Flupyradifurone: a brief profile of a new butenolide insecticide

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Abstract

BACKGROUND: The development and commercialization of new chemical classes of insecticides for efficient crop protection measures against destructive invertebrate pests is of utmost importance to overcome resistance issues and to secure sustainable crop yields. Flupyradifurone introduced here is the first representative of the novel butenolide class of insecticides active against various sucking pests and showing an excellent safety profile.

RESULTS: The discovery of flupyradifurone was inspired by the butenolide scaffold in naturally occurring stemofoline. Flupyradifurone acts reversibly as an agonist on insect nicotinic acetylcholine receptors, but is structurally different from known agonists as shown by chemical similarity analysis. It shows a fast action on a broad range of sucking pests as shown in laboratory bioassays and exhibits excellent field efficacy on a number of crops using different application methods including foliar, soil, seed treatment and drip irrigation. It is readily taken up by plants and translocated in the xylem as demonstrated by phosphor imaging analysis. Flupyradifurone is active on resistant pests including cotton whiteflies and is not metabolized by recombinantly expressed CYP6CM1, a cytochrome P450 conferring metabolic resistance to neonicotinoids and pymetrozine.

CONCLUSION: The novel butenolide insecticide flupyradifurone shows unique properties and will become a new tool for integrated pest management (IPM) around the globe as demonstrated by its insecticidal, ecotoxicological and safety profile.

Keywords: Flupyradifurone, Sivanto®, nicotinic acetylcholine receptor agonist, CYP6CM1, metabolic resistance, resistance management, sucking pests, butenolide

1 INTRODUCTION

The discovery and development of new chemical classes of insecticides is of utmost importance to keep key invertebrate pests under economic damage thresholds and thus to guarantee sustainable yields of commodities in order to supply affordable food for a growing world population.[1] The task of discovering a new class of insecticides or a new mode of action these days seems to be reserved for a fairly low number of R&D-based companies, particularly considering the recent consolidation process in the crop protection industry.[2] However between 1990 and 2010 the costs for the development of a new insecticide increased by more than 100% to approx. $250 million, and the average development and registration process takes almost 10 years.[2] To bear this in mind one will inevitably understand that during the last two decades strong cost/benefit measures have been implemented in industry and that these days 140,000 compounds need to be screened for a new product on the market, a fact in strong contrast to 1 out of 20,000 compounds 35 years ago.[2,3] However, new selective insecticides compatible with modern integrated pest management principles addressing the regulatory needs for an improved toxicological and environmental profile will stepwise replace older chemistry suffering from resistance development in many invertebrate pests frequently targeted by indispensable chemical treatments in some agricultural settings. The implementation of resistance management strategies based on mode of action rotation including new chemical classes/modes of action is a prerequisite in modern applied agriculture as promoted by the Insecticide Resistance Action Committee (IRAC).[4] Particularly hemipteran insect species such as aphids, whiteflies, stink bugs and psyllids are known to be among the most destructive pests in the world, transmitting plant pathogenic viruses/bacteria and causing feeding damage on numerous annual and perennial crops. Such pests are not yet accessible by trait technologies, so there is a need for corrective treatments to keep them under economic damage thresholds. Some sucking pest species have developed resistance to virtually all chemical classes of insecticides introduced to control them.[5] In order to diversify the toolbox necessary for appropriate resistance management measures in alternation with established compounds, new chemical classes of insecticides need to be introduced.[6]

In search of new chemical scaffolds leading to novel chemical classes of insecticides - particularly for sucking pest control - the natural product stemofoline (Fig. 1; **1**), isolated from the leaves and stem of the oriental medicinal plant *Stemona japonica* (Blume) Miq. (Stemonaceae) and known as a potent agonist of insect nicotinic acetylcholine receptors (nAChRs), was considered as a good starting point.[7,8] Stemofoline shows fast-acting insecticidal, antifeedant, and repellent activities, but

its activity is significantly lower than commercial products acting on insect nAChRs.[9-11] Therefore, **1** was broadly used as a potent lead structure in order to identify novel active ingredients for modern crop protection.[11]

Recently the design of smaller molecules has focused mainly on the stemofoline 2,6-methanofuro[2,3,4-gh]pyrrolizine cage structure. Based on this cage structure the class of pyridinyl-cyanotropanes were found, for example the *in vivo* highly active 3-(5-chloro-3-pyridinyl)-8-(2,2,2-trifluoroethyl)-8-azabicyclo[3.2.1]octane-3-carbonitrile (Fig. 1, **2a**; R = CH_2CF_3), which is bio-activated in larval *Heliothis virescens* (Fabricius) by cleavage of the *N*-(2,2,2-trifluoroethyl) residue (Fig. 1, **2b**; R = H).[12,13] When studying the impact of the stemofoline head group to the efficacy of **1**, we became interested in the 5-ring butenolide scaffold as a starting point for the preparation of new bioactive scaffolds and finally the identification of the biologically active enaminocarbonyl compound class (**I**; Fig. 1).

At the same time, our concept was strongly supported by molecular modeling investigations with **1** and relevant nAChR ligands containing similar structural fragments like the azoles (**II**) and 5-ring and 6-ring heterocycles (**III**) as outlined in Figure 2.[14-16] By stepwise chemical optimization of enaminocarbonyl compounds (**I**) via forming different active butenolide subclasses like (**IV**; Z = O) and (**V**; A = Het) the butenolide lead structures (**VI**; R^1 = CH_2CHR-F) were obtained (Fig. 3; in all structures of subclasses **IV**-**VIII** changes are highlighted by frames). Further exploration of substituted *N*-containing 5- and 6-ring heterocycles in (**VI**) resulted in butenolides (**VII**; Het = X, Y, Z or W-substituted 3-pyridinyl), which could be optimized by the introduction of the 6-chloropyridin-3-yl moiety (X, Y, Z, W = H) to form highly active insecticides such as (**VIII**). Finally, the introduction of a second fluorine atom into the *N*-2-fluoroethyl side chain of (**3**, R = H) led to the discovery of flupyradifurone (Fig. 3; **4**, R = F).[17]

Compared with already commercialized nAChR agonists like *N*-cyano-amidines (acetamiprid, thiacloprid), nitroenamines (nitenpyram), *N*-nitro-guanidines (imidacloprid, clothianidin, thiamethoxam or dinotefuran) or sulfoximines (sulfoxaflor), the butenolide flupyradifurone (**4**, Z = O) contains a different pharmacophore system as a new bioactive scaffold (Fig. 4; Table 1) and was chosen for further global development by Bayer CropScience under the trade name Sivanto® (SL 200 g l^{-1} (soluble liquid)).[11] Flupyradifurone has a favorable toxicological and ecotoxicological safety profile (Table 2) and is under global development for foliar, drench and seed treatment applications, particularly against sucking pest insects in numerous agricultural and horticultural crops (Table 3). It received its first commercial registrations in Central America (Guatemala and Honduras) in April

2014 and is classified by IRAC as a new chemical subgroup 4D (butenolides) within the latest published version of the IRAC mode of action classification system (www.irac-online.org).

The present paper briefly reports on the chemistry incl. chemical synthesis and similarity relations to established pharmacophores addressing the nAChR. It reflects on the profile and some of the technical properties of flupyradifurone such as mode of action, translocation in plants, insecticidal efficacy against selected sucking pests in laboratory and field tests, speed of action, and the lack of metabolic cross-resistance to neonicotinoids *in vivo* and *in vitro* by using recombinantly expressed CYP6CM1, a cytochrome P450 known to confer resistance to neonicotinoids and pymetrozine.[18]

2 EXPERIMENTAL METHODS

2.1 Chemical synthesis

All reagents were purchased from commercial suppliers and used without further purification. 4-[(2-fluoroethyl)amino]furane-2(5*H*)-one (**X;** R = H) or 4-[(2,2-difluoroethyl)amino]furane-2(5*H*)-one (**X;** R = F) can be synthesized by a described route: 2-fluoroethylamine hydrochloride or 2,2-difluoroethylamine and tetronic acid (**5**). *N*-[(6-chloropyridin-3-yl)methyl]-2,2-difluoroethane-1-amine (**XI**; R = F) was synthesized by a described route: 2-chloro-5-chloromethylpyridine and 2,2-difluoroethane-1-amine. Figure 5 shows general synthetic pathways to butenolides (**VII**) *via* tetronic acid (**5**) or 5-ring lactone intermediates (**X**) by two different methods A and B.[19] Starting with **5** the butenolides (**VII**) can be prepared either by treatment with 2-fluoro-containing ethylamine (R = H, F) following *N*-alkylation of the intermediates (**X**) with 2-chloro-5-chloromethylpyridine (Method A) or by coupling of **5** with secondary amines of type (**XI**) (Method B). Representative preparation methods of the insecticidally active butenolides (**VII**) and are given below. [^{1}H] NMR spectra were recorded in deutero-acetonitrile (CD_3CN), with tetramethylsilane as the internal standard using a Bruker Avance 600 instrument (600 MHz) (Bruker Corporation, Billerica, MA).

*2.1.1 4-[[(6-Chloropyridin-3-yl)methyl](2-fluoroethyl)amino]furan-2(5H)-one (**3**; general procedure for butenolides VII according to Method A)*

1.00 g (6.89 mmol) of 4-[(2-fluoroethyl)amino]furane-2(5*H*)-one (**X;** R = H) and 0.55 g (13.78 mmol) of a 60 % dispersion of sodium hydride in mineral oil in tetrahydrofuran (200 ml) were heated under reflux for 2h. After cooling to room temperature, 2.23 g (13.78 mmol) of 2-chloro-5-chloromethylpyridine were added, and the mixture was heated under reflux for further 4 hours. The reaction mixture was cooled to room temperature, and methanol was added. After concentration of

the reaction mixture under reduced pressure, the residue was taken up in ethyl acetate and the mixture was washed successively twice with 1N aqueous hydrochloric acid, twice with 1N aqueous sodium hydroxide solution and once with saturated sodium chloride solution. The organic phase was then dried over sodium sulfate and concentrated under reduced pressure. Purification of the residue by column chromatography on silica gel (silica gel 60 – Merck, particle size: 0.04 to 0.063 mm) using the mobile phase mixture ethyl acetate:cyclohexane (9:1) resulted in 949 mg (50 % of theory) of 4-[[6-chloropyridin-3-yl)methyl](2-fluoroethyl)amino]furan-2(5*H*)-one (**3**).

[^{1}H] NMR (CD_3CN, δ, ppm): 3.50 (td, 2H), 4.50 (s, 2H), 4.57 (dt, 2H), 4.65 (s, 1H), 4.79 (s, 2H), 7.38 (d, 1H), 7.65 (dd, 1H), 8.28 (d, 1H).

*2.1.2 4-[[(6-Chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino]furan-2(5H)-one (**4**; flupyradifurone, Sivanto®; general procedure for butenolides VII according to Method B)*

On a water separator, 21.90 g (106.0 mmol) of *N*-[(6-chloropyridin-3-yl)methyl]-2,2-difluoroethane-1-amine, 14.85 g (148.4 mmol) of tetronic acid (**5**) and 183 mg (1.1 mmol) of 4-toluenesulfonic acid (*p*-TSA) in toluene (250 ml) were heated under reflux for 2 h. The reaction mixture was concentrated under reduced pressure, the residue was then taken up in ethyl acetate and the mixture was washed successively twice with 1N aqueous hydrochloric acid, twice with 1 N aqueous sodium hydroxide solution and once with saturated sodium chloride solution. The organic phase was dried over sodium sulfate and concentrated under reduced pressure. Purification of the residue by column chromatography on silica gel (silica gel 60 – Merck, particle size: 0.04 to 0.063 mm) using the mobile phase ethyl acetate resulted in 15.9 g (52 % of theory) of 4-[[6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino]furan-2(5*H*)-one (**4**).

[^{1}H] NMR (CD_3CN, δ, ppm): 3.59 (td, 2H), 4.51 (s, 2H), 4.76 (s, 1H), 4.80 (s, 2H), 6.03 (tt, 1H), 7.38 (d, 1H), 7.64 (dd, 1H), 8.28 (d, 1H).

2.2 Cheminformatics to compute chemical similarity relations

The full 30x30 similarity matrix was calculated for a set of compounds (Fig. 7), employing Tanimoto indices computed on Unity fingerprints using Sybylx2.0.[20] The full similarity table can also be interpreted as a connection table, connecting all pairs of molecules with a certain similarity value. Inspired by methods from social network analysis, the similarity derived connection table reveals cliques (or clusters) of related compounds, when only connections with a minimum similarity value are considered. In a first step, all pairs of compounds with a Tanimoto index greater than 0.5 were considered, resulting in five disconnected graphs, i.e. one for nicotinoids, one for sulfoximines, one

for butenolides, one for furanyl-substituted neonicotinoids, and one big cluster comprising all other neonicotinoid derivatives. In order to arrive at a single, connected graph, the nearest neighbour with a Tanimoto $\leq$ 0.5 was searched for each member of the four small clusters. Only the pairs with the highest similarities were kept. Using the software Mathematica-suite (Wolfram Research, Inc., Mathematica, Version 10.0, Champaign, IL) the resulting connection tables were visualised using the "Spin Electric Embedding" scheme, which treats the graph as a kind of 2D molecule and minimized assumed repulsive forces between all vertices.

2.3 Mode of action

2.3.1 Receptor binding studies

Radioligand [^{3}H]imidacloprid displacement studies were conducted according to established protocols by using membranes isolated from frozen (-80°C) housefly (*Musca domestica* L.) heads.[21,22] Briefly: Five g heads were homogenized in 100 ml 0.1 M K-phosphate buffer, pH 7.4, 320 mM sucrose, 1 mM EDTA using an Ultra Turrax at 4°C. After centrifugation for 15 min at 1200 g and 4°C, the pellet was re-suspended and centrifuged again. Both supernatants were combined and filtered through Miracloth and the filtrate was subsequently centrifuged at 105.000g for 60min at 4°C. The resulting pellet was re-suspended in buffer and adjusted to approx. 0.5 mg protein ml^{-1}. The assay was conducted in a total volume of 1 ml, consisting of 850 µl homogenate, 50 µl [^{3}H]imidacloprid (25.000 dpm; 1.406 GBq µmol^{-1}) in 0.1 M K-phosphate buffer, pH 7.4 containing 1 g l^{-1} BSA and 5 % ethanol (0,25% final concentration). After 5 min different flupyradifurone concentrations were added (1000, 100, 10, 3, 1, 0.3, 0.1, 0.01 nM; containing up to 0.1% DMSO). After incubation for 60 min at 22°C while shaking, the samples were filtered through pre-wetted Whatman GF/C glass fiber filters followed by 2 rinses with 3 ml ice-cold 0,1M K-phosphate buffer (pH 7.4). Subsequently the filters were dried (55°C, 40 min), and 3.5 ml scintillation cocktail was added. After 16h at room temperature the samples were subjected to liquid scintillation counting.

2.3.2 Electrophysiology

Electrophysiological recordings using whole cell voltage clamp technology were done on isolated neurons of *Spodoptera frugiperda* (J.E. Smith) (fall armyworm) as recently described.[23] Briefly: Isolated ganglia were treated with 4 mg L^{-1} dispase, incubated for 5 min at 37°C, centrifuged, and re-suspended in culture buffer by gentle aspiration with a fire-polished pasteur pipette, with slight modifications as described elsewhere.[24] Cell somata were plated onto glass cover slips previously coated with concanavalin-A (400 µg ml^{-1}) and laminin (4 µg ml^{-1}). The cells were kept at room

temperature. Electrophysiological recordings were done with the whole-cell voltage clamp technique at a holding potential of -70 mV as described elsewhere.[25] The external bath contained 150 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 150 mM trehalose, and 10 mM Hepes, pH 7.4 (adjusted with NaOH). The (internal) pipette solution contained 100 mM CsF, 50 mM CsCl, 10 mM Cs-EGTA, 1 mM $CaCl_2$, 150 mM trehalose, and 10 mM Hepes, pH 7.4 (adjusted with CsOH). Flupyradifurone was applied at different concentrations to the cells using the U-tube reversed flow technique.[26] Currents were measured with an L/M-EPC 7 patch clamp amplifier (List, Darmstadt, Germany). Current records were low-pass Bessel filtered at 315 Hz and digitized at 1 kHz sample rate. The dose response curve was fitted by the Hill equation. All currents were normalized to mean amplitudes elicited by 10 µM acetylcholine before and after test concentrations were applied.

2.4 Plant uptake and translocation studies

Uptake and distribution of [pyridinylmethyl-^{14}C]-labelled flupyradifurone (specific activity 4.37 MBq mg^{-1}; purity >99%) formulated as SL200 (containing radiolabelled flupyradifurone at 13.4 KBq mg^{-1}) was qualitatively studied in 21 day old tomato plants (*Solanum lycopersicum* L.) after soil drench application at 24 mg a.i./plant in 5 l pots in dry sandy loam at 80-90% water holding capacity. Plants were maintained at 60-70% relative humidity and a temperature of 21°C and 16°C during day (14h light) and night time (8h), respectively. At sampling dates (1, 3, 7, 14 and 24 days after application) shoots were cut near the soil surface, pressed between several layers of filter paper and dried for 24 h at 50°C. After drying the shoots (leaves) were exposed to a phosphor imaging plate placed into a Fuji-Fujix BAS cassette 2040. Cassettes were then stored for 6 days in a lead shielding box at ambient temperature. Afterwards the phosphor imaging plates were measured with a Fujifilm BAS 2500 scanner and obtained images were analysed by AIDA Image Analyzer 4.14 software (Raytest GmbH, Germany). In a second set of experiments droplets of [pyridinylmethyl-^{14}C]flupyradifurone SL200 formulation were either applied to the midrib of leaf 7 (25000 Bq $droplet^{-1}$) or directly onto the center pinnate leaves of leaf 8 (1000 Bq $droplet^{-1}$) of a tomato plant. Four days after application plants were subjected to the same preparation and imaging procedure as described above.

2.5 Bioassays

2.5.1 Speed of action and suppression of aphid honeydew excretion

Green peach aphid, *Myzus persicae* (Sulzer) used in this bioassay were taken from an anholocyclic insecticide susceptible laboratory strain (NS) maintained on cabbage (*Brassica oleracea* L.) at 22°C, 60-70% relative humidity and 16:8 (h) photoperiod. In order to check the speed of action of flupyradifurone (formulated as 200 SL) we measured the suppression of honeydew excretion of *M.*

persicae feeding on oilseed rape (*Brassica napus* L.) leaves after foliar treatment at a rate of 75 g a.i. ha^{-1}. As a comparison a treatment with thiamethoxam (Actara®, Syngenta, 25 g a.i. ha^{-1}) was included in the trial. Ten day old greenhouse grown oilseed rape plants (6 leaves) were infested with a mixed population of 100 aphids feeding on the abaxial side of the leaves. Plants were treated 50 cm above canopy with a purpose-build linear track sprayer with a flat fan nozzle SS 8003-E at above-mentioned rates using a water volume of 300 l ha^{-1}. Each treatment was replicated twice; control plants were treated with water only. The treated plants were placed at 22°C and 60-70% relative humidity under neon light. Filter papers were mounted under each leaf during pre-application and post-application phases and replaced every 30 min for up to 240 min. Filter papers were then sprayed with ninhydrin at 1g l^{-1} acetone and dried for 5 min at 100°C. The area of purple honeydew spots was determined using standard image analysis software based on Open CV Halcon library (Bayer Technology Services GmbH, Leverkusen), plotted per plant and compared to water treated controls at different elapsed time intervals.

2.5.2 Aphid leaf-dip bioassays

All insecticide susceptible aphid species tested were derived from laboratory cultures grown without insecticide selection pressure for at least 10 years. A leaf-dip bioassay procedure was recently designed to test diverse insecticidal classes of chemistry under the same conditions in 6-well tissue culture plates or purpose-built ventilated Petri-dishes.[27] Wells of 6-well tissue culture plates or Petri-dishes were filled with 2 ml Agar (10 g l^{-1})). Several plates/Petri-dishes were usually prepared and stored at 4°C until use. Plates/Petri-dishes were equilibrated to room temperature and leaf discs (Ø 30 mm) were cut out of the first and second fully expanded true leaves from three-week old plants such as *B. oleracea* (*M. persicae*), *Humulus lupulus* L. (*Phorodon humuli* (Schrank)), *Gossypium hirsutum* L. (*Aphis gossypii* (Glover)) and *Lactuca sativa* L. (*Nasonovia ribisnigri* (Mosley)). The leaf discs were then dipped into serial aqueous dilutions of active ingredient in 0.02 % (w/v) Triton X-100, and after drying on tissue paper the leaf discs were transferred to 6-well plates/Petri-dishes. Flupyradifurone was tested at least thrice with up to six replicates per concentration. Ten adult aphids were confined to each well. Mortality was scored after 72h unless otherwise stated. Aphids unable to move and showing strong symptoms of poisoning were scored as dead.

A similar set-up was used to test imidacloprid in comparison to flupyradifurone with metabolically resistant clones of *M. persicae* (F03-09) and *P. humuli* (Hallertau). *M. persicae* clone F03-09 was derived from a *M. persicae* strain collected in oilseed rape in France in 2009, and *P. humuli* clone Hallertau was derived from a field strain collected in German hops (Hallertau) in 2011.

2.5.3 Whitefly bioassays

Insecticide susceptible strains of *Bemisia tabaci* (Gennadius) (SUD-S) and *Trialeurodes vaporariorum* (Westwood) (S-6230) were taken from laboratory cultures maintained without insecticide selection for at least ten years. Whitefly adult leaf-dip bioassays followed an established experimental protocol.[28] Briefly: Cotton leaf discs (3.5 cm in diameter) were dipped for 5 s into insecticide solutions (prepared in aqueous Triton X-100, 0.02% w/v) diluted to the required concentration, or into the diluent alone for controls. Leaf discs were then air-dried on tissue paper and subsequently laid on an agar bed (12 g l^{-1}), held within a plastic Petri dish or 6-well tissue culture plate. Adult females were then placed in the dish and confined using a close-fitting ventilated lid. Bioassays consisted of three replicates per concentration, each with a group of 20-30 female insects. They were maintained at 22-24°C, with adult mortality scored after 72 h.

The same bioassay type was used to compare the efficacy of imidacloprid and flupyradifurone against neonicotinoid-resistant strains of *B. tabaci,* i.e. biotype Q (strain Q, collected in Almeria, Spain, 2007) and biotype B (strain B, collected in Unai, Brazil, 2009). Furthermore an imidacloprid-resistant strain of *T. vaporariorum* collected in 2007 on greenhouse ornamentals in the Netherlands was tested for flupyradifurone cross-resistance.

2.5.4 Field trials against lettuce aphids

As an example to show the field performance of flupyradifurone against aphids seven field trials in lettuce against *N. ribisnigri* conducted in the EU maritime centre zone were analysed and combined. The trials were conducted in Belgium, France, Germany and the Netherlands at BBCH stage 15-19 using flupyradifurone (SL 200) at 125 g a.i. ha^{-1} applied by foliar spray using a knapsack sprayer equipped with flat-fan nozzles and water volumes between 300 and 916 l ha^{-1}. Commercial formulated standards such as pymetrozine (200 g a.i. ha^{-1}), thiacloprid (96 g a.i. ha^{-1}) and spirotetramat (75 g a.i. ha^{-1}) were included in all trials for comparison. Treatments in each trial were replicated 3-4 times and aphid infestations were above economic threshold levels. Assessments of efficacy were done at different elapsed time intervals after spraying by scoring surviving aphids. Percentage control was calculated in relation to the aphid infestation level of control plots.

2.6 Baculovirus-mediated P450-expression and insecticide metabolism

B. tabaci full length CYP6CM1 sequence (GenBank accession no GQ214539) and *Drosophila melanogaster* (Meigen) NADPH CPR (cytochrome P450 reductase) sequence (GenBank accession no Q27597) were obtained by gene synthesis and each was inserted into pDEST8 expression vector (Invitrogen) for baculoviral expression. The heterologous expression in SF9 cells using baculovirus

transfection and preparation of microsomes was described elsewhere.[18, 29] CYP6CM1 activity was confirmed by its dealkylation ability of various fluorescence substrates such as 7-ethoxycoumarin (data not shown) and the successful hydroxylation of imidacloprid to 5-hydroxy imidacloprid as described recently.[30]

2.6.1 Metabolism of insecticides by CYP6CM1

Metabolism of technical insecticides including imidacloprid, pymetrozine, flupyradifurone, spirotetramat and sulfoxaflor (obtained in-house; purity each >99%) was assayed by incubation of the recombinant CYP6CM1/NADPH CPR microsomes (0.2 mg ml^{-1} total protein content) in 0.1 M potassium phosphate buffer with NADPH-regenerating system (Promega; 1.3mM NADP+, 3.3mM glucose-6-phosphate, 3.3mM $MgCl_2$, 0.4 U/mL glucose-6-phosphate dehydrogenase) and insecticide (20 µM) at 30°C for 4 h. The total assay volume was 200 µl using three replicates for each data point. Microsomes without NADPH served as a control. The assay was quenched by the addition of acetonitrile (to 50% final concentration), centrifuged for 10min at 3000g and the supernatant subsequently analyzed by tandem mass spectrometry. Recovery rates of active ingredients using microsomal fractions without co-factor were normally close to 100%. All samples obtained from insecticide metabolism assays were analysed for parent compound degradation using ultra performance liquid chromatography [Waters Acquity UPLC System (Eschborn, Germany)] mass spectrometry utilizing a TSQ Vantage triple quadrupole instrument with H-ESI II source (Thermo, Dreieich, Germany) operating in positive ion mode as recently described.[29]

2.7 Statistics

In radioligand binding studies I_{50}-values (concentration of unlabelled ligand displacing 50% of [^{3}H]imidacloprid from its binding site) were calculated using a 4 parameter logistic non-linear fitting routine (GRAPHPAD-PRISM 5, www.graphpad.com). Lethal concentration (LC) values were calculated from log-dose probit-mortality regressions using Polo PC (LeOra Software, California). Whenever necessary mortality figures were corrected using Abbotts formula.[31] Resistance ratios were calculated by dividing the LC_{50}-value of the susceptible reference strain with the LC_{50}-value of the resistant strain.

3. RESULTS

3.1 Mode of action

The novel butenolide insecticide flupyradifurone acts on insect nAChRs as shown in radioligand binding studies conducted with tritiated imidacloprid, a well-known nAChR agonist out of the different chemical class of neonicotinoids (Fig. 6). Flupyradifurone displaces [^{3}H]imidacloprid bound to *M. domestica* nAChRs from its binding site with nanomolar affinity, and an I_{50}-value of 2.38 ± 1.93 nM was calculated. Whole-cell current responses after application of flupyradifurone and acetylcholine in a single neuron isolated from *S. frugiperda* CNS suggest reversible binding and are shown in Fig. 6. Flupyradifurone activates endogenously expressed insect nAChRs and acts as a partial agonist with a relative agonist efficacy of 0.56 relative to the amplitude elicited by 1mM acetylcholine. Using the same experimental set up imidacloprid exhibits a relative efficacy of 0.15 relative to acetylcholine (data not shown). The flupyradifurone dose response curve fitted by the Hill equation revealed a Hill coefficient of n_H 0.97 ± 0.090 and suggests a single binding site (EC_{50}-value = 2.3 µM for half maximal activation of nAChRs).

3.2 Chemical similarity analysis

Flupyradifurone is shown to address insect nAChRs in a similar way as other commercialized chemical classes of insecticides such as sulfoximines, nicotinoids and neonicotinoids. In order to check its chemical relatedness to known effectors of insect nAChRs, an analysis in terms of Tanimoto indices of a given set of compounds for each subclass was conducted (Fig. 7). The resulting graph visualizes all pairs of compounds with a similarity of more than 50%, plus some additional, lower similarities to create a connected graph. This graph may be read in analogous way as graphs from social network analysis, revealing cliques of tightly connected, and thus related, individuals. Here, “individuals” are chemical structures and “relations” are defined in terms of Tanimoto index. The majority of neonicotinoid compounds included in the analysis, form a large clique, or cluster suggesting high chemical relatedness, with the exception of the tetrahydrofuryl (methyl)-substituted dinotefuran derivatives, which are clearly separated from heteroaromatic chloropyridyl- and chlorothiazolyl-substituted neonicotinoids, albeit they have the same pharmacophore system (N-nitroguanidine). Sulfoximines also clearly cluster outside the neonicotinoid substructures as well as the nicotine group. The novel butenolide chemistry represented by flupyradifurone and its derivatives also forms a separate cluster in terms of Tanimoto similarity, highlighting its chemical difference to neonicotinoids and other nAChR agonists.

3.3 Plant uptake and translocation

Uptake and translocation of [pyridinylmethyl-^{14}C]-labelled flupyradifurone was qualitatively studied in tomato plants using different treatment regimes, i.e. drench, stem and foliar application.

Phosphor-imaging analysis of tomato plants harvested one day after drench application revealed a fast root uptake and even distribution of [^{14}C]-flupyradifurone equivalents within the entire plant via xylem translocation (Fig. 8a). Highest concentrations of [^{14}C]-flupyradifurone equivalents are seen 7 and 14 days after drench application, whereas 24 days after application a marked decline is observed. Furthermore the phosphor-imaging autoradiographs clearly indicate the accumulation of flupyradifurone in the distal parts of the leaves at different time intervals after drench application, thus suggesting apoplastic transport via the xylem and the absence of significant phloem translocation (Fig. 8a). Flupyradifurone applied to the midrib of tomato plants or true leaves is also readily taken up and translocated via the transpiration stream (Fig. 8b), clearly indicating the systemic properties of the compound after foliar application.

3.4 Speed of action

The translaminar speed of action of flupyradifurone was investigated by checking the suppression of honeydew excretion of *M. persicae* feeding on treated oilseed rape plants. Flupyradifurone applied at recommended rates leads to a quick feeding (honeydew excretion) stop within 2 hours after spray application even on aphids feeding on the abaxial leaf side and not directly exposed to spray droplets (Fig. 9). Two days after application of flupyradifurone all aphids were dead (data not shown). The action of flupyradifurone is superior to thiamethoxam which was included as a commercial standard in the same experiment.

3.5 Cross-resistance assessment and efficacy against aphids and whiteflies

Log-dose probit-mortality data of flupyradifurone against a number of strains of different aphid and whitefly species is shown in table 4. Endpoint mortality was reached 72h after leaf-dip application in all cases and calculated LC_{50}-values were often lower than those obtained for imidacloprid using the same bioassay set-up, indicating the high insecticidal activity of flupyradifurone particularly against strains resistant to imidacloprid (table 4). Some resistant strains included in the bioassays such as the *B. tabaci* strains Q-type and B-type exhibit extremely high resistance ratios against imidacloprid, but virtually no or low resistance ratios to flupyradifurone, indicating a lack of high-level cross-resistance between neonicotinoids and the new butenolide insecticide flupyradifurone (table 4; Fig. 10). Adult females of strain B-type collected in Unai, Brazil do not respond at all to imidacloprid concentrations of 1000 mg l^{-1}, whereas flupyradifurone at 16 mg l^{-1} resulted in 50% adult mortality, and 100% nymph mortality (data not shown).

3.6 Field trial results

Flupyradifurone (200 SL) provided excellent field control of lettuce aphids at rates of 125 g ha^{-1} after foliar application and shows at all assessment intervals an insecticidal potential greater than pymetrozine and thiacloprid included as commercial standards at rates of 200 g ha^{-1} and 96 g ha^{-1}, respectively. Flupyradifurone provided the highest level of control against lettuce aphids at 6-10 days after application, i.e. 96% efficacy. Table 5 summarizes the results obtained in seven field trials in the EU maritime zone. Flupyradifurone provided levels of control greater than spirotetramat at 2-3 and 6-10 days after application, but spirotetramat at 75 g ha^{-1} shows superior control 13-15 days after application.

3.7 CYP6CM1 mediated metabolism

Microsomal preparations containing *B. tabaci* CYP6CM1 functionally expressed in Sf9 cells do not metabolize flupyradifurone, but show significant degradation of imidacloprid and pymetrozine (Fig. 11). However, imidacloprid is different from flupyradifurone as it belongs to the neonicotinoid class of chemistry. Two more insecticides of distinct chemical classes, spirotetramat (ketoenols) and sulfoxaflor (sulfoximines) were also incubated with the same microsomal preparations and in both cases no detoxification was observed. After a microsomal incubation period of 4h at 30°C quantitative UPLC-MS analysis revealed a degradation of 23 ± 1% and 70 ± 6% for imidacloprid and pymetrozine, respectively, whereas flupyradifurone, spirotetramat and sulfoxaflor were not depleted at all (Fig. 11). Both imidacloprid and pymetrozine were hydroxylated by CYP6CM1 overexpressed in Sf9 cell microsomes (data not shown). No metabolization was observed in the absence of NADPH and all data shown in Fig. 11 were corrected for possible substrate depletion observed in Sf9 cells infected with baculovirus not expressing CYP6CM1.

4. Discussion

New chemical classes of insecticides are considered as one of the cornerstones in modern applied entomology in order to guarantee sustainable yields and to combat the development and spread of insecticide resistance, particularly in sucking pests such as aphids and whiteflies, which are known to include some of the most destructive global crop pest species within the order Homoptera.[32-33] Flupyradifurone is a new IPM suitable insecticide developed by Bayer CropScience which will be globally launched starting in 2014 as a new chemical option for the control of agricultural and horticultural pests, especially sucking pests of the order Homoptera. Some parameters of its chemical, ecotoxicological and safety profile have been outlined in tables 1-3 in the introductory part of this paper as well as in a recent regional conference paper in Italy.[34] The present paper aims to

introduce flupyradifurone to a broader scientific audience and provide first sets of experimental data concerning its chemical and biological properties.

Flupyradifurone was shown to act on insect nAChRs by both radioligand binding studies using housefly head membranes and electrophysiological recordings using isolated insect neurons. As shown by alternating acetylcholine and flupyradifurone applications on the very same neuron it reversibly binds to and activates endogenous insect nAChRs, similar to other commercial insecticidal compounds such as sulfoxaflor, the neonicotinoids and nicotinoids.[11, 35] However, although flupyradifurone shares the same mode of action with those chemical classes mentioned above, it is chemically different as it is the first nAChR insecticide containing the stemofoline-derived (natural compound) butenolide pharmacophore as a new bioactive scaffold agonistically addressing insect nAChRs. Its distinct chemical nature among commercial nAChR agonists is demonstrated here by a computational cheminformatics driven approach, i.e. the analysis of chemical similarity relations (Tanimoto indices) between all commercially known classes of nAChR agonists and some non-commercial, but close derivatives. Butenolide substructures cluster separate from neonicotinoid insecticides, sulfoximines and nicotinoids. The neonicotinoid cluster includes 2-halogen-thiazol-5-yl methyl- as well as 6-chloropyridin-3-yl methyl-substituted neonicotinoids, but not dinotefuran which has a non-aromatic tetrahydrofuryl(methyl) substituent, however the N-nitroguanidine pharmacophore system remains the same as in imidacloprid, clothianidin and thiamethoxam, thus justifying its recent pharmacophore-based classification as a neonicotinoid insecticide.[11, 35] In terms of pharmacophore substructures also sulfoximines are clearly distinct from neonicotinoids as recently shown,[36] likewise the nicotine derivatives due to their basic nature.[37] It is interesting to note that chlorothiazolyl- and chloropyridyl-substituted neonicotinoids are still similar enough in terms of Tanimoto indices to form one large chemical group (Fig. 7). The distinct chemical structure of the novel butenolide pharmacophore and the lack of metabolic cross-resistance of flupyradifurone led to the formation of a new subgroup (4D) classification within the IRAC mode of action classification scheme, a tool for setting up resistance management strategies based on mode of action rotation.[38] In that scheme neonicotinoids, nicotine and sulfoxaflor are separated as subgroups 4A, 4B and 4C, respectively. A structure-based sub-classification clearly supported by the chemical similarity analysis conducted here. However, noteworthy to mention here that subgroup rotation is only advisable if no other alternatives of different mode of action groups are available in certain agricultural settings.

Flupyradifurone shows excellent activity against different aphid and whitefly species in laboratory bioassays and LC_{50}-values for all tested susceptible strains are in a similar range as published for

other insecticides commercialized to control particularly sucking pests, e.g. neonicotinoids and homopteran feeding blockers.[39-41] Flupyradifurone acts quite fast on sucking pests as exemplified by a translaminar study on the suppression of honeydew excretion in green peach aphid, feeding on the abaxial site of adaxially treated oilseed rape leaves. Within a short time interval most of the aphids stopped feeding and died two days later, suggesting a high potential of flupyradifurone to prevent the transmission of plant pathogenic viruses at recommended field rates. The good translaminar action is supported by phosphor-imaging results of translocation studies with radiolabelled flupyradifurone, which is readily taken up by plant roots, leaves and stems shortly after application, suggesting a good systemic action once translocated *in planta*. It is mainly translocated in the xylem as shown by its accumulation in distal leaf regions when taken up by the leaf lamina, roots and stems. Such a pattern has recently also been demonstrated for other xylem systemic insecticides such as thiacloprid and cyantraniliprole.[42, 43]

Field trials against lettuce aphids known to be difficult to control due to their feeding sites deep in the canopy of lettuce plants demonstrated its excellent efficacy when compared to market standards such as thiacloprid, pymetrozine and thiamethoxam. This finding is strongly supported by recently published field results against other sucking pests such as *Dysaphis plantaginea* and *Aphis pomi* in apples, *A. gossypii, M. persicae, T. vaporariorum* and *B. tabaci* in vegetables, as well as *Empoasca flavescens* and *Scaphoideus titanus* in grapes.[34] The authors showed that flupyradifurone in many field trials is equal or better than standard insecticides against many economically important sucking pest species. Flupyradifurone can be foliarly applied even during flowering as it shows no adverse effects on actively foraging honeybees in long-term field trials in oilseed rape when applied at rates as high as 205 g ha^{-1} (table 2).

Cross-resistance studies using resistant strains of aphids and whiteflies revealed that flupyradifurone lacks metabolic cross-resistance to the neonicotinoid insecticide imidacloprid. However, flupyradifurone should not be used against *M. persicae* carrying a R81T target-site mutation in the nAChR ß1-subunit as recently described for populations collected in peaches in Spain and France.[5, 11] Particularly neonicotinoid resistant strains of *B. tabaci* belonging to groups MEAM-1 (middle-east Asia minor 1; B-type) and MED (Mediterranean, Q-type) were of interest in our studies, as both are known to express high metabolic resistance by overexpressing a specific detoxification enzyme, CYP6CM1.[44] The overexpression of this cytochrome P450 is known to confer extremely high resistance to a number of neonicotinoid insecticides and pymetrozine.[44, 45] The B-type strain of *B. tabaci* originating from Brazil and tested in this study shows a resistance ratio of >1300-fold against imidacloprid, and a strong overexpression of CYP6CM1 (data not shown). Flupyradifurone exhibited

virtually no cross-resistance in this strain (RR = 3) and therefore we investigated the potential of CYP6CM1 to metabolize flupyradifurone when functionally expressed in an insect cell line. Similar studies have been conducted earlier and clearly demonstrated the potential of CYP6CM1 to detoxify neonicotinoids such as imidacloprid by hydroxylation.[30,45] The metabolism studies presented here revealed that functionally expressed CYP6CM1 does not detoxify flupyradifurone, but imidacloprid and pymetrozine as already shown earlier.[18] Thus demonstrating that flupyradifurone is resistant to the major CYP6CM1-based metabolic mechanism conferring neonicotinoid resistance in whiteflies. Similar to flupyradifurone two other chemically distinct commercial insecticides, i.e. sulfoxaflor and spirotetramat were also not detoxified, highlighting the dual specificity of CYP6CM1 to some neonicotinoids such as imidacloprid and pymetrozine, but not other sucking pest active insecticides which are therefore potential partners in resistance management programmes. However sulfoxaflor was already shown earlier to resist detoxification by another cytochrome P450, cyp6g1 of *Drosophila melanogaster* which converts imidacloprid to 5-hydroxy-imidacloprid.[46] Our results support the usefulness of flupyradifurone as a new tool in resistance management strategies, even against sucking pest species such as B- and Q-type *B. tabaci* expressing metabolic resistance to neonicotinoid insecticides.

5 CONCLUSIONS

Flupyradifurone is a new butenolide insecticide discovered and developed by Bayer CropScience offering a high potential for the rapid control of some of the most destructive sucking pest species in many crops. It is shown to have an excellent ecotoxicological and safety profile fulfilling today´s regulatory requirements for a modern IPM suitable insecticide which offers versatile application methods to provide best possible pest/crop solutions in modern applied agriculture. It will be globally launched under the name Sivanto® prime starting in 2014 and is a new resistance management tool shown to offer potential to control resistant whitefly and aphid species expressing metabolic mechanisms of resistance against neonicotinoid insecticides.

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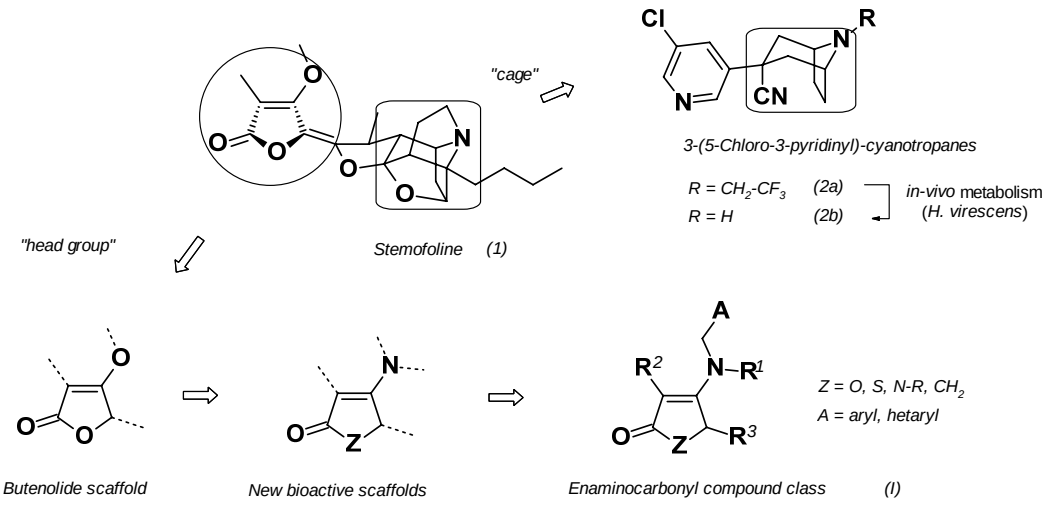


Figure 1. Natural product stemofoline (**1**) as lead structure for novel ligands, *e.g.* **2a, b** and (**I**).

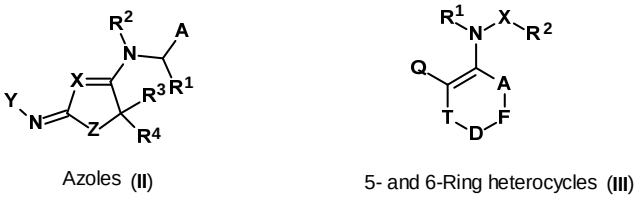


Figure 2. Relevant nAChR ligands (**II**) and (**III**) as lead structures for butenolide chemistry.

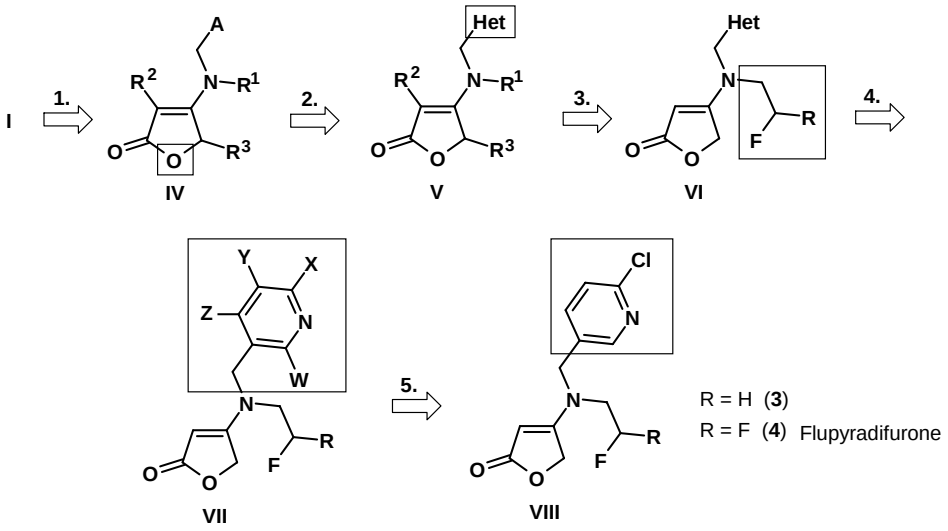


Figure 3. Stepwise chemical evolution of the enaminocarbonyl compound (**I**) *via* the active butenolide subclasses (**IV**)-(**VIII**) resulted in discovery of flupyradifurone (**4**, Sivanto®).

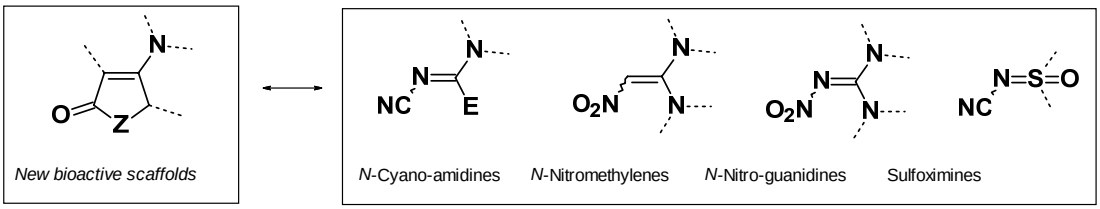


Figure 4. New bioactive scaffold vs pharmacophore systems of known nAChR agonists.

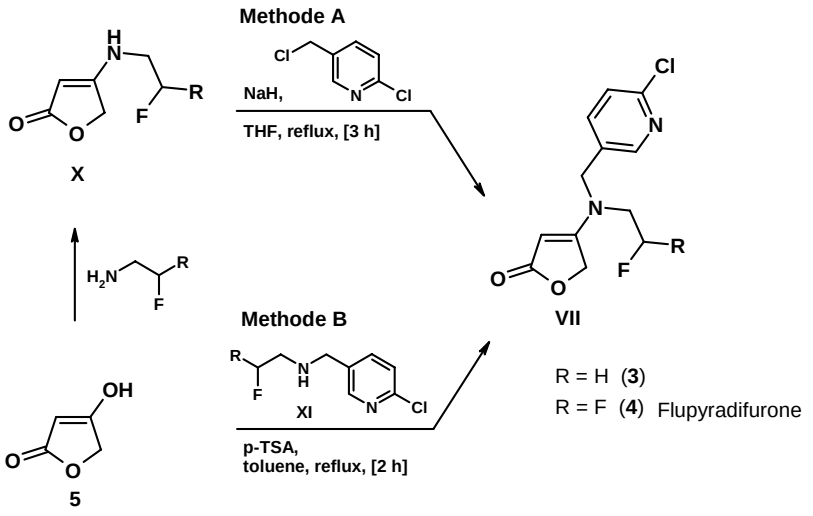


Figure 5. General synthetic pathways to butenolides (**VII**) by the methods A and B.

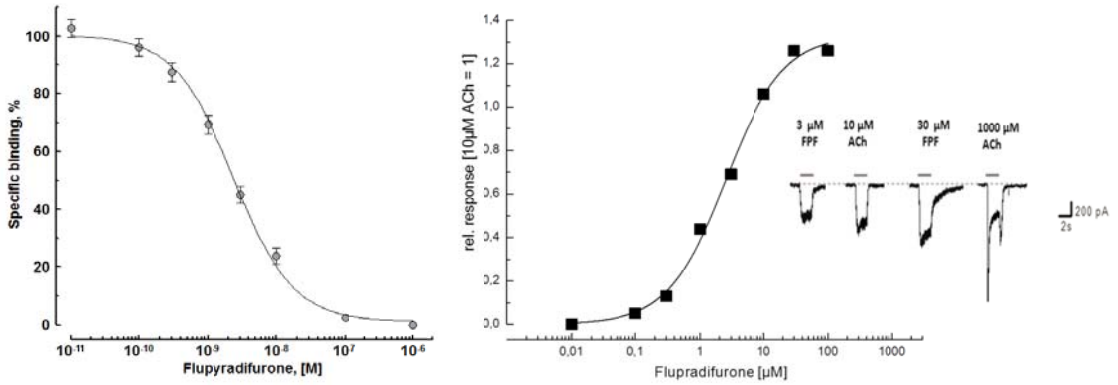


Figure 6. (Left) Binding affinity of flupyradifurone to nicotinic acetylcholine receptors in head membrane preparations of *Musca domestica* using [^{3}H]imidacloprid as a probe in radioligand displacement studies. Data are mean values ± SEM (n=3). (Right) Electrophysiological whole cell current responses of a neuron isolated from the central nervous system of *Spodoptera frugiperda* after application of different concentrations of flupyradifurone (FPF) and acetylcholine (ACh) (holding potential -70mV).

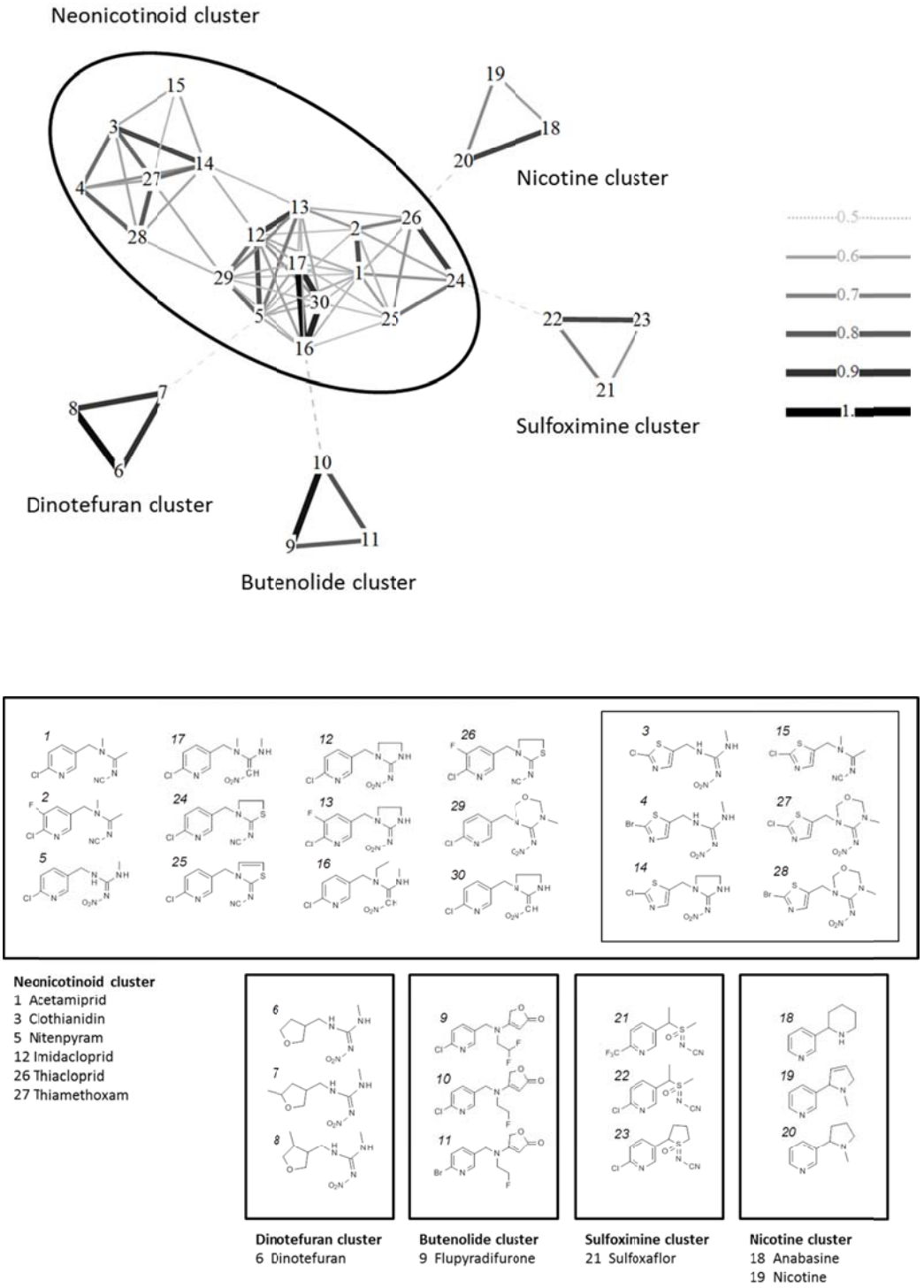


Figure 7. Chemoinformatic analysis of chemical similarity relations (Tanimoto indices) between different structural classes of nicotinic acetylcholine receptor agonists and their derivatives revealed structure-based clusters. Line thickness and grey level encode Tanimoto similarity values. Solid lines are used for similarities greater than 50%. In order to avoid a disconnected graph, the fourth highest similarity relationship for each of the small, triangular shaped clusters is given as a dashed line.

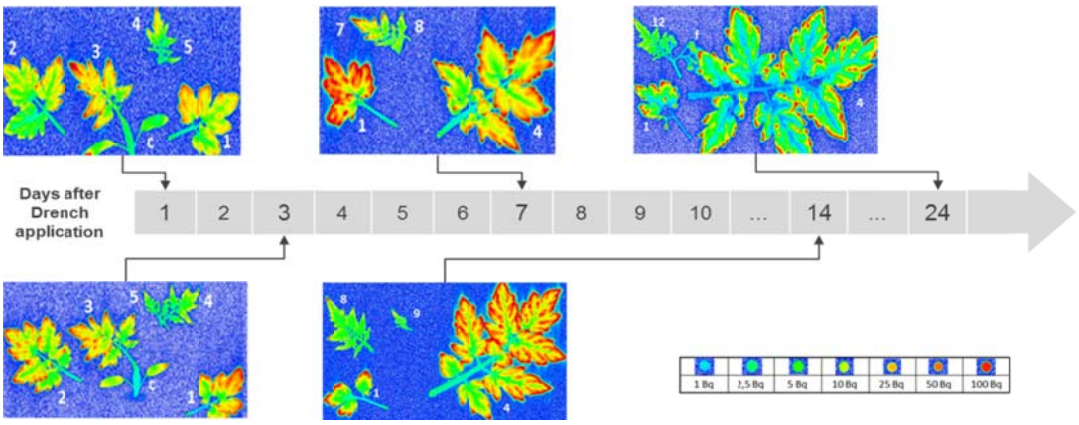


Figure 8a. Translocation of SL200 [^{14}C]flupyradifurone a.i. equivalents at different elapsed time intervals in tomato plants after root uptake via drench application. Numbers and letters indicate leaf positions: c=cotyledons, leaf 1=oldest leaf, 12=youngest leaf, f=flowers.

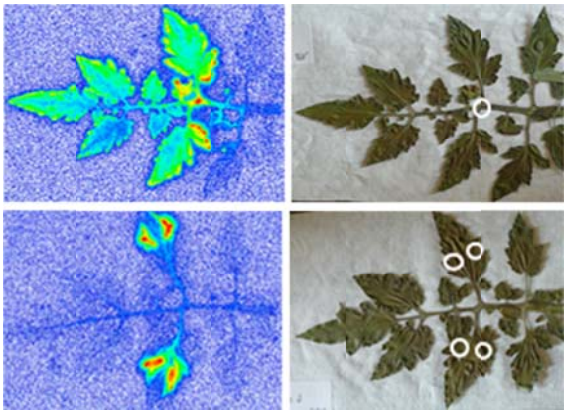


Figure 8b. Uptake and translocation of SL200 [^{14}C]flupyradifurone a.i. equivalents into tomato plants 4 days after stem and foliar application, respectively. Droplet application points are indicated by white circles.

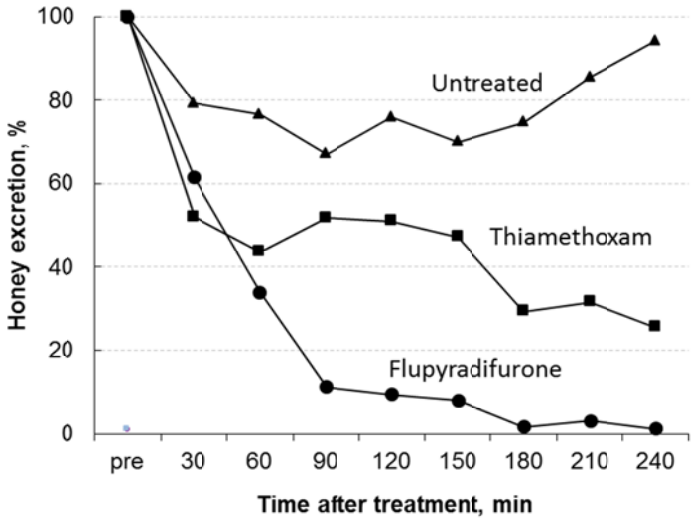


Figure 9. Kinetics of suppression of honeydew excretion in *Myzus persicae* feeding on oilseed rape and foliarly treated with flupyradifurone (Sivanto® 200SL, 75 g a.i./ha) and thiamethoxam (Actara®, 25 g a.i./ha). Control plants remain untreated. Percentage suppression of honeydew excretion is relative to honeydew excretion levels pre-application.

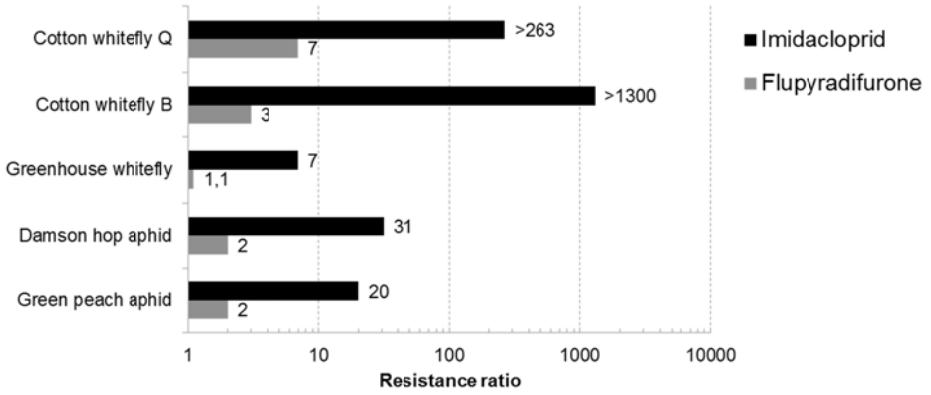


Figure 10. Comparison of resistance ratios for flupyradifurone and imidacloprid in different strains of selected sucking pest species. All data are based on leaf-dip tests using adults (72h).

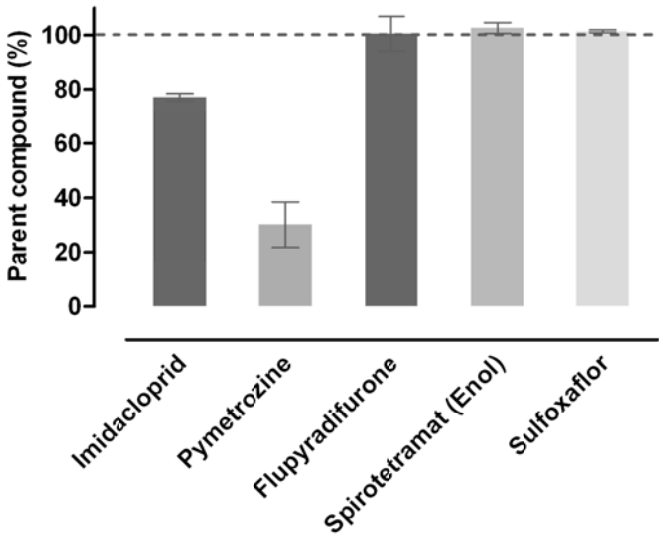


Figure 11. Detoxification of different insecticides (20 µM; 30°C; 4h; 200 µl) by CYP6CM1 of *Bemisia tabaci* functionally expressed in Sf9 cells using a baculovirus transfection system.

Table 1. Identity and physico-chemical properties of flupyradifurone

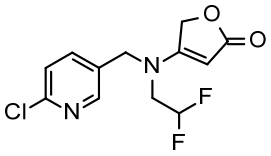
Common name (ISO)	flupyradifurone
Chemical name (IUPAC)	4-[[(6-chloropyridine-3-yl)methyl](2,2-difluoroethyl)amino]furan-2(5H)-one
Structural formula	
Empirical formula	$C_{12}H_{11}Cl\ F_2N_2O_2$
Molecular weight	288.68 g mol^{-1}
Appearance	solid powder
Colour	white to beige
Odour	weak, not characteristic
Melting point	69°C
Boiling point	no boiling point at atmospheric conditions
Vapour pressure	9.1 x 10^{-7} Pa (20°C)
Density	D=1.43 (purity 99.4%)
Solubility (at 20°C)	water 3.2 gL^{-1} (pH 4) water 3.0 gL^{-1} (pH 7)
	n-heptane 0.0005 gL^{-1}
	methanol >250 gL^{-1}
Partition coefficient	log P_{ow} = 1.2 (at 25°C and pH 7)

Table 2. Mammalian toxicological and ecotoxicological properties of technical flupyradifurone on non-target organisms

Acute oral toxicity, rat LD_{50}	>300 mg kg^{-1}, <2000 mg kg^{-1} body weight
Acute dermal toxicity, rat LD_{50}	>2000 mg kg^{-1} body weight
Acute inhalation toxicity, rat LC_{50}	>4671 mg m^{-3}
Skin irritation	Non-irritant
Eye irritation	Non-irritant
Mutagenicity	Not a mutagen
Carcinogenicity	Not a carcinogen
Reproductive & developmental toxicity	No concern
Birds (bobwhite quail) acute oral LD_{50}	232 mg kg^{-1} body weight
Fish (rainbow trout) acute LC_{50}	>74.2 mg L^{-1}
Aquatic invertebrates (*Daphnia*) acute EC_{50}	>77.6 mg L^{-1}
Aquatic plants (algae) EC_{50}	>80 mg L^{-1}
Earthworm (*Eisenia*) 14 days LC_{50}	193 mg kg^{-1} dry weight soil
Honeybee acute contact LD_{50}	>100 µg bee^{-1}
Honeybee acute oral LD_{50}	1200 ng bee^{-1}
Honeybee foliage residue	No effects at 205 g ha^{-1}
Honeybee long-term field studies at 205 g a.i./ha in oilseed rape (full bloom and bees actively foraging)	No adverse effects
Bumblebee acute contact LD_{50}	>100 µg bee^{-1}

Table 3. Selected crops and pests targeted by flupyradifurone applications

Crop	Pest	Application method
Vegetables/	Whiteflies	Foliar/drench
Potatoes	Aphids	
	Potato psyllid	
Pome fruits	Aphids	Foliar
	Scales	
	Psyllids	
	Sawfly	
Grapes	Leafhoppers	Foliar/drench
	Grapevine Mealybug	
Citrus	Asian citrus psyllid	Foliar/drench
	Citricola scale	
	Citrus thrips	
Cotton	Cotton aphid	Foliar
	Lygus bugs	
Soybean	Aphids	Seed treatment
Coffee	Coffee leafminer	Foliar/drench
Cocoa	Cocoa mirids	
Hops	Damson hop aphid	Foliar
Ornamentals	Aphids	Foliar
	Whiteflies	

Table 4. Log-dose probit-mortality data of flupyradifurone against different strains of aphid and whitefly species in leaf-dip laboratory bioassays (72h). Strains Roznava (*P. humuli*), NS (*M. persicae*), SUD-S (*B. tabaci*) and 6230 (*T. vaporariorum*) are insecticide susceptible laboratory reference strains. Strains Hallertau (*P. humuli*), F03-09 (*M. persicae*), Q- and B-type (*B. tabaci*) and NL-07 (*T. vaporariorum*) are field strains expressing moderate to high resistance to neonicotinoids.

Species / Strain	Flupyradifurone		Imidacloprid	
	LC_{50} mg L^{-1}	CL95%	LC_{50} mg L^{-1}	CL95%
Nasonovia ribisnigri	0.38	0.32-047	0.27	0.095-0.87
Phorodon humuli:				
Roznava	0.040	0.059-0.077	0.14	0.12-0.14
Hallertau	0.087	0.064-0.12	4.4	2.2-8.9
Aphis gossypii	3.2	2.8-3.6	13	10-18
Myzus persicae:				
NS	0.32	0.19-0.54	0.24	0.11-0.40
F03-09	0.64	0.18-2.5	4.7	3.8-5.9
Bemisia tabaci:				
SUD-S	5.5	0.33-4.9	0.76	0.47-1.2
Q-type (Almeria, Spain)	40	22-56	>200	nd
B-type (Unai, Brazil)	16	9.6-23	>1000	nd
Trialeurodes vaporariorum				
6230	4.8	3.8-6.7	22	5.4-38
NL-07	4.3	2.1-7.4	140	47-540

Table 5. Summary of seven lettuce field trials conducted in the EU central zone comparing flupyradifurone and commercial standards against lettuce aphids (*Nasonovia ribisnigri*)

Compound	Rate (g ha^{-1})	Efficacy (%), days after application		
		2-3	6-10	13-15
Flupyradifurone	125	86	96	83
Pymetrozine	200	74	79	57
Thiacloprid	96	84	88	72
Spirotetramat	75	65	90	98