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Flupyrimin: A Novel Insecticide Acting at the Nicotinic Acetylcholine Receptors

Yasumichi Onozaki,[†] Ryo Horikoshi,[†] Ikuya Ohno,[†] Shigeki Kitsuda,[†] Kathleen A. Durkin,[‡] Tomonori Suzuki,[§] Chiaki Asahara,[§] Natsuko Hiroki,[§] Rena Komabashiri,[§] Rikako Shimizu,[§] Shogo Furutani,[¶] Makoto Ihara,[¶] Kazuhiko Matsuda,[¶] Masaaki Mitomi,[†] Shinzo Kagabu,[∥] Katsuhito Uomoto,[†] and Motohiro Tomizawa*[,§]

[†]Agricultural and Veterinary Research Laboratories, Agricultural and Veterinary Division, Meiji Seika Pharma Co., Ltd., Yokohama, Kanagawa 222-8567, Japan

[‡]College of Chemistry, University of California, Berkeley, California 94720-1460, United States

[§]Faculty of Life Sciences, Tokyo University of Agriculture, Setagaya, Tokyo 156-8502, Japan

[¶]Faculty of Agriculture, Kindai University, Nara 631-8505, Japan

[∥]Faculty of Education, Gifu University, Gifu 501-1193, Japan

Supporting Information

ABSTRACT: A novel chemotype insecticide flupyrimin (FLP) [*N*-[(*E*)-1-(6-chloro-3-pyridinylmethyl)pyridin-2(1*H*)-ylidene]-2,2,2-trifluoroacetamide], discovered by Meiji Seika Pharma, has unique biological properties, including outstanding potency to imidacloprid (IMI)-resistant rice pests together with superior safety toward pollinators. Intriguingly, FLP acts as a nicotinic antagonist in American cockroach neurons, and [^{3}H]FLP binds to the multiple high-affinity binding components in house fly nicotinic acetylcholine (ACh) receptor (nAChR) preparation. One of the [^{3}H]FLP receptors is identical to the IMI receptor, and the alternative is IMI-insensitive subtype. Furthermore, FLP is favorably safe to rats as predicted by the very low affinity to the rat $\alpha4\beta2$ nAChR. Structure−activity relationships of FLP analogues in terms of receptor potency, featuring the pyridinylidene and trifluoroacetyl pharmacophores, were examined, thereby establishing the FLP molecular recognition at the *Aplysia californica* ACh-binding protein, a suitable structural surrogate of the insect nAChR. These FLP pharmacophores account for the excellent receptor affinity, accordingly revealing differences in its binding mechanism from IMI.

KEYWORDS: *pyridinylidene and trifluoroacetyl pharmacophores, flupyrimin, nicotinic acetylcholine receptor, nicotinic insecticide*

INTRODUCTION

The nicotinic acetylcholine (ACh) receptor (nAChR) is the prototypical ligand-gated ion channel responsible for rapid excitatory neurotransmission and therefore is a crucial target for insecticide action.[1−3] The binding site interactions of nicotinic insecticides and related analogous ligands have been defined with mollusk ACh binding protein (AChBP), which is an appropriate surrogate of the extracellular ligand-binding domain of the nAChR by chemical/structural biology approach with adequate resolution to understand the recognition properties of the drug-binding region.[4−9] The defined three-dimensional binding site structure in the nicotinic ligand-bound state subsequently facilitates the molecular design of novel insecticidal compounds with unique pharmacophores.[10−17]

Nicotine (NIC) or anabasine (Figure 1) in tobacco extract was, for centuries, the best available agent to control piercing-sucking insect pests: i.e., primary generation of nicotinic insecticides. On the other hand, the search for unusual structures and optimization successfully led to the discovery of second generation neonicotinoid insecticides represented here by imidacloprid (IMI), acetamiprid (ACE), clothianidin (CLO), and dinotefuran. Neonicotinoids are utilized throughout the world, accounting for more than one-fourth of the global insecticide market.[1−3] Currently, novel nicotinic compounds flupyradifurone (FPF), triflumezopyrim (TFM), and dicloromezotiaz, arbitrarily classified here as new generation, are introduced into the market (Figure 1).[18−21] Meanwhile, neonicotinoid-resistant insect pests are recently devastating rice fields in south, southeast, and east Asian countries.[22,23] Furthermore, safety toward nontarget and/or beneficial organisms is a key requirement to develop agrochemicals. In the aforementioned circumstances, Meiji Seika Pharma discovered a novel chemotype insecticide flupyrimin [FLP, *N*-[(*E*)-1-(6-chloro-3-pyridinylmethyl)pyridin-2(1*H*)-ylidene]-2,2,2-trifluoroacetamide], providing important biological properties featuring outstanding insecticidal potency to the resistant rice pests as well as superior safety toward beneficial organisms, including pollinators.[24]

The present investigation consists of three goals. The first aim is to introduce insecticidal properties of FLP and reveal the physiological effects on insect neurons. Second, the present report characterizes the binding interactions of FLP with the insect nAChRs in comparison with those of the other nicotinic

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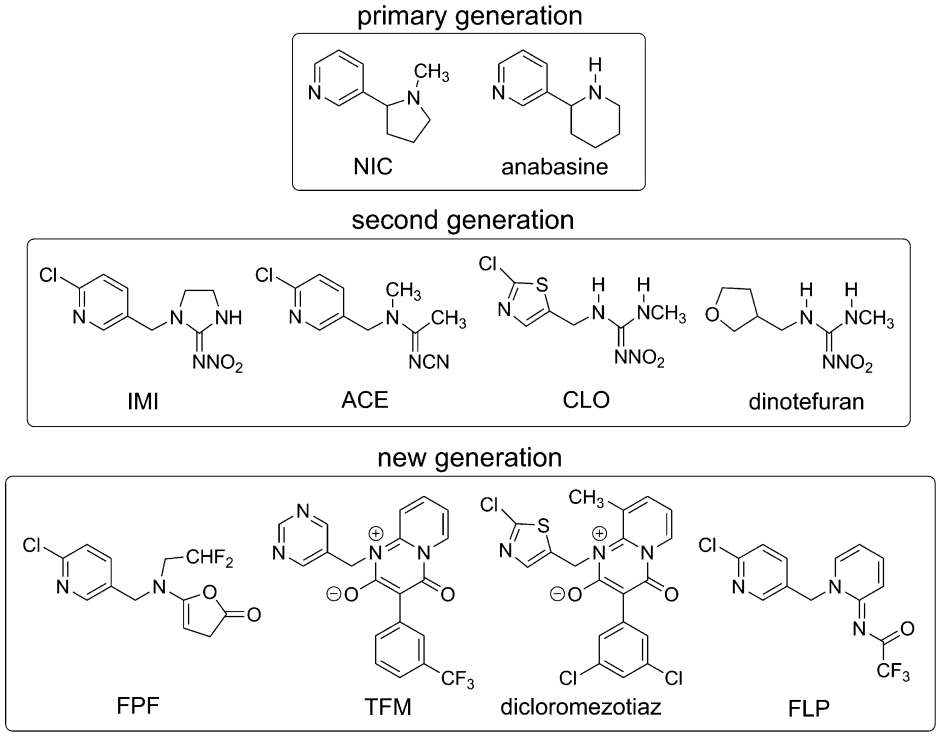

Figure 1. Chemical structures of nicotinic insecticides competing with the endogenous agonist ACh at the insect nAChRs. The nicotinic agents are categorized under the following three groups: i.e., botanical NIC and anabasine as primary generation nicotinic insecticides; synthetic neonicotinoids (represented here by IMI, ACE, CLO, and dinotefuran) as second generation, and afterward, new generation compounds (FPF, TFM, diclomezotiaz, and FLP) were discovered.

insecticides. Finally, we predict the FLP molecular recognition at the insect nAChR homologue based on structure−activity relationships (SARs) of the FLP analogues focusing on the pharmacophoric pyridinylidene and trifluoroacetyl moieties.

MATERIALS AND METHODS

Chemicals. Radiosynthesis of [^{3}H]FLP (90 Ci/mmol) was performed by IZOTOP (Budapest, Hungary), and [^{3}H]IMI (17 Ci/mmol) was available from our previous report[14] (Figure 2). (−)-[*N*-Methyl-^{3}H]NIC ([^{3}H]NIC) (80 Ci/mmol) was obtained from PerkinElmer (Boston, MA). (−)-NIC tartrate, IMI, ACE, and CLO were purchased from Wako Pure Chemical (Osaka, Japan). (±)-Epibatidine (EPI) dihydrochloride was available from Sigma (St. Louis, MO). FPF and TFM were prepared by reported methodologies.[18−20] FLP (**1**) and the analogues considered here are listed in Figure 2. Compounds **3**−**10** and **12**−**17** were available from our previous papers,[10,14,24] and compounds **11** and **19**−**21** were synthesized according to described procedures.[25−27]

*N-[(E)-1-(6-Chloro-3-pyridinylmethyl)piperidin-2(1H)-ylidene]-2,2,2-trifluoroacetamide (**2**).* To a stirred solution of 1-[(6-chloro-3-pyridinyl)methyl]piperidine-2(1*H*)-imine[28] (233 mg, 1.00 mmol) in $CHCl_3$ (15 mL) was slowly added trifluoroacetic anhydrate (441 mg, 2.10 mmol) followed by *N*,*N*-diisopropyl ethylamine (452 mg, 3.50 mmol) on an ice−water bath. The mixture was stirred overnight at the ambient temperature. Water (30 mL) was added to the reaction mixture and extracted with $CHCl_3$ (15 mL × 2); the combined organic phase was washed successively with 1% HCl aq., 10% Na_2CO_3 aq., and finally with water and dried. The solvent was removed in vacuo and the product **2** was isolated as white solid by silica gel column chromatography (hexane:EtOAc 1:1): yield, 37 mg (6%); mp 65−67 °C; NMR ($CDCl_3$), δ_H 1.80 (2H, *m*), 1.85 (2H, *m*), 3.06 (2H, *t*, *J* = 6.3 Hz), 3.44 (2H, *t*, *J* = 6.3 Hz), 4.82 (2H, *s*), 7.33 (1H, *d*, *J* = 8.0 Hz), 7.75 (1H, *dd*, *J* = 8.0 Hz, *J* = 2.3 Hz), 8.35 (1H, *d*, *J* = 2.3 Hz); δ_C 19.2, 22.2, 29.5, 48.8, 51.1, 115.5 (*q*, J_{C-F} = 288.8 Hz), 124.8, 130.2, 139.6, 149.7, 151.7, 164.7 (*d*, J_{C-F} = 35.9 Hz), 172.1; δ_F −76.1. ESI-HRMS for $C_{13}H_{13}ClF_3N_3O$: calcd (+H$^+$), 320.0777; found, 320.0774.

*N-[(E)-1-(6-Chloro-3-pyridinylmethyl)pyridin-2(1H)-ylidene]-2-iodoacetamide (**18**).* To a stirred solution of 1-[(6-chloro-3-pyridinyl)-methyl]pyridine-2(1*H*)-imine[24] (100 mg, 0.45 mmol) in CH_3CN (10 mL) was added 2-iodoacetyl chloride (102 mg, 0.50 mmol). The reaction mixture was stirred at room temperature for 2 h, and EtOAc (30 mL) was added, and then washed sequentially with 1 N HCl aq. (10 mL × 3) and 1N NaOH aq. (10 mL × 3). The organic phase was dried and evaporated in vacuo. The residue was applied onto silica gel column chromatography (EtOAc:MeOH 1:1) to obtain the product **18** as brown solid: yield, 40 mg (23%); mp 116−118 °C (decom.); NMR ($CDCl_3$), δ_H 3.88 + 4.17 (2H, 2*s*), 5.46 (2H, *s*), 6.64 (1H, *t*, *J* = 13.5 Hz), 7.31 + 7.33 (1H, 2*d*, *J* = 4.3 Hz, *J* = 4.3 Hz), 7.58 (2H, *m*), 7.81 (1H, *m*), 8.25 + 8.33 (1H, 2*d*, *J* = 8.9 Hz, *J* = 9.1 Hz), 8.46 (1H, *t*, *J* = 4.5 Hz); δ_C 47.2, 52.7, 112.5, 121.7, 124.6, 124.7, 130.1, 137.6, 139.2, 140.5, 149.4, 149.5, 151.7. ESI-HRMS for $C_{13}H_{11}ClIN_3O$: calcd (+H$^+$), 387.9713; found, 387.9716.

*N-[(E)-1-(6-Chloro-3-pyridinylmethyl)pyridin-2(1H)-ylidene]-2-benzamide (**22**).* Similar to the above (**18**), compound **22** (as yellow solid) was obtained using benzoyl chloride. Yield, 35 mg (20%); mp 115−117 °C; NMR ($CDCl_3$), δ_H 5.53 (2H, *d*, *J* = 4.1 Hz), 6.54−6.55 (1H, *m*), 7.28−7.30 (1H, *m*), 7.38−7.47 (3H, overlap *m*), 7.53−7.63 (2H, overlap *m*), 7.72 (1H, *m*), 8.16 (2H, *d*, *J* = 8.3 Hz), 8.36 (1H, *dd*, *J* = 8.3 Hz, *J* = 2.7 Hz), 8.45 (1H, broad *s*); δ_C 52.5, 111.4, 121.8, 124.8, 128.0, 129.3, 130.7, 131.2, 137.7, 138.7, 138.8, 139.7, 149.2, 151.6, 158.9, 174.4. EI-HRMS for $C_{18}H_{14}ClIN_3O$: calcd, 323.0825; found, 323.0810.

Insecticidal Activity. Insecticidal activities of test compounds against brown planthopper (*Nilaparvata lugens*), white-backed planthopper (*Sogatella furcifera*), small brown planthopper (*Laodelphax striatella*), rice green leafhopper (*Nephotettix cincticeps*), cotton aphid (*Aphis gossypii*), greenhouse whitefly (*Trialeurodes vaporariorum*), rice leaf bug (*Trigonotylus caelestialium*), western flower thrips (*Frankliniella occidentalis*), diamondback moth (*Plutella xylostella*), rice leaf beetle (*Oulema oryzae*), and house fly (*Musca*

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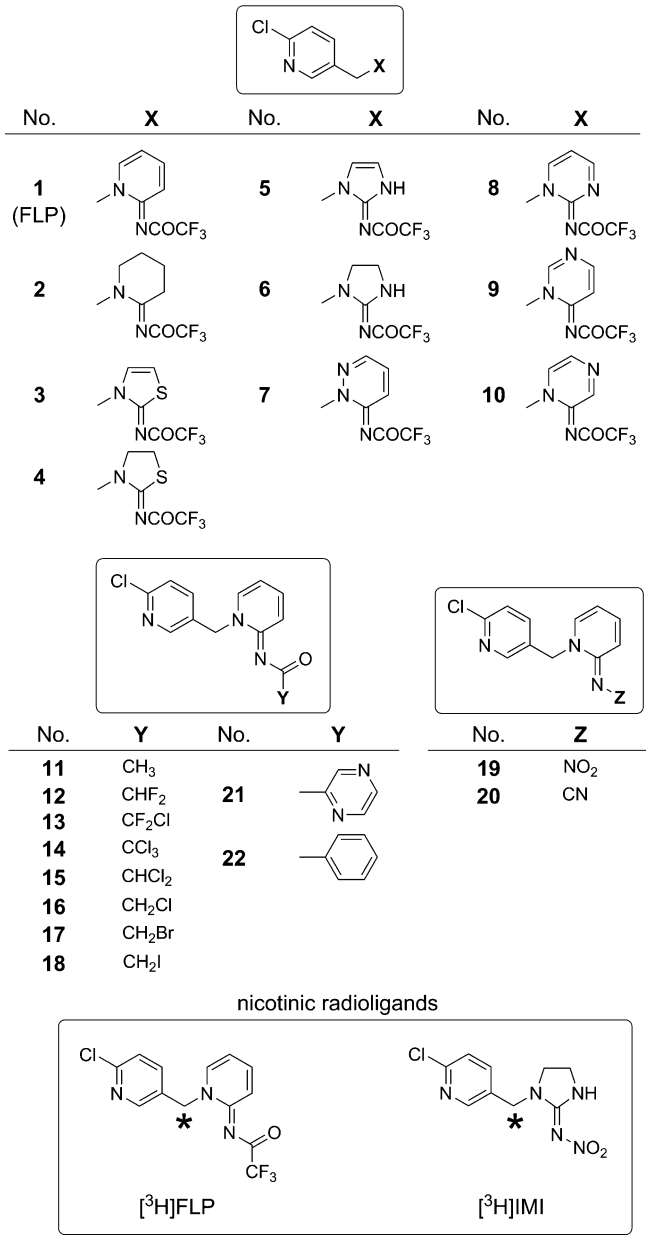

Figure 2. Chemical structures of FLP analogues and of two radiolabeled nicotinic insecticides [^{3}H]FLP and [^{3}H]IMI (asterisks indicate positions of tritium) considered in the present investigation.

demestica) were evaluated according to our laboratory procedures (see Supporting Information for details).[24] Acute toxicities toward honeybee (*Apis mellifera*), bumblebee (*Bombus terrestris*), and hornfaced bee (*Osmia cornifrons*) were examined by Eco-Science Corp. (Nagano, Japan).

Electrophysiology. The abdominal nerve cord (from the terminal abdominal ganglion to the sixth ganglion) was removed from male adult American cockroaches (*Periplaneta americana*) and cultured as described earlier.[29] The action of ACh or FLP was recorded electrophysiologically using the whole-cell patch-clamp technique.[30]

Radioligand Binding. [^{3}H]FLP or [^{3}H]IMI binding to the native *Musca* brain nAChRs was performed according to Tomizawa et al.[31] Mammalian $\alpha4\beta2$ nAChR was expressed by baculovirus system. The rat $\alpha4$ or $\beta2$ subunit cDNA from Origene Technologies (Rockville, MD) was inserted into pBacPAK8 at the *Xho*I/*Eco*RI site or *Bam*HI/*Xho*I site, respectively. Transfection of Sf21 cells, plaque selection, recombinant virus amplification, and infection and harvesting of cells were carried out according to instruction manual for the BacPAK Baculovirus Expression System (Clontech Laboratories, Mountain View, CA). Monolayer cell culture was coinoculated with the $\alpha4$ and $\beta2$ recombinant virus at each multiplicity of infection of 10. The Sf21 cells were harvested 72 h after infection. Receptor preparation and [^{3}H]NIC binding were performed as described by D'Amour and Casida.[32] IC_{50} values (molar concentrations of test chemicals necessary for 50% displacement of specific radioligand binding) and binding parameters [dissociation constant (K_D); maximal binding capacity (B_{max}); Hill coefficient (n_H)] were calculated by SigmaPlot 13 software (SYSTAT Software, San Jose, CA).

Calculations. Docking and molecular dynamics (MD) simulations of FLP were performed with the *Aplysia californica* AChBP structure in its IMI-bound state (PDB code 3C79)[7] as described in our previous papers.[11,12,17] A single water molecule in the receptor (which is well-known to form bridging H-bonds between pyridine nitrogen of IMI and loop E amino acids) was included. The docking was made with a pair of (+)- and (−)-face subunit chains. Then, AChBP-FLP was subject to MD simulations such that the active site region can fully explore conformational space. This required backbone constraints due to the structural limitations of a pair of (+)- and (−)-chains model rather than a full pentameric system. Subsequently, a sample frame from the MD simulation was selected for detailed density function theory (DFT) calculations according to Cerón-Carraco et al.[33] The relevant amino acids and a single water molecule within 4 Å of the docked FLP were individually calculated for an interaction energy at a higher DFT level [M06-2X/CC-PVTZ(-F)+ with counterpoise correction].

RESULTS

Biological Activities. Insecticidal activities of FLP, IMI, and other commercial standard insecticides against diverse target pests are given in Table 1. FLP showed higher activities against *Nilaparvata*, *Sogatella*, *Plutella*, *Oulema*, and *Musca* than IMI and comparable activities to those of IMI against *Laodelphax*, *Nephotettix*, *Trigonotylus*, and *Frankliniella* (by foliar or topical application), although IMI and dinotefuran were more potent than FLP toward *Aphis and Trialeurodes*. FLP exhibited higher efficacy against *Sogatella* than sulfoxaflor, similar activity toward *Nilaparbata* to fipronil and *Musca* to

Table 1. Insecticidal Activities of FLP against Various Insect Species Compared with Those of IMI and Other Standard Insecticides

		LC_{90} or LD_{90}[b]		
target pests	stage (application)[a]	FLP	IMI	other standard[c]
N. lugens[d]	2nd larva (f)	4.5	46	5.3 (F)
S. furcifera[d]	2nd larva (f)	1.2	9.1	4.5 (SF)
L. striatella[d]	2nd larva (f)	1.1	1.2	
N. cincticeps	2nd larva (f)	0.2	0.2	
A. gossypii	1st larva (f)	0.3	0.04	
T. vaporariorum	adult (f)	>100	1.0	
T. caelestialium	2nd larva (f)	0.6	1.0	0.1 (D)
F. occidentalis	1st larva (f)	20	20	0.1 (E)
P. xylostella	2nd larva (f)	0.9	41	0.01 (SP)
O. oryzae[d]	adult (t)	0.4	1.5	
M. domestica	adult (t)	0.07	3.0	0.08 (P)

[a]Applied via foliar (f) or topical (t). [b]Unit for foliar (f) or topical (t) given in ppm or μg/female, respectively. [c]Other standard insecticides used are fipronil (F), sulfoxaflor (SF), dinotefuran (D), emamectin benzoate (E), spinosad (SP), and permethrin (P). [d]*Nilaparvata*, *Sogatella*, *Laodelphax*, and *Oulema* collected in Kumamoto (2010), Odawara (2011), Odawara (2001), and Gotemba (2012), respectively, in Japan.

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permethrin. FLP had lower potency against *Trigonotylus*, *Franklinitella*, and *Plutella* than dinotefuran, emamectin, and spinosad. Notably, FLP exhibited a 29- or 41-fold higher activity compared to those of IMI and 5- to 7-fold higher than fipronil against other field populations of *Nilaparvata* [collected in Kagoshima (2014 and 2015), Japan] (by foliar application) (Table 2). Furthermore, FLP showed efficacies against

Table 2. Insecticidal Potencies of FLP in Comparison with Those of IMI and Fipronil against Field Populations of *Nilaparvata* and *Laodelphax* Collected in Japan

	LC_{90}, ppm (foliar application)		
population	FLP	IMI	fipronil
	Nilaparvata		
Kumamoto 2010	4.5	46	5.3
Kagoshima 2014	3.5	100	19
Kagoshima 2015	3.0	124	20
	LD_{90}, μg/seedling (soil drench application)		
	FLP	IMI	fipronil
	Nilaparvata		
Kumamoto 2010	26	117	46
Kagoshima 2014	45	470	98
Kagoshima 2015	40	510	36
	Laodelphax		
Odawara 2001	34	169	6
Oita 2013	9	283	>200
Odawara 2016	40	93	>200
Ibaraki 2016	21	197	313

planthoppers even by soil drenching application more stable than those of IMI and fipronil. FLP had a potency 5- or 13-fold higher compared to that of IMI and similar to that of fipronil against the same field populations of *Nilaparvata*. Against *Laodelphax* [collected in Odawara (2001 and 2016), Oita (2013), and Ibaraki (2016), Japan], FLP was 2- to 31-fold more active than those of IMI and over 5- to 22-fold higher against the populations collected in the past few years than those of fipronil, whereas fipronil had high activity only against the Odawara 2001 population. In the ecotoxicological aspect, IMI was highly toxic to *Apis* and *Bombus* via oral and/or topical route administration,[34−36] whereas FLP was harmless to the pollinators (Table 3). FLP also showed low toxicity against *Osmia*.

Electrophysiological Response. Inward current after application of 10 μM ACh was clearly evoked in the dissociated terminal abdominal ganglion neurons of *Periplaneta* (Figure 3). In contrast, 10 μM FLP [and even at 100 μM (data not shown)] produced no inward current, and subsequent washing with saline for 2 min gave a complete recovery in response to 10 μM ACh. Surprisingly, a robust blocking activity was observed when 1 nM FLP was applied for 1 min prior to 10 μM ACh treatment. Moreover, the action was not recovered even by a 6 min successive wash with saline. IC_{50} value of FLP, for the inhibitory effect on the ACh-induced current amplitude, was 0.32 nM (Figure 3).

Table 3. Comparative Acute Toxicity between FLP and IMI to Adult Pollinators

			LD_{50} or LC_{50}	
pollinator	application	unit	FLP	IMI[c]
A. mellifera[a]	oral	μg/bee	>53	0.04
	topical	μg/bee	>100	0.02
B. terrestris[a]	topical	μg/bee	>100	0.02
O. cornifrons[b]	foliar	ppm	>100	unavailable

[a]Examined according to guideline from the Japanese Ministry of Agriculture, Forestry, and Fisheries; lethality data was obtained four days after treatment. [b]Diluted solution of suspension concentrate formulation (10% FLP) was applied to strawberry pots, and adults were then released (lethality was observed three days after treatment). [c]Data from refs 34−36.

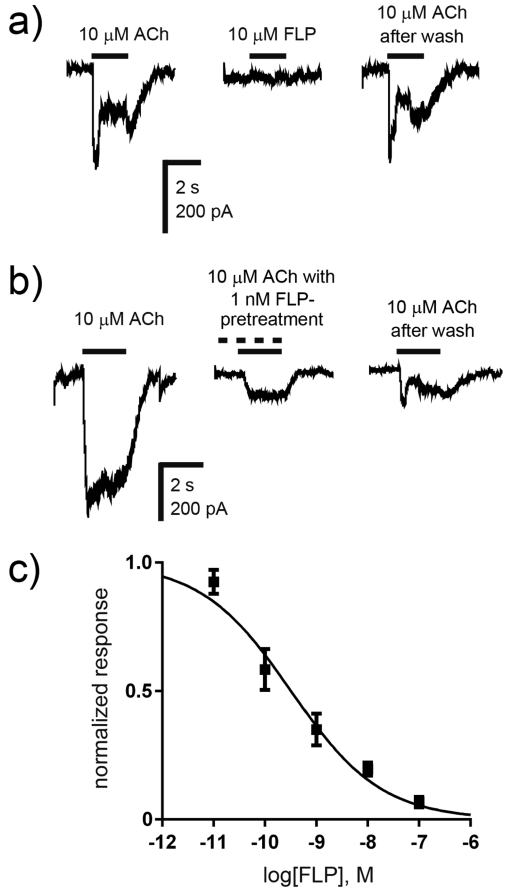

Figure 3. Typical electrophysiology responses (using whole-cell patch-clamp technique) produced by ACh and FLP (treated for 2 s) in the dissociated terminal ganglion neurons of *P. americana* (panel a). Responses to 10 μM ACh, 10 μM FLP, and then 10 μM ACh after a 2 min wash with saline (left, middle, and right, respectively). In addition, no inward current was observed when 100 μM FLP was applied (data not shown). Blocking effect of FLP on the ACh-evoked response (panel b). Responses to 10 μM ACh alone, 10 μM ACh with 1 nM FLP pretreatment (for 1 min prior to ACh application), and then 10 μM ACh after a 6 min wash with saline (left, middle, and right, respectively). Concentration−response curves for the blocking action of FLP [IC_{50} 0.32 ± 0.13 nM (n = 3)] on the ACh-induced current amplitude of *Periplaneta* thoracic neurons (panel c).

SARs. The present SAR study in terms of binding potency to the *Musca* nAChR and insecticidal activity against *Laodelphax* focused on the functions of diene π-electrons on the heterocyclic ring (compounds **1**−**10**) and of halogen atom(s) (compounds **1** and **11**−**20**) (Table 4). Relative to nAChR potency (indicated by IC_{50} value), FLP (**1**) with diene π-electrons on the ring showed binding affinity greater than that of the corresponding saturated analogue (**2**). Similarly,

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Table 4. SARs of FLP Analogues in Terms of Their Potencies as Displacers of [^{3}H]FLP (1 nM) Binding to the *Musca* nAChR and Their Insecticidal Activities against the *Laodelphax*

no.	binding potency IC_{50}, nM ± SD (n = 3)	insecticidal activity[a] LD_{90}, μg/seedling
1 (FLP)	4.1 ± 0.1	0.03
2	92 ± 10	>20
3	6.4 ± 0.9	>20
4	48 ± 2	>20
5	50 ± 2	>20
6	80 ± 1	>20
7	240 ± 30	>20
8	120 ± 19	>20
9	170 ± 17	20
10	820 ± 120	>20
11	980 ± 70	>20
12	6.6 ± 0.5	0.25
13	5.2 ± 1.8	0.25
14	16 ± 2	>20
15	6.0 ± 1.0	>20
16	14 ± 1	>20
17	35 ± 11	5
18	7.1 ± 0.3	>20
19[b]	2.1 ± 0.1	0.1
20	1.1 ± 0.1	20

[a]Examined by root-soaking application method (collected in Odawara, 2001). [b]Potency of **19** with a nitroimine moiety against the field *Nilaparvata* (Kumamoto, 2010) diminished, while trifluoroacetyl FLP retained the activity (data not shown).

unsaturated thiazoline (**3**) or imidazoline (**5**) analogues had an enhanced affinity relative to that of the equivalent saturated thiazolidine (**4**) or imidazolidine (**6**) analogues, respectively. Compounds with an extra nitrogen atom on the dihydropyridine ring: i.e., dihydropyridazine (**7**); dihydro-1,3-pyrimidine (**8**); dihydro-1,5-pyrimidine (**9**); and dihydropyrazine (**10**), which have the decreased ring electron density, were essentially inactive. *N*-Acetyl analogue (CH_3) (**11**) had a 240-fold lower affinity compared to that of the corresponding *N*-trifluoroacetyl (CF_3) FLP (**1**). Interestingly, the other *N*-haloacetyl analogues providing CHF_2, CF_2Cl, CCl_3, $CHCl_2$, CH_2Cl, CH_2Br, and CH_2I substituents (**12**–**18**) retained the affinity of FLP (**1**). *N*-Nitro (**19**) and *N*-cyano (**20**) analogues were also very potent as *Musca* nAChR ligands. Regarding insecticidal activity against the *Laodelphax* (indicated by dose giving 90% lethality, LD_{90}), compounds with high binding affinity to the insect nAChR were clearly insecticidal (**1**, **12**, **13**, **17**, and **19**), although some other high affinity compounds unfortunately gave low insecticidal activity (**3**, **14**, **15**, **16**, **18**, and **20**). Compounds with low receptor potency (**2** and **4**-**11**) were not insecticidal as predicted. Interestingly, **19** was insecticidal but **20** was somehow less toxic.

Binding properties. Saturation isotherm of [^{3}H]FLP binding to the *Musca* receptor was compared with that of [^{3}H]IMI (Figure 4). For [^{3}H]FLP, multiple binding sites were revealed: i.e., K_D values of 0.27 and 6.5 nM; B_{max} values 170 and 210 fmol/mg protein; n_H 0.66. In contrast, a single binding component was detected for [^{3}H]IMI: i.e., K_D 2.2 nM; B_{max} 220 fmol/mg protein; n_H 0.98.

Actions of diverse nicotinic agents at the [^{3}H]FLP receptor were considered in comparison with those at the [^{3}H]IMI receptor. IC_{50} values for all agents listed in Table 5 showed

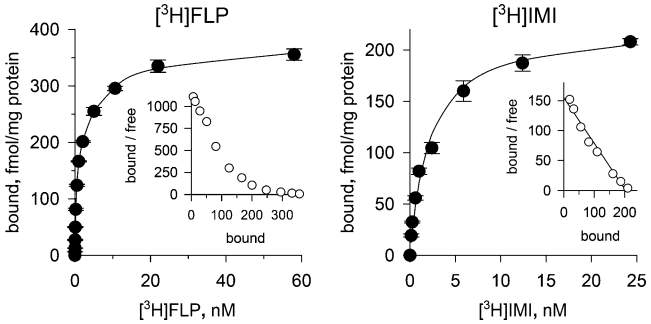

Figure 4. Saturation isotherm and Scatchard plot (insert) for specific [^{3}H]FLP (left) or [^{3}H]IMI (right) binding to the native *M. domestica* brain nAChRs. The saturation curve has error bar for each data point (±SD, n = 3), and the Scatchard plot is displayed as representative data from three individual experiments. Specific binding is the difference in radioactivity in the absence and presence of 20 μM FLP for [^{3}H]FLP and 20 μM IMI for [^{3}H]IMI. Nonspecific bound was less than 10% of total bound for both radioligands, although nonspecific bound at higher [^{3}H]FLP concentrations (22 and 58 nM) gave 15 and 27%, respectively, of total bound. For [^{3}H]FLP, multiple binding components were detected: i.e., the binding parameters (±SD, n = 3) are K_D values 0.27 ± 0.04 and 6.5 ± 1.9 nM; B_{max} values 170 ± 19 and 210 ± 10 fmol/mg protein; n_H 0.66 ± 0.03. In contrast, a single binding site is revealed for [^{3}H]IMI: i.e., the binding parameters (±SD, n = 3) are K_D 2.2 ± 0.2 nM; B_{max} 220 ± 4 fmol/mg protein; n_H 0.98 ± 0.04.

Table 5. Potencies of Diverse Nicotinic Agents as Displacers of [^{3}H]FLP (1 nM) or [^{3}H]IMI (5 nM) Binding to the *Musca* nAChRs

ligand	[^{3}H]FLP binding		[^{3}H]IMI binding	
	IC_{50} (nM ± SD, n = 3)	n_H	IC_{50} (nM ± SD, n = 3)	n_H
	FLP analogues			
1 (FLP)	4.1 ± 0.1	0.99	1.0 ± 0.2	0.96
11	980 ± 70	1.1	1000 ± 330	0.98
21	1.6 ± 0.3	0.98	1.6 ± 0.5	0.96
22	82 ± 5	0.92	77 ± 12	0.92
	others			
NIC	22 000 ± 4,100	0.75	6200 ± 440	0.94
IMI	28 ± 4	0.78	8.4 ± 2.6	0.99
ACE	39 ± 3	0.78	16 ± 3	0.98
CLO	23 ± 2	0.60	8.4 ± 0.6	1.0
FPF	33 ± 9	0.53	19 ± 12	0.93
TFM	46 ± 3	0.65	37 ± 10	0.64

fundamentally the same relationship in both assay systems. As an index for binding property, n_Hs of FLP analogues with varied *N*-acyl moieties (**1**, **11**, **21**, and **22**) assayed by [^{3}H]FLP were consistent to those examined by [^{3}H]IMI: i.e., n_H around 1.0, indicating no cooperativity. Whereas, other agents (NIC, IMI, ACE, CLO, and FPF) assayed by [^{3}H]FLP gave n_H 0.53–0.78, suggesting negative cooperativity. The five ligands had n_H around 1.0 in [^{3}H]IMI binding. Noteworthy, TFM somehow showed negative cooperativity (n_H 0.64–0.65) in both assay systems.

Simultaneous dual radioligand binding experiments (Table 6) were conducted in which the simultaneous binding or direct competition of two radioligands was examined in the same *Musca* brain receptor preparation. This method can provide direct evidence that two radioligands bind either to distinct sites (or receptor subtypes) or to the identical domain (or closely coupled sites).[37−39] On the basis of saturation

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Table 6. Simultaneous Dual Radioligand Bindings in *Musca* nAChRs

radioligand	assay level (nM)[a]	specific binding (dpm/mg protein)[b]	dual binding (% of expected)[c]
[^{3}H]FLP	1	50 000 ± 2400	79.4
[^{3}H]IMI	20	13 000 ± 450	20.6
[^{3}H]FLP + [^{3}H]IMI	1 + 20	28 100 ± 750	44.6
[^{3}H]FLP	20	96 000 ± 3100	88.6
[^{3}H]IMI	20	12 400 ± 610	11.4
[^{3}H]FLP + [^{3}H]IMI	20 + 20	94 800 ± 2500	87.5

[a]Concentrations are at least 3.1-fold greater than that of the K_D values of both radioligands, i.e., at or near saturation. [b]Mean ± SD (n = 3−6). Specific binding is the difference in radioactivity in the absence and presence of 10 μM FLP for [^{3}H]FLP and 10 μM IMI for [^{3}H]IMI or an appropriate combination of two unlabeled ligands for simultaneous dual radioligand bindings. [c]Expected is the theoretical total of 100% defined as the sum of the dpm/mg protein for each individual radioligand.

experiments (as given in Figure 4), concentration of [^{3}H]FLP (1 or 20 nM) is at or near saturation for the high affinity site (K_D 0.27 nM) or the low affinity site (K_D 6.5 nM), respectively. Also, 20 nM [^{3}H]IMI is a 9.1-fold higher than the K_D value (2.2 nM). [^{3}H]FLP (1 nM) and [^{3}H]IMI (20 nM) bindings were found to be 79.4 and 20.6%, respectively, of that for the theoretical total of 100%. The two radioligands together conferred only 44.6% of the expected value. Therefore, there is clear interference by one radioligand in the binding of the other one. [^{3}H]FLP and [^{3}H]IMI (both at 20 nM) bindings gave 88.6 and 11.4%, respectively, of that for the theoretical total of 100%. Interestingly, simultaneous use of the both radioligands at the above condition recovered 87.5% of the expected total value of 100%: i.e., the two radioligands mostly bind to distinct sites. Accordingly, high affinity [^{3}H]FLP binding site (saturable by 1 nM) overlaps with that for [^{3}H]IMI, and they compete with each other at the same binding place. In contrast, low affinity [^{3}H]FLP binding component (saturable by 20 nM) is an independent receptor from that of the aforementioned one. Further, IC_{50} value of α-bungarotoxin (α-BGT) as a displacer of [^{3}H]FLP binding to the *Musca* receptor preparation was 860 nM or of FLP in [^{125}I]α-BGT binding was 300 nM, suggesting that FLP is essentially insensitive to the α-BGT receptor.

Remarkably, [^{3}H]FLP (100 nM) gave no specific binding (2.4%) to the recombinant rat $\alpha4\beta2$ nAChR, whereas [^{3}H]NIC (5 nM) specifically bound (97%) (Table 7). Furthermore, FLP had quite low binding affinity (1100 nM) to the $\alpha4\beta2$ receptor (assayed by [^{3}H]NIC as reporter molecule) compared with those of two representative nicotinic alkaloids (−)-NIC and (±)-EPI (2.8 and 0.09 nM, respectively) (Table 8). FLP was not highly toxic to rats via oral and dermal routes (Table 8 footnote).

Table 7. Radioligand Binding Profiles of Recombinant Rat $\alpha4\beta2$ nAChR

		specific binding[a]	
radioligand	assay level (nM)	fmol/mg protein[b]	%[c]
[^{3}H]FLP	100	69 ± 33	2
[^{3}H]NIC	5	1300 ± 50	97

[a]Specific binding was defined as the difference in radioactivity in the absence and the presence of 100 μM FLP or 10 μM (−)-NIC for [^{3}H]FLP or [^{3}H]NIC, respectively. [b]Mean ± SD (n = 4−6). [c]Specific binding relative to total binding.

Table 8. Potency of Nicotinic Ligands as Displacers of [^{3}H]NIC (5 nM) Binding to the Recombinant Rat $\alpha4\beta2$ nAChR

ligand[a]	IC_{50}, nM ± SD (n = 3)
FLP[b]	1100 ± 50
(−)-NIC	2.8 ± 0.5
(±)-EPI	0.09 ± 0.02

[a]As with FLP, FPF and TFM were also inactive (IC_{50}s 2700 ± 590 and 2400 ± 210 nM, respectively). [b]Relative to toxicity to rats, LD_{50} of FLP via oral or dermal route was 300 < LD_{50} ≤ 2000 or >2000 mg/kg, respectively.

Binding Site Interactions. In silico FLP binding site interactions were established with *Aplysia* AChBP (as a suitable structural surrogate for the insect nAChR extracellular ligand-binding domain) based on the present SAR findings and previous chemical and structural biology investigations (Figure 5).[4−8] The FLP chloropyridinyl chlorine contacts with loop E A107 and M116, and a water-mediated H-bonding is formed among the chloropyridine nitrogen, I106, and I118. The trifluoroacetyl three fluorine atoms contact with C226−227, S189, Y55, Q57, and I118 via H-bonding or hydrophobic interaction. The = NC(O) oxygen contacts Y55 OH or S189/C190 backbone NH. The two π-electron systems on the FLP pyridinylidene make π-stacks with W147 and Y188. The pairwise interaction energies between FLP and each of relevant amino acids configuring the FLP-binding pocket in *Aplysia* AChBP were calculated at higher DFT level (Table 9). Loop B W174 and loop C C190−191 show strong interactions. Loop E I118, loop C Y188, and a water are also substantial. Loop D Q57 or Y55 makes a certain contribution, while loop C S189 or loop E M116 takes a plausible interaction.

DISCUSSION

Exploration of novel chemotype nicotinic insecticide, undergoing atypical interaction with the target and preventing from detoxification, may confer unique biological properties such as substantial potency to the neonicotinoid-resistant pests[22,23,40−43] and favorable safety toward pollinators. The present report introduces a new agent FLP, discovered by Meiji Seika Pharma, which has excellent insecticidal potency particularly against the rice insect pests, including the IMI-insensitive populations. Resistance to IMI and other neonicotinoids in major target rice pests *Nilaparvata* and *Laodelphax* are primarily attributable to enhanced detoxification by cytochrome P450(s).[22,23,40,41] Surprisingly, an IMI-resistant peach-potato aphid (*Myzus persicae*) (highly resistant FRC strain) with a R81T mutation (on *Myzus* β1 loop D) conserves a single high affinity [^{3}H]IMI binding site (K_D 4 nM and B_{max} 2000 fmol/mg protein).[43] It should be underscored that FLP is much less toxic to bees compared with IMI and other neonicotinoids in acute toxicity evaluation.[44] Further, FLP shows a favorable mammalian safety as predicted, in part, by the low affinity to the recombinant rat $\alpha4\beta2$ receptor, a major nAChR subtype expressed in the vertebrate brain.

Regarding binding properties, [^{3}H]FLP binds to the multiple receptor subtypes with comparable B_{max} values in *Musca*. Interestingly, one of these is conserved as the [^{3}H]IMI-sensitive subtype. The alternative [^{3}H]FLP site is possibly an extra

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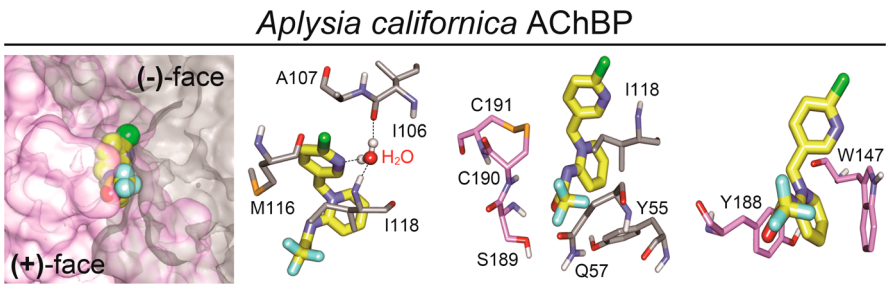

Figure 5. Structural model for FLP binding site interactions with the sea slug (*A. californica*) AChBP, a suitable structural surrogate for the insect nAChR ligand-binding domain.[1,2,5−7] FLP is embedded in the binding pocket localized at an interface between two subunits displayed in surface representation [pink and silver for AChBP (+)- and (−)-face subunits, respectively] (left). The FLP binding site interactions are exhibited in three viewpoints: i.e., chloropyridine moiety and loop E region with a water molecule (second from left); trifluoroacetyl pharmacophore and diverse amino acids from loops C, D, and E (second from right); diene π-electrons of the ring and aromatic amino acids on loops B and C (right). Relevant amino acids in pink or silver are from (+)- or (−)-face subunit, respectively.

Table 9. Interaction Energies of FLP with Key Amino Acids Configuring the Binding Cavity

Aplysia AChBP amino acid	interaction energy (kcal/mol)
W147	−13.7
Y188	−5.39
S189	−1.78
C190−191	−7.05
Y55	−2.26
Q57	−2.98
I118	−5.63
M116	−1.72
water	−4.14

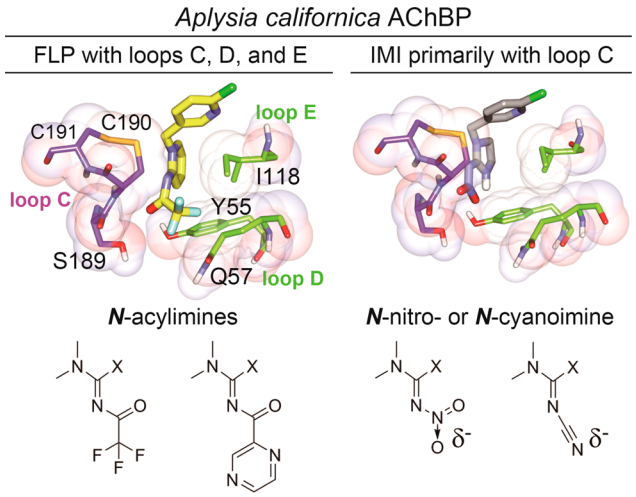

Figure 6. Schematic representation of the unique binding interaction of FLP C(O)CF_3 pharmacophore with the *Aplysia* AChBP loops C, D, and E amino acids (upper left), thereby emphasizing an obvious contrast to that of IMI NO_2 tip oxygen with the loop C amino acids (PDB 3C79)[7] (upper right). *Aplysia* AChBP is a suitable structural surrogate of the insect nAChR ligand-binding domain.[1,2,5,6] *N*-Acylimine pharmacophore (lower left) docks, in an adequate depth, with the interfacial niche between loops C, D, and E, whereas *N*-nitro- or *N*-cyanoimine electronegative tip (lower right) primarily interacts with the loop C region.[5,7,11,17]

subtype (IMI-insensitive). Thus, FLP may act at diverse insect nAChR subtypes. NIC, three neonicotinoids (IMI, ACE, and CLO), and two new insecticides FPF and TFM act on the high affinity [^{3}H]FLP site in a different way from that of FLP analogues with extended *N*-acylimine pharmacophore. In contrast, these six nicotinic agents and the FLP analogues competitively act at the [^{3}H]IMI site. These observations suggest that a binding subsite or niche embracing the *N*-acylimine pharmacophore of FLP analogues is inconsistent with that for neonicotinoid *N*-nitro/cyanoimine moiety, yet a tiny part of the whole *N*-acyl niche serves as the interaction point with the *N*-nitro/cyano tip. FPF presumably shares the same cavity (or closely coupled subsites) for neonicotinoids. On the other hand, TFM may interact in a distinct way from that of neonicotinoid or FLP with *Musca* nAChRs, although TFM binds to the *Myzus* [^{3}H]IMI site in a competitive manner.[19] FPF shows agonistic action in fall armyworm *Spodoptera frugiperda* neurons,[18] whereas TFM acts as a nicotinic antagonist in *Periplaneta*.[19] In the present investigation, FLP also exhibits strong antagonist activity. However, CLO elicits agonist response in the *Periplaneta* neurons.[45] Therefore, FLP is undoubtedly distinct in its physiological response from FPF and neonicotinoids.

IMI and FLP are similar in providing a common 6-chloropyridin-3-ylmethyl moiety, whereas they are crucially different in their pharmacophoric systems. The neonicotinoid is coplanar between the guanidine or amidine plane and the nitro or cyano substituent, thereby yielding electronic conjugation to facilitate partial negative charge (δ^-) flow toward the tip oxygen or nitrogen atom.[10,46] The electronegatively charged oxygen or nitrogen forms H-bonding primarily to loop C amino acids (Figure 6).[5−7,33] Thus, the conjugated electronic system serves as a critical neonicotinoid pharmacophore. In fact, nitro/cyanoimine neonicotinoid shows high affinity to the hybrid nAChR(s) consisting of insect α (*Myzus* α2 or *Drosophila* α2) and mammalian β (rat β2) subunits.[38] Once *Drosophila* α2 subunit of a hybrid receptor (*Drosophila* α2/chicken β2) is replaced by chicken α4 subunit, response to IMI is completely wiped out.[47] Chimera hybrid receptor configured by insect α and vertebrate β2 subunits, wherein insect loop D sequence is inserted, modestly enhances IMI-elicited agonist responses (EC_{50}s a few fold better than that of the wild type).[47,48] In sharp contrast, FLP trifluoroacetyl fluorine atoms variously interact with loops C, D, and E amino acids via H-bonding(s) and/or van der Waals contact(s). Thus, the long extended FLP trifluoroacetyl pharmacophore is anchored, in an adequate depth, to the interfacial cavity consisting of loops C, D, and E. Correspondingly, analogous *N*-acylimine (e.g., compound **21** with a pyrazine ring) type pharmacophore is nestled in the aforementioned niche.[11] Furthermore, diene π-electrons on the heterocyclic ring make π-stacking interactions with aromatic

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amino acids from loops B and C, conferring a sufficient stabilization energy.[49] Therefore, the above two unique FLP pharmacophores play individual and decisive role(s) on the molecular recognition at the binding surface. Further SAR investigations of FLP analogues based on electrophysiology response and radioligand binding would be warranted to fully explain FLP mode of insecticidal action.

In summary, FLP, as a new chemotype nicotinic insecticide discovered by Meiji Seika Pharma, exhibits remarkable biological properties featuring outstanding potency to neonicotinoid-insensitive rice insect pests and superior safety toward pollinators. Intriguingly, FLP acts on the insect nAChRs as an antagonist via a recognition manner different from those of the other nicotinic insecticides.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b02924.

Details on insecticidal activity evaluation: i.e., the origin and the stage of insect pests examined and data observation point after treatment and assay methodologies (against *Laodelphax* by root dipping and against *Nilaparvata* and *Laodelphax* by foliar application and soil drenching) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: mt204882@nodai.ac.jp; Phone +81-3-5477-2529; Fax +81-3-5477-2655.

ORCID

Motohiro Tomizawa: 0000-0002-5579-4114

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

ACh, acetylcholine; AChBP, ACh binding protein; ACE, acetamiprid; α-BGT, α-bungarotoxin; CLO, clothianidin; DFT, density function theory; FLP, flupyrimin; FPF, flupyradifurone; IMI, imidacloprid; MD, molecular dynamics; NIC, nicotine; nAChR, nicotinic ACh receptor; SAR, structure−activity relationship; TFM, triflumezopyrim

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Supporting Information for:

Flupyrimin: A novel insecticide acting at the nicotinic acetylcholine receptors

Yasumichi Onozaki,[†] Ryo Horikoshi,[†] Ikuya Ohno,[†] Shigeki Kitsuda,[†] Kathleen A. Durkin,[‡] Tomonori Suzuki,[§] Chiaki Asahara,[§] Natsuko Hiroki,[§] Rena Komabashiri,[§] Rikako Shimizu,[§] Shogo Furutani,[¶] Makoto Ihara,[¶] Kazuhiko Matsuda,[¶] Masaaki Mitomi,[†] Shinzo Kagabu,[∥] Katsuhito Uomoto,[†] and Motohiro Tomizawa[§,*]

[†]Agricultural and Veterinary Research Labs, Agricultural and Veterinary Division, Meiji Seika Pharma Co., Ltd., Yokohama, Kanagawa 222-8567, Japan

[‡]College of Chemistry, University of California, Berkeley, California 94720-1460, United States

[§]Faculty of Life Sciences, Tokyo University of Agriculture, Setagaya, Tokyo 156-8502, Japan

[¶]Faculty of Agriculture, Kindai University, Nara 631-8505, Japan

[∥]Faculty of Education, Gifu University, Gifu 501-1193, Japan

Supporting Table 1. Summary for the origin and the stage of insect pests considered in this paper and data observation point after treatment

pest	origin (Japan)	method	stage	observation point (day after treatment)
Nilaparvata lugens	field populations: Kumamoto (2010), Kagoshima (2014 and 2015)	foliar	2nd instar	7
Sogatella furcifera	Odawara (2011)	foliar	2nd instar	7
Laodelphax striatella	Odawara (2001)	foliar	2nd instar	7
Nephotettix cincticeps	susceptible*	foliar	2nd instar	7
Aphis gossypii	susceptible*	foliar	1st instar	3
Trialeurodes vaporariorum	Odawara (2002)	foliar	adult	3
Trigonotylus caelestialium	Odawara (1995)	foliar	1st instar	3
Frankliniella occidentalis	susceptible*	foliar	1st instar	3
Plutella xylostella	Yokohama (1991)	foliar	2nd instar	3
Oulema oryzae	Gotemba (2012)	topical	adult	2
Musca domestica	Yumenoshima 3rd	topical	adult	2
Nilaparvata lugens	field populations: Kumamoto (2010), Kagoshima (2014 and 2015)	soil drenching	2nd instar	7
Laodelphax striatella	field populations: Odawara (2001 and 2016), Oita (2013), Ibaraki (2016)	soil drenching	2nd instar	7

*Purchased from Sumika Technoservice Corp. (Takarazuka, Japan).

Methodology for insecticidal activity evaluation

Laodelphax by root dipping

The *Laodelphax*, employed for the present SAR study, was collected in Odawara, Japan in 2001 and reared in our laboratory without insecticide selection. Wheat seedlings with 2 days after seeding were dipped with the roots into a solution of each test compound at a designed concentration of 10% acetone/water solution. After dipped for 3 days, 10 second instar larvae were released onto each seedling. Thereafter, the seedlings infested with *Laodelphax* were left to stand in a thermostatic chamber (16 h light and 8 h dark) at 25°C. Four days after the release, the larvae were observed on survival or death.

Nilaparvata and Laodelphax by foliar application and soil drenching

The solution of FLP at a predetermined concentration prepared to 50% (for foliar) or 10% (for soil drenching) acetone/water (containing 0.05% Tween 20) was applied to 3 weeks old rice seedlings grown in pot. The treated seedlings were air dried for foliar tests or placed for 3 days for soil drenching, following which 10 second-instar larvae were released onto each seedling. The seedlings and larvae were then held in an incubation chamber at 25° C. (16 h period of light, 8 h dark period). Seven days after released, the numbers of alive and dead insects were counted.