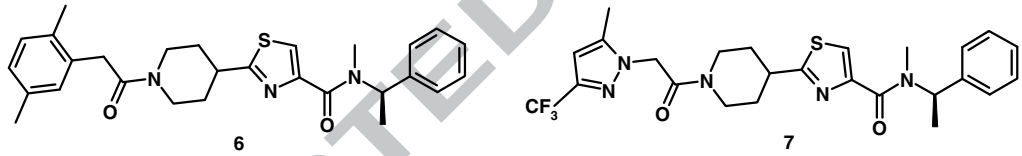


Figure 2. Structures for bis-amides **6** and **7.**

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 revealed that an unsubstituted piperidine ring was optimal. Replacing the piperidine ring with a piperazine ring gave analogs with good activity, but poor photo stability, most likely due to extended conjugation of the extra nitrogen lone pair. Replacing the piperidine ring with a homopiperidine ring or a tetrahydropyridine ring lowered activity, while replacing the piperidine ring with a pyrrolidine ring or an azetidine ring eliminated activity. Ring-opened analogs were not active.

High activity could be retained by replacing the thiazole ring with other 5-membered ring heterocycles, as shown in Figure 3, 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.

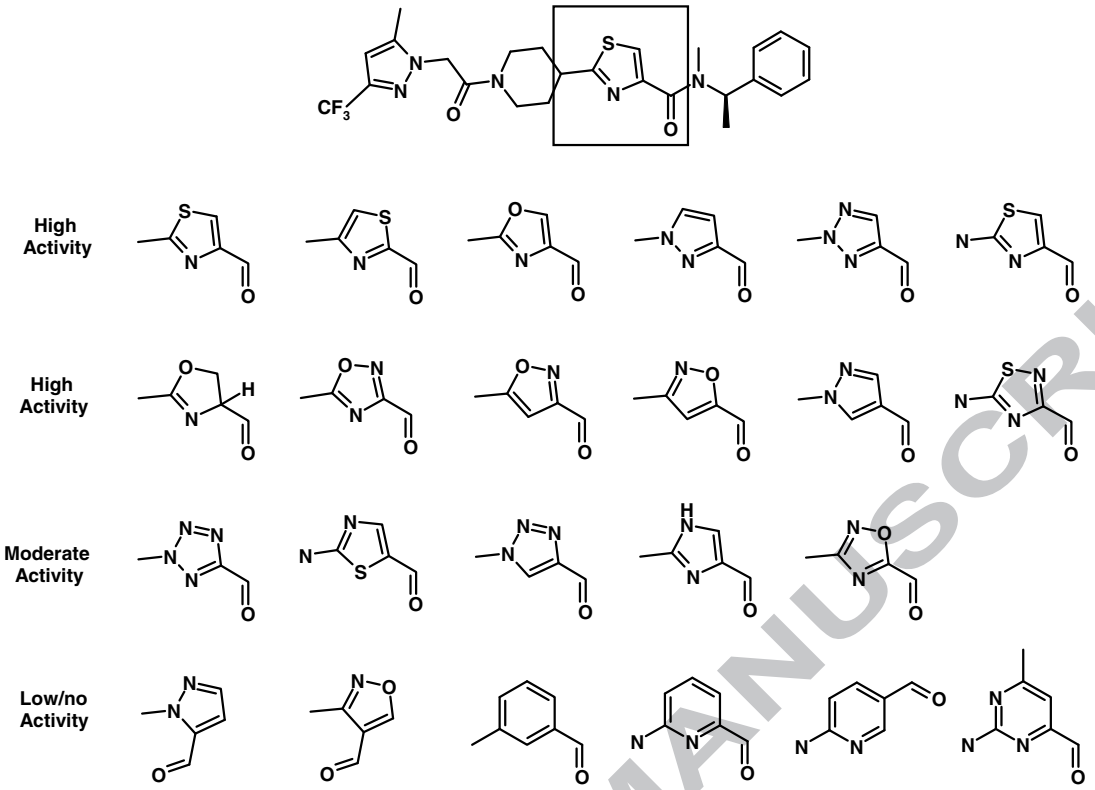


Figure 3. Thiazole moiety SAR.

2.3 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 **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 α-methyl, the *N*-methyl onto the phenyl and the α-methyl onto the phenyl as outlined in Figure 4. The first two modifications were unproductive, but the third resulted in a remarkable boost in activity.

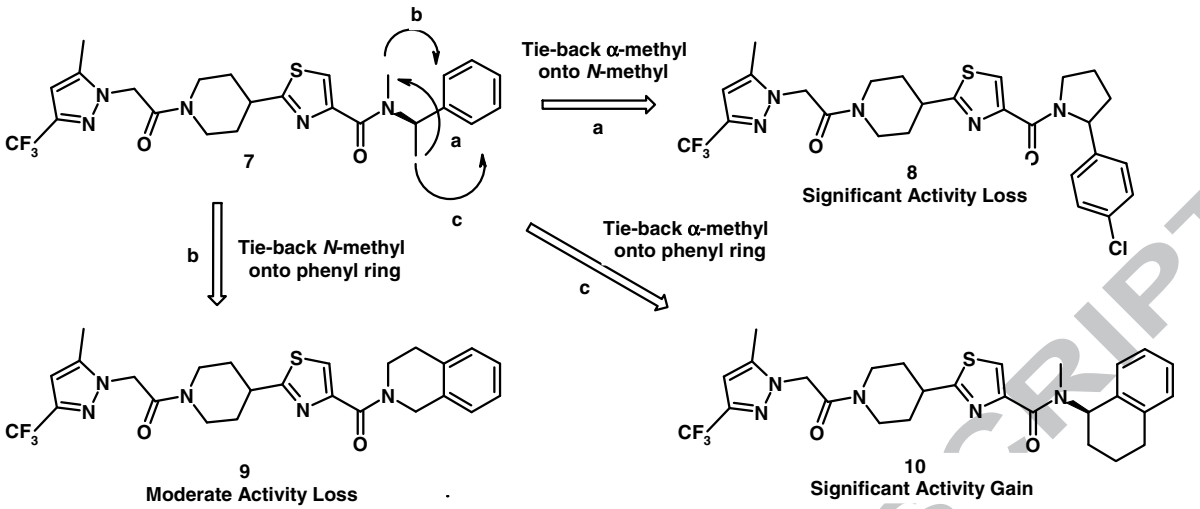


Figure 4. Restricting conformations near the chiral center.

The tetraline amide **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 (Table 1). Bis-amide **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 PLB and GDM at 100 gai/ha, outperforming commercial fungicides.[5]

With this approach validated, we turned our attention to the amide bond. Amide **7** exists as a mixture of two rotamers, clearly observable in its NMR spectrum and requiring a temperature of 110 ^{o}C for signal coalescence. We envisioned preparing the two isomeric imidazolines **11** and **12** as our best probe compounds, but also prepared the phenyl isoxazoline **13** as a potential benzyl amide bioisostere (see Figure 5). This was fortuitous, since both **11** and **12** were inactive, while isoxazoline **13** again showed a remarkable boost in activity over compound **7**.

Like tetraline amide **10**, racemic isoxazoline **13** was 1000-fold more active than starting Compound **1** (Table 1). The (*R*)-enantiomer of **13** was more active than the corresponding (*S*)-enantiomer. Restricting the rotation of the phenyl ring by tying it back onto the 5-position of the isoxazoline ring gave the spiro analog **14** (Figure 5) and another significant boost in activity. Spiro 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 (Table 1). Field tests demonstrated its superior performance versus the leading commercial oomycete fungicides.[6,7]

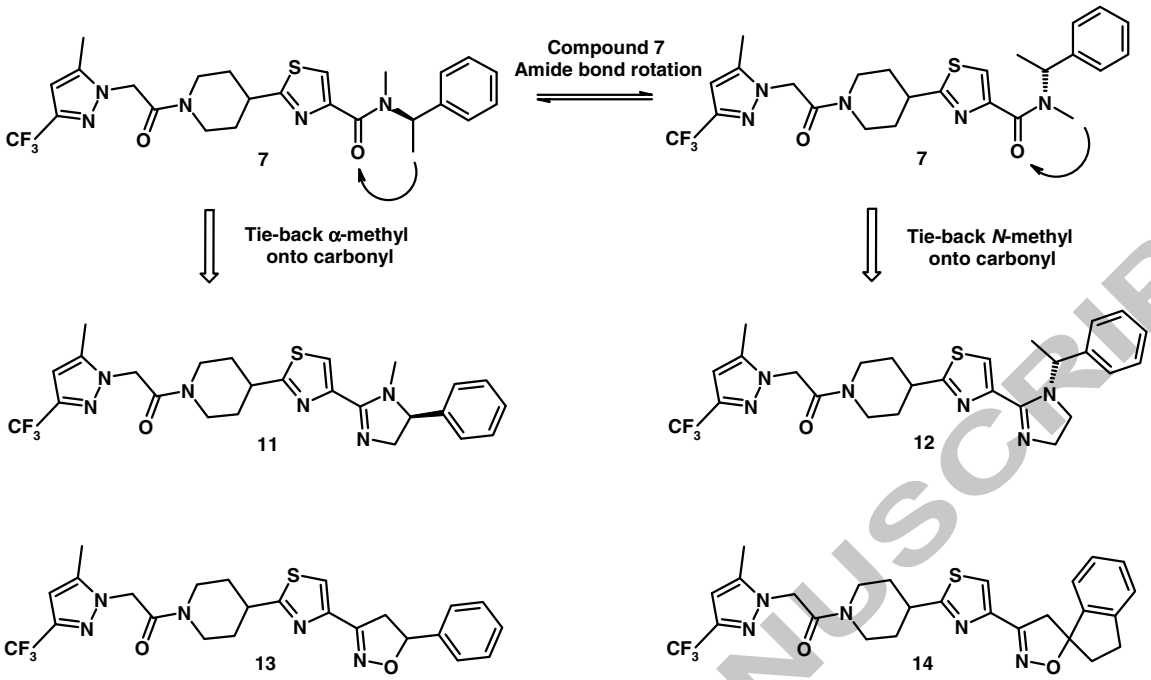


Figure 5. Amide bond bioisosteres. Imidazolines **11** and **12** are not active. Isoxazolines **13** and **14** have unbroken activity at 0.4 and 0.08 ppm, respectively.

2.4 Selecting the optimal candidate

An intensive effort looking at a wide range of alternate amide bioisosteres led to many active chemotypes[8,9] (Figure 6), but none were more active than the isoxazoline chemotype. The corresponding isoxazole and the homologous dihydrooxazine were also quite active.

The piperidinyl thiazole isoxazolines can be readily prepared as shown in Scheme 2 for phenyl isoxazoline **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-piperidine thioamide **2** forms the thiazole **18** followed by amine deprotection and coupling with the pyrazole acetic acid **19** to afford **13** in high yield.

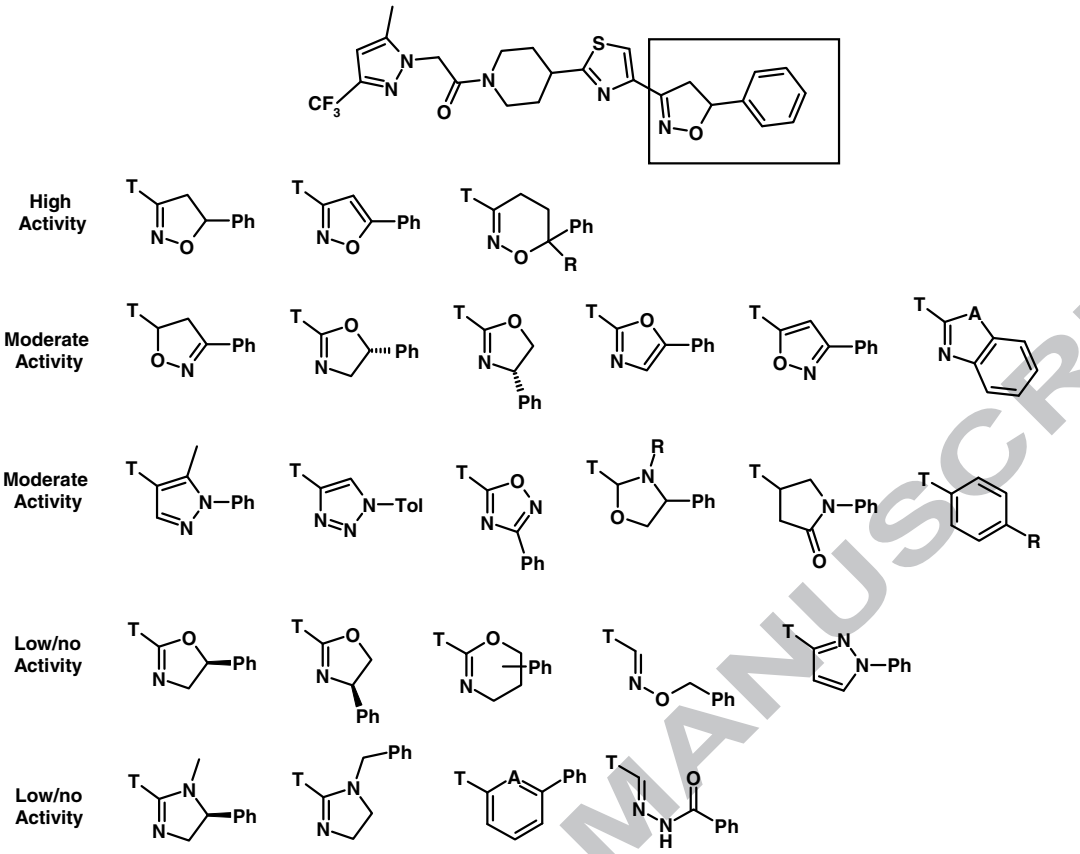
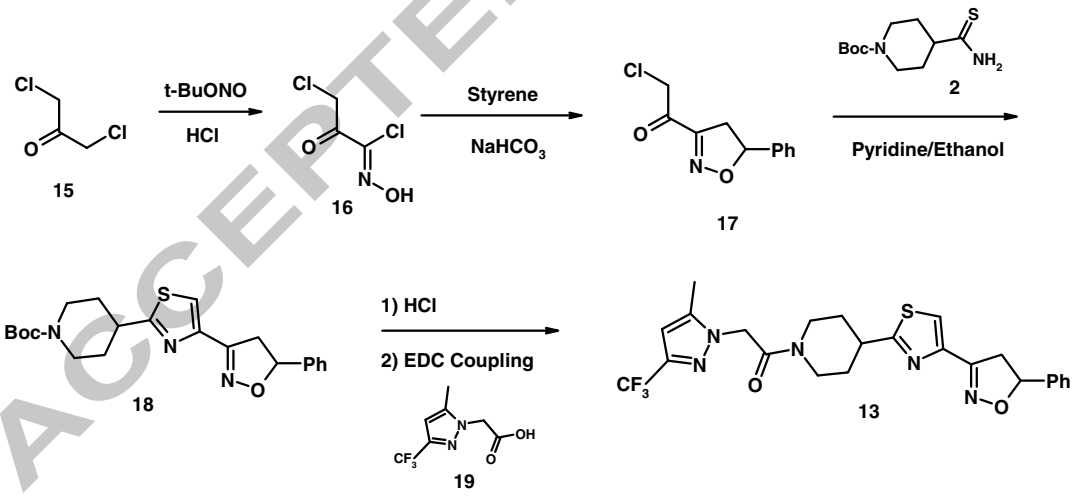


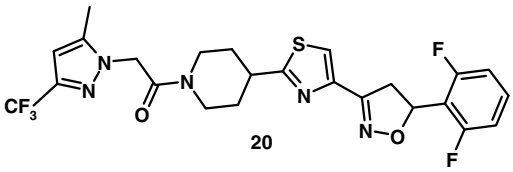
Figure 6. Isoxazoline moiety SAR.



Scheme 2. Synthesis of compound **13**.

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 **14** being among the best. The phenyl ring of **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 **13** to give analogs with unprecedented levels of activity. For example, Figure 7 depicts the 2,6-difluorophenyl analog **20**, which showed a 10,000-fold increase in activity versus bis-amide **1**, with > 90% control of preventative and curative TLB and curative GDM observed at an application rate of 0.02 ppm (Table 1).[6]



20

Figure 7. Compound **20**: unbroken activity at 0.02 ppm.

Table 1
Potency progression observed during optimization

Compound	Lowest rate required[a] (ppm)	Relative activity[b]
1	200	1
6	40	5
7	10	20
10	0.4	1,000
13	0.4	1,000
14	0.08	2,500
20[c]	0.02	10,000

[a]Lowest compound application rate required to provide > 90 percent control of preventative and curative TLB and curative GDM relative to the untreated control plants. [b]Relative activity increase compared to Compound **1**. [c]Oxathiapiprolin.

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.[10] 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.[11]

With so many chemotypes with high intrinsic activity, it soon became apparent that the key challenge would be finding the best of the best to move forward into development. We had a wide 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,[12] which necessitated finding the most stable polymorph for each one to assess formulation characteristics and biological efficacy.

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 toxicological and field efficacy testing. All of the candidates performed well in the field with the 2,6-difluorophenyl analog **20** (Figure 7) being selected as the commercial development candidate and has been assigned the common name of oxathiapiprolin.

2.5 Biological activity

Compound **20** was evaluated in the field for control of the major oomycete diseases of grapes, potatoes and vegetables. It 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. Compound **20** consistently outperformed all of the commercial disease control standards.

Compound **20** 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.

Compound **20** 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 grow and expand and new leaves as they emerge and grow.

Compound **20** has an excellent toxicity profile, with low oral, neuro, developmental, dermal, aquatic, avian and bee toxicity.

2.6 Mechanism of action

Initial work to determine the mechanism of action of the bis-amides focused on comparisons with known fungicides and tests to determine if they acted via known mechanisms were all negative. Confocal microscopy using a version of tetraline amide **10** tethered to a fluorescent tag showed localization to the nucleus, Golgi and endoplasmic reticulum. Ligand localization could be prevented by high concentrations of active analogs but not by other known fungal classes.

A bis-tritiated version of isoxazoline **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.

At this point two parallel efforts were undertaken to identify the target protein using the model oomycete *Phytophthora capsici*.[13,14] A biochemical approach used a tethered version of isoxazoline **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

ultraviolet light and resistant mutants were selected with growth media infused with isoxazoline **14** or **20**. 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 . Additionally, transformation of *P. capsici* cells with a plasmid encoding a mutated OSBP gene conferred resistance to compound **20**.

OSBP represents a novel target for oomycete disease control. The cellular role of this protein family is not well understood.[15] Studies to provide definitive evidence for the cellular role of OSBP in *Phytophthora* and the exact mechanism of compound **20**'s cytotoxicity are still underway.

3. Conclusions

Compound **20** 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 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.

Compound **20** has a novel site of action, binding strongly to an oxysterol binding protein with an, as yet, unknown cellular function. Compound **20** has the perfect combination of attributes to provide outstanding oomycete disease control, has been assigned the common name of oxathiapiprolin 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 valuable tools for growers in their efforts to combat these diseases.

4. Experimental Section

4.1 Chemistry

All new compounds were characterized by standard spectroscopic methods. ^{1}H NMR spectra were recorded on a Varian Unity 400 spectrometer at 400 MHz using deuterated solvent and tetramethylsilane as an internal standard. Chemical shifts are reported in ppm downfield from the standard (δ= 0.00). All reactions were carried out under anhydrous conditions under an inert atmosphere (nitrogen or argon) with commercially available dry solvents. All the compounds described below are known and these and alternate preparations for the compounds in Table 1 can be found in references 4 and 8. A crystal structure for form B of Compound **20** can be found in reference 12.

4.1.1. Preparation of 3-chloro-N-hydroxy-2-oxo-propanimidoyl chloride (16)

To a solution of 1,3-dichloroacetone (**15**, 150 g, 1.12 mol) in 2 M solution of hydrogen chloride in diethyl ether (600 mL) was added *t*-butyl nitrite (126 g, 1.1 mol) over 45 minutes keeping the temperature below 26 °C with a cold water bath. The reaction was allowed to stir overnight at ambient temperature and the mixture was concentrated under reduced pressure. *n*BuCl was added and the mixture concentrated under reduced pressure again. The resulting solid was slurried in *n*BuCl and collected under filtration to give a 41.3 g of a white solid. The filtrate was concentrated under reduced pressure to about half volume to give a second crop of white solid (25.8 g), and the filtrate was further concentrated to give

a third crop (26.5 g). The solids were combined to give 3-chloro-*N*-hydroxy-2-oxo-propanimidoyl chloride (**16**, 93.6 g, 0.60 mol, 53%). ^{1}H NMR (DMSO-d_6) δ4.96 (s, 2H), 13.76 (s, 1H).

4.1.2. Preparation of 2-chloro-1-(4,5-dihydro-5-phenyl-3-isoxazolyl)ethanone (17)

To a mixture of styrene (6.79 g, 65.3 mmol) and powdered sodium bicarbonate (32.1 g 0.38 mol) in acetonitrile (100 mL), 3-chloro-*N*-hydroxy-2-oxo-propanimidoyl chloride (**16**, 10 g, 64.1 mmol) was added in 10 portions over 20 minutes. The reaction mixture was then stirred for additional 1 h and filtered. The filtered solid was rinsed with acetonitrile, and the combined filtrates were concentrated under reduced pressure to leave an oil, which was triturated first with hexanes and then with 1-chlorobutane to give 2-chloro-1-(4,5-dihydro-5-phenyl-3-isoxazolyl)ethanone (**17**, 13.6 g, 61 mmol, 95%) as a white solid. ^{1}H NMR ($CDCl_3$) δ3.13 (m, 1H), 3.66 (m, 1H), 4.96 (s, 2H), 5.83 (m, 1H), 7.34–7.44 (m, 5H).

4.1.3. Preparation of 1,1-dimethylethyl 4-[4-(4,5-dihydro-5-phenyl-3-isoxazolyl)-2 thiazolyl]-1-piperidinecarboxylate (18)

A mixture of 1,1-dimethylethyl 4-(aminothioxomethyl)-1-tetrahydropyridine-carboxylate (**2**, 2.4 g, 10 mmol), 2-chloro-1-(4,5-dihydro-5-phenyl-3-isoxazolyl)ethanone (**17**, 2.3 g, 10 mmol), pyridine (1.6 g, 20 mmol) and tetrabutylammonium bromide (0.65 g, 2 mmol) in ethanol (20 mL) was refluxed for 2.5 h and cooled in an ice/water bath. The resulting precipitate was filtered, washed with cold ethanol and air dried to give 1,1-dimethylethyl 4-[4-(4,5-dihydro-5-phenyl-3-isoxazolyl)-2 thiazolyl]-1-piperidinecarboxylate (**18**, 2.85 g, 6.9 mmol, 69%) as a white solid. 1H NMR (CDCl3) δ1.47 (s, 9H), 1.7 (m, 2H), 2.1 (m, 2H), 2.85 (m, 2H), 3.2 (m, 1H), 3.45 (m, 1H), 3.84 (m, 1H) 4.2 (br s, 2H), 5.75 (m, 1H), 7.25–7.40 (m, 5H), 7.61 (s, 1H).

4.1.4. Preparation of 4-[4-[4,5-dihydro-5-phenyl-3-isoxazolyl]-2-thiazolyl]-1 [[5-methyl-3 (trifluoromethyl)-1H-pyrazol-1-yl]acetyl]piperindine (13)

To a solution of 1,1-dimethylethyl 4-[4-(4,5-dihydro-5-phenyl-3-isoxazolyl)-2 thiazolyl]-1-piperidinecarboxylate (**18**, 0.815 g, 1.97 mmol) in dichloromethane (50 mL) was added a solution of hydrogen chloride in diethyl ether (2 M, 10 mL, 20 mmol). The reaction mixture was stirred at room temperature for 1 h to give a gummy precipitate. Methanol was added to dissolve the precipitate, and the reaction mixture was stirred for an additional 1 h. The reaction mixture was concentrated under reduced pressure and partitioned between ethyl acetate and saturated aqueous sodium bicarbonate solution, and the organic layer was dried ($MgSO_4$) and concentrated to give the free amine as a clear oil (0.31 g), which solidified on standing.

A mixture of the resulting free amine (0.31 g, 1.0 mmol), 5-methyl-3-(trifluoromethyl)-1H-pyrazole-1-acetic acid (**19**, 0.208 g, 1.0 mmol), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (0.25 g, 1.3 mmol), triethylamine (150 uL, 1.08 mmol) and a catalytic amount of 1-hydroxy-benzotriazole hydrate (~1 mg) in dichloromethane (5 mL) was swirled to form a vortex and held at room temperature for 16 h. The reaction mixture was diluted with dichloromethane (10 mL), and washed with 1 N aqueous hydrochloric acid and saturated aqueous sodium bicarbonate solution. The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to give 4-[4-[4,5-dihydro-5-phenyl-3-isoxazolyl]-2-thiazolyl]-1 [[5-methyl-3-(trifluoromethyl)-1H-pyrazol-1-yl]acetyl]piperindine (**13**, 0.47 g, 0.93 mmol, 93%) as a white foam. A sample crystallized from ethanol melted at 109 – 113 ^{o}C. 1H NMR (CDCl3) δ1.8 (m, 2H), 2.2 (m, 2H), 2.32 (s, 3H), 2.9 (m, 1H), 3.3 (m, 2H), 3.42 (m, 1H),

3.85 (m, 1H) 4.05 (m, 1H), 4.55 (m, 1H), 4.98 (m, 2H), 5.75 (m, 1H), 6.33 (s, 1H), 7.25–7.42 (m, 5H), 7.63 (s, 1H).

4.2 Biology

Compounds were first dissolved in acetone in an amount equal to 3% of the final volume and then suspended at the desired concentration (in ppm) in acetone and purified water (50/50 mix) containing 250 ppm of the surfactant Trem® 014 (polyhydric alcohol esters). Spraying a 200 ppm test suspension to the point of run-off on the test plants was the equivalent of a field rate of 500 g / hectare.

4.2.1 *Phytophthora infestans* on tomatoes (tomato late blight; TLB) preventative assay

The test suspension was sprayed to the point of run-off on tomato seedlings. The following day the seedlings were inoculated with a spore suspension of *Phytophthora infestans* (the causal agent of tomato late blight) and incubated in a saturated atmosphere at 20 °C for 24 h, and then moved to a growth chamber at 20 °C for 5 days, after which disease ratings were made.

4.2.2 *Phytophthora infestans* on tomatoes (tomato late blight; TLB) curative assay

Tomato seedlings were inoculated with a spore suspension of *Phytophthora infestans* (the causal agent of tomato late blight) and incubated in a saturated atmosphere at 20 °C for 17 h. After a short drying period, the test suspension was sprayed to the point of run-off on the tomato seedlings and then moved to a growth chamber at 20 °C for 4 days, after which disease ratings were made.

4.2.3 *Plasmopara viticola* on grapes (grape downy mildew; GDM) curative assay

Grape seedlings were inoculated with a spore suspension of *Plasmopara viticola* (the causal agent of grape downy mildew) and incubated in a saturated atmosphere at 20 °C for 24 h. After a short drying period, the test suspension was sprayed to the point of run-off on the grape seedlings and then moved to a growth chamber at 20 °C for 5 days, after which the test units were placed back into a saturated atmosphere at 20 °C for 24 h. Upon removal, disease ratings were made.

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