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Original Article

Unexpected plant-systemic insecticidal properties of alkylene-tethered bis-imidacloprid derivatives

Masaru MORI,[†] Mami KIKUCHI,[†] Ikuya OHNO[††] and Shinzo KAGABU[†††,*]

[†] *Research Center, Kureha Corporation, 16 Ochiai, Nishiki-machi, Iwaki, Fukushima 974–8686, Japan*
[††] *The United Graduate School of Agricultural Science, Gifu University, Gifu 501–1193, Japan*
[†††] *Department of Chemistry, Faculty of Education, Gifu University, Gifu 501–1193, Japan*

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Alkylene-tethered bis-imidacloprid (bis-IMI) derivatives were evaluated for insecticidal activity after foliar dipping, foliar spraying and under systemic conditions. Insecticidal activity on the dipped leaves was dependent on the tether length, and heptamethylene (C7) and octamethylene (C8) derivatives, their best compounds, almost completely controlled the green peach aphid (*Myzus persicae*) at 10 mg l^{-1}, while all divalent compounds were weak against the tobacco cutworm (*Spodoptera litura*). C6 and C7 derivatives were examined for residual and systemic activity. Complete control of the green peach aphid by both compounds was observed on evaluation 72 hr after insect release on the eleventh day after foliar treatment, and the C7 derivative showed distinguished systemic activity against the brown plant hopper (*N. Lugens*) and the green peach aphid. The bis-IMIs should be disqualified as insecticidal candidates on virtual screening criteria owing to its high log *P* value, large molecular mass and excessive numbers of hydrogen-bond acceptors and rotatable bonds. Additional criteria may be necessary to account for the plant-mobile insecticidal behavior of such novel structures as bis-IMIs.

Keywords: bioavailability, bis-imidacloprid, Lipinski rule, neonicotinoid insecticides, systemicity.

Introduction

Lead generation is the first gateway to a promising pharmaceutical and insecticidal candidate. Virtual screening methods have been recognized as a powerful tool to improve the odds of identifying first-class drugs in the medicinal area.[1,2] Lipinski's Rule of Five has become a standard protocol among the developed methods to extract drug-like molecules from vendor listings.[3] This rule qualifies physicochemical parameters for orally bioavailable drugs, *i.e.*, molecular mass ≤500, log *P* ≤5, H-bond donors ≤5, and H-bond acceptors ≤10. Tice has confirmed that the Rule of Five for medicines can be adapted in principle for pesticides based on its application to 136 developed post-emergence herbicides and 243 insecticides, and has proposed amended criteria for insecticides.[4]

Since the first product, imidacloprid (IMI), was discovered, a number of molecular modifications have been attempted of the neonicotinoid insecticide class to find potential effectors.[5–10] New chemical entities seem to be designed based on the binding model referring to the existing products; consequently, most are compliant with the Lipinski rule. Recently, we devised a unique neonicotinoid structure of alkylene-tethered twin neonicotinoids and reported their biological properties.[11–17] These new molecules obviously violate the Rule of Five. For example, the hexamethylene bis-IMI derivative, whose insecticidal potency is comparable to that of IMI after injection into American cockroaches,[11] has a large molecular mass of 594, H-bond acceptors as many as 12, and excessive 13 rotatable bonds. Also, the heptamethylene homolog displays high binding affinity to the nicotinic acetylcholine receptor.[16] Would these compounds be disqualified as insecticidal candidates? How can we then legitimize their potent biological behavior? There may be an argument that the prevailing selection rule is applicable primarily to the pharmacokinetic process for a test substance to reach the target site rather than the binding site interaction. In other words, the rule addresses chiefly the properties of an insecticide regarding dermal permeation of the insect body and successive distribution in the insect body fluid. Also, in the relation to behavior in plants, it defines the optimal pharmacokinetic properties for

* To whom correspondence should be addressed.
Tel.: (+81) 58–232–1180;
E-mail: kagabus@gifu-u.ac.jp
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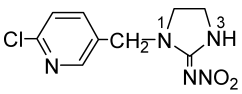
the entry of a toxicant into plant tissue through the root and successive apoplastic translocation or penetration through leaves followed by basipetal movement.

This argument prompted us to examine the biological profile of Lipinski rule outliers under conditions involving bioavailability-related factors, *i.e*., in drench application and soil treatment. As a result, to our surprise, some divalent neonicotinoids showed systemic insecticidal activity. This study discusses the unique insecticidal behavior of divalent neonicotinoids in relation to their chemical and physical features.

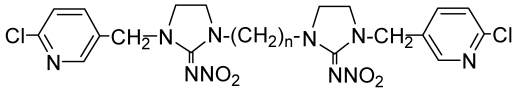
Materials and Methods

1. Test Compounds

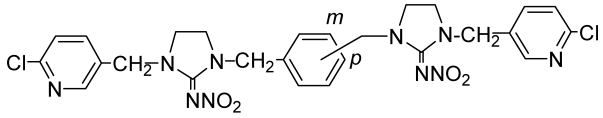
The test compounds in Fig. 1 were prepared according to published procedures.[11–13,17)]



1 (imidacloprid)



2. n=2 **3.** n=3 **4.** n=4 **5.** n=5 **6.** n=6
7. n=7 **8.** n=8 **9.** n=9 **10.** n=10 **11.** n=12



12. *m*; **13.** *p*

Fig. 1. Imidacloprid and bis-imidacloprid derivatives.

2. Insecticidal assays

Insecticidal assays were carried out by Aburahi AgroResearch Co., Ltd. (Kouka, Shiga, Japan). Green peach aphid, brown plant hopper and tobacco cutworm were collected from Chinese cabbage, rice and aroid, respectively, in the field of Aburahi AgroResearch. Diamondback moth was purchased from Sumika Techno Service Corporation (Hyogo, Japan). Green peach aphid and diamondback moth were maintained in-house on Chinese cabbage, and brown plant hopper on rice seedling. Tobacco cutworm was reared with an artificial diet (Insecta LF; Nosan Corporation, Kanagawa, Japan). These insects were maintained without exposure to insecticides.

2.1. Insecticidal activity against the green peach aphid (Myzus persicae) after foliar dipping

Two Chinese cabbage leaves were dipped for several seconds into an aqueous test solution until the leaf surface was wet. The test solution contained 10 or 100 mg l^{-1} of ingredient in 0.2% (v/v) acetone and 0.2% (v/v) ethanol with an appropriate volume of commercial surfactant (Shin-Rinoh®; Nihon Noyaku Co., Ltd., Osaka, Japan). After drying, the leaves were placed in a plastic dish (9 cm diameter). Ten adult aphids were released into the dish, which was covered with a lid. The dishes were placed into an incubator at 25°C, and mortality was determined after 120 hr. The average percentage of the number of dead insects in three replications of the test was defined as the mortality (Table 1).

2.2. Insecticidal activity against the tobacco cutworm (Spodoptera litura) after foliar dipping

Cabbage leaves were dipped into the test solution described above and placed in a plastic dish. Ten second-instar tobacco cutworm larvae were released into the dish, which was covered with a lid. The incubation condition, period, and method of activity assessment followed the description given in 2.1 (Table 1).

2.3. Tests for residual activity of insecticide after foliar treatment

Test solutions containing 10 or 100 mg l^{-1} of an active ingredient were prepared as described in 2.1. The solutions were then sprayed onto rice or cabbage seedlings in a pot. On the third, seventh, and eleventh days after treatment, twenty brown plant hoppers (*Nilaparvata lugens*) for rice, and twenty diamondback moths (*Plutella xylostella*) or twenty adult green peach aphids for cabbages were released. Activity was assessed 72 hr after each release according to the description given in 2.1 (Fig. 2).

Table 1. Insecticidal activity after foliar dipping

Compounds	Mortality (%)[a)]			
	Green peach aphid		Tobacco cutworm	
	100 mg l^{-1}	10 mg l^{-1}	100 mg l^{-1}	10 mg l^{-1}
1[b)]	100	100	97(±2)	17(±2)
2	97(±3)	23(±6)	20(±5)	0
3	27(±6)	13(±5)	80(±10)	13(±6)
4	7(±4)	0	57(±12)	7(±2)
5	13(±6)	0	40(±9)	0
6	23(±7)	10(±4)	83(±12)	27(±5)
7	97(±2)	93(±2)	85(±10)	23(±5)
8	97(±2)	83(±12)	80(±9)	27(±7)
9	93(±2)	77(±13)	57(±10)	3(±1)
10	43(±7)	27(±7)	27(±4)	0
11	50(±9)	19(±6)	0	0
12	57(±10)	33(±8)	47(±9)	3(±2)
13	97(±2)	97(±2)	47(±9)	3(±2)
Control[c)]	5(±2)	0		

[a)] SE (n=3) is in parentheses. [b)] Imidacloprid. [c)] 0.4% Acetone + 0.4% ethanol + surfactants in water (see, text).

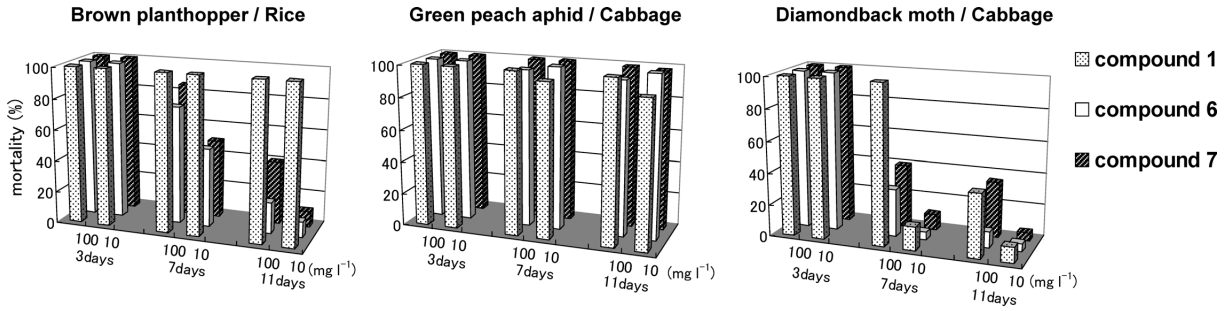


Fig. 2. Residual insecticidal activity after foliar treatment. Mortality was evaluated at 72 hr when the insects were released on the third, seventh, and eleventh days after the application of a sample solution of 100 and 10 mg l^{-1} to the host plants. Error bar (SE 0–20%, $n=3$) is not displayed since it would visually obscure the data.

2.4. Tests for systemic activity of insecticide after drench application

The test solutions containing 100 mg l^{-1} of an active ingredient were prepared as described in 2.1. Rice seedlings at the second leaf stage were collected from a nursery box and soil on the plant was rinsed off completely with water. Two or three rice seedlings were then bound up and the roots were dipped in the test solution until the end of the test. On the third, seventh and fourteenth days after treatment, ten brown plant hoppers were released, and the number of dead insects was counted after 24, 48 and 72 hr (Fig. 3).

2.5. Tests for systemic activity of insecticide after soil treatment

An aqueous test solution containing 1,000 mg l^{-1} active ingredient, 1% (v/v) DMSO, and an appropriate amount of commercial surfactant (Shin-Rinoh®) was prepared. Ten milliliters of the test solution, corresponding to 10 mg of active ingredient, were poured onto the soil surface around the cabbage seedling stumps. On the third, seventh, and eleventh days after treatment, ten diamondback moth larvae and ten green peach aphids were released. Insecticidal activity was assessed 24, 48, and 72 hr after each release for diamondback moths and 72 hr for green peach aphids (Fig. 4).

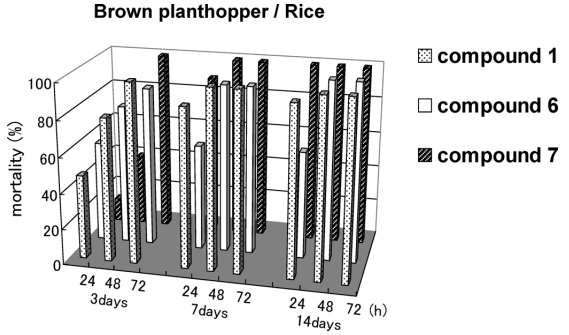


Fig. 3. Systemic insecticidal activity after drench application on rice. Rice seedling roots were dipped into a test solution of 100 mg l^{-1}. Mortality was evaluated 24, 48 and 72 hr when brown planthoppers were released on the third, seventh, and fourteenth days after dipping treatment. Error bar (SE 0–20%, $n=3$) is not displayed since it would visually obscure the data.

3. Calculation of the parameters

Moriguchi log *P* values (*m* log *P*) were calculated according to published procedures.[18] The number of H-bond donors was obtained by counting the numbers of OH and NH bonds in each molecule. H-bond acceptors are the sum of the numbers of nitrogen and oxygen atoms, as described by Lipinski *et al.*,[3] except for the nitrogen atom in NO_2 in the present study. The number of rotatable bonds was the sum of single bonds in the acyclic moieties, except N–O bonds in the NO_2 group as well as C–H, C–Cl and N–H bonds (Table 2).

Results

1. Insecticidal activity after foliar dipping (Table 1)

Test plant leaves were dipped in the aqueous solution of an active ingredient and dried before the insects were released. The mortality evaluated 120 hr after infestation depended on the tether length. Hepta- to nonamethylene-tethered compounds (**7**–**9**) had high efficacy toward the green peach aphid at 10 mg l^{-1}. The other twin compounds with a shorter or longer tether had lower activity at this dose. The result that the potency of hexamethylene derivative (**6**) was modest was unexpected because it has comparable potency to IMI (**1**) after injection into the American cockroach, and blocking potential for cockroach central nerves,[11,14] and high binding affinity to the [^{3}H]IMI site of housefly head nAChR.[11] Another telling result is that *p*-xylenyl derivative (**13**) was obviously superior to the *m*-isomer (**12**). On the other hand, none of the tested compounds, independent of the tether length, showed significant activity against the tobacco cutworm at 10 mg l^{-1}, which coincided with the general profile of neonicotinoids, being highly active against sucking insects and relatively weak against lepidopterans.[19]

Insecticidal intoxication in the sucking aphids occurs through a series of processes, ingestion of the toxicant from the plant vascular system, subsequent movement of the toxicant through the insect body fluid to the target site, and ultimately interaction with the receptor. The insecticidal result demonstrates that some outliers in Lipinski's criteria possess

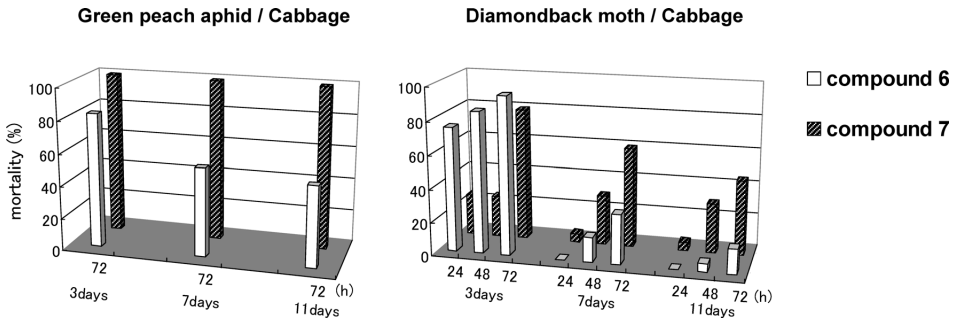


Fig. 4. Systemic insecticidal activity after soil treatment on cabbage. The test solution was poured onto the soil surface around the cabbage seedling stumps, and mortality was evaluated after 72 hr for green peach aphids or 24, 48, and 72 hr when diamondback moths were released on the third, seventh, and eleventh days after soil treatment. Error bar (SE 0–20%, $n=3$) is not displayed since it would visually obscure the data.

not only high intrinsic activity related to the target site action but also satisfactory bioavailable properties involved with mobility inside the insect body.

2. Tests for residual activity after foliar treatment (Fig. 2)

Hexamethylene and heptamethylene divalent compounds (**6**, **7**) showed comparable activity against the brown plant hopper on rice to IMI on the third day of infestation, but activity declined over time. These twin compounds exhibited excellent residual action against the green peach aphid by foliar application of 10 mg l^{-1} of an active ingredient solution on cabbage. Complete control by both compounds was observed 72 hr after the insects were released on the third, seventh and eleventh days after ingredient treatment. They also showed a good residual effect on the diamondback moth on cabbage on the third day of infestation after 10 mg l^{-1} treatment, but here the effect also diminished rapidly after the seventh day. A similar decaying tendency against this lepidoptera insect was observed with IMI treatment at 10 mg l^{-1} dose.

Since non-ionized lipophilic compounds generally do not move much basically through the plant,[20,21] most of the sprayed compounds remain on the leaves or within the vascular system. Considering that compounds **6** and **7** have no activity by topical treatment (unpublished result using the housefly), lethal action by contact is unrealistic; therefore, the insecticidal action most likely occurred by sucking the toxicant from the phloem. The results of the residual effect experiment suggest that active ingredients were continually transported from the leaf surface to the vascular system.

Table 2. Tice's criteria and molecular features of a set of tethered molecules

Compounds	Molecular mass	$m \log P$	H-bond donors	H-bond acceptors	Rotatable bonds
—[a)]	≥150, ≤500	≥0, ≤5.0	≤2	≥1, ≤8	≤12
1[b)]	255.7	1.88	1	6	3
2	537.4	4.13	0	12	9
3	551.4	4.35	0	12	10
4	565.4	4.56	0	12	11
5	579.4	4.77	0	12	12
6	593.5	4.97	0	12	13
7	607.5	5.18	0	12	14
8	621.5	5.37	0	12	15
9	635.6	5.57	0	12	16
10	649.6	5.76	0	12	17
11	677.6	6.14	0	12	19
12	613.5	5.69	0	12	10
13	613.5	5.69	0	12	10

[a)] Tice's criteria for insecticides. [b)] Imidacloprid.

3. Tests for systemic activity after drench application (Fig. 3)

One trait of IMI is its outstanding systemic property.[8,19,22] We conducted an experiment to examine the systemic behavior of the twin compounds using brown planthoppers on rice seedlings, whose roots were dipped in an aqueous solution of 100 mg l^{-1} of the active ingredient before insect infestation. Unexpectedly, compound **6** significantly controlled and **7** completely controlled the insects 72 hr after each infestation on the third, seventh, and fourteenth days after dipping, demonstrating that substantial amounts of these molecules were absorbed into the roots of the rice from the aqueous solution, reaching the endodermis surrounding the stele followed by movement into the vascular tissues, and were ultimately transported into the shoot with the transpiration stream.

4. Tests for systemic activity after soil treatment (Fig. 4)

Insecticidal activity against the green peach aphid and the diamondback moth was examined when the sample solution was applied to the soil surface around the cabbage seedling stumps. In this experiment, the concentrations of the active ingredients in the soil-water available to plant roots would be lower than in the experiment in the hydroponic system due to partitioning of chemicals in soil organic matter. Complete control of the green peach aphid by compound **7** was observed on the eleventh day of infestation, indicating that root uptake of the divalent compound from the soil and subsequent xylem translocation actually occurred and that the lethal dose was constantly provided for over eleven days under these conditions. The insecticidal activity of hexamethylene compound **6** was inferior to the higher homologue. Significant activity of both compounds against the diamondback moth was observed on the third day of infestation following soil treatment, but mortality fell over time.

Discussion

Three different tests revealed that some divalent compounds show not only high intrinsic activity but also sufficient bioavailability. The Rule of Five admits a maximum mass limit of 500 for "druggable" agents. Increased molecular size lowers solubility and renders poor penetration through membranes. The average molecular mass is 324 for marketed insecticides,[4] which agrees with the selection rule for insecticidal candidates; on the other hand, the tested twin compounds deviated from this criterion; for example, compound **7** exceeds the mass limit of 500 by as much as 100 (Table 2). The present results of its high insecticidal activity suggest that exceeding the molecular mass should not be necessarily detrimental to activity.

The lipophilicity/hydrophilicity of a molecule is an important pharmacokinetic criterion for drug effectiveness. One of the most prevalent quotients is the partition coefficient between octanol and water (P_{ow}). Briggs *et al.*[23] observed that apoplastic movement in barley is optimal in chemicals with a P_{ow} of 1.8 and the concentration in the transpiration stream declines markedly for more polar and lipophilic compounds. Uchida[24] estimated a P_{ow} of 2.9–4.3 for suitable derivatives of isoprothiolane against the white-backed planthopper on rice. Because of our compounds' extremely low solubility both in water and octanol, we could not obtain reliable P_{ow} values, and we therefore calculated Moriguchi's values ($m \log P$)[18] on Tice's suggestion for general utility.[4] The statistics indicate that the current insecticides are centered in an $m \log P$ range of 1.7 to 3.5 with an average of 2.6,[4] while the values of compounds **6** and **7** are around 5 (Table 2). Nevertheless, they afforded high systemic activity.

There may be some uncertainty in calculated P_{ow} values, because the calculation is performed under the assumption that a set of subject molecules has the same stereoelectronic relationship; however, some cases are known where a slight structural variation can result in an unexpected solubility change. For example, the introduction of a methyl group to the 3*N* position of IMI did not augment the lipophilicity of IMI as ordinary cases do, but conversely enhanced water solubility by as much as 15 times, and we have ascribed this anomaly to a distortion of the conjugate coplanarity of IMI by alkylation.[25,26] To check the water solubility of the present divalent molecules, we compared the NMR spectra of a solution that was prepared by filtrating a 1 ml D_2O suspension containing 0.1 mmol hexamethylene bis-IMI with 3*N*-methyl IMI. None of the peaks due to the C6 derivative was discernable, while peaks due to 3*N*-methyl IMI were detectable (unpublished results). This experiment proved that an NMR observable amount of the C6 derivative was not dissolved in D_2O, and suggests that the unusual water-solubility enhancement by alkyl bridging is not the case for bis IMIs.

Large numbers of H-bond acceptors and donors often cause insolubility of the molecule in many solvents due to the formation of intermolecular H-bonds in the crystal lattice. All of the presented twin molecules bear 12 H-acceptors. Actually, these compounds have only limited solubility in solvents. The number of proposed H-bond acceptors for expected insecticides is ≥ 1 and ≤ 8, and the bis-derivatives far exceed this range.

Very flexible compounds are rarely potent pesticides because of their entropy disadvantage upon binding to the receptor. Entropy terms are highly related to the number of rotatable bonds in a molecule, and the rotatable bond numbers in compounds **6** and **7** are beyond the upper limit of 12 of the selection rule. As previously mentioned, they have high biological potency when injected or in binding to nAChR, or as nerve-blocking agents.[11,14,16]

As discussed, these twin molecules are disqualified in each respective criterion for drug-like molecules despite the marked insecticidal potency. A prodrug concept could be an explanation to warrant a contradiction of the selection rule. Thus, lipophilic divalent molecules would be capable of cuti-

cle penetration or dermal permeation, and once they are absorbed, they decompose enzymatically to regenerate rather hydrophilic IMI inside a plant, or in the insect midgut. In fact, Yamamoto *et al.* found that *N*-methyl IMI was partly demethylated *in vitro* by a mixed function oxidase system.[27] Also, there could be some decomposition by soil microorganisms for divalent compounds; however, the extent of IMI regeneration through these biodegradation processes would presumably be limited in the present case, as far as we see the dependency of the insecticidal potency upon the alkylene-tether length and the obvious superior biological potencies of 3*N,3N′*-alkylene divalent compounds to those of 3*N*-alkyl IMI derivatives[28] even though the enzymatic susceptibility of a molecule should not be affected significantly by the alkyl-chain length or by the difference between the mono-substituted alkyls and α,ω-di-substituted alkylenes.

Further, there are some examples to which the general rule cannot apply because of the unique biological mechanism. A well-known outlier is glyphosate, which exhibits distinguished herbicidal activity despite its exceptionally low $m\log P$ value of –2.37. For glyphosate, a transport mechanism different from the lipophilic pathway of the majority of herbicides, *i.e.* the active ingredient penetrates the leaf with a transporter, has been advocated.[29] In addition, ionizable molecules such as glyphosate could not follow the rule as do neutral molecules, considering that the solubility and $\log P$ of ionic molecules are pH-dependent.[30]

The divalent neonicotinoids elicit excitatory toxicity signs similar to monovalent compounds.[11,14,16] They are not ionic and bear the essential specific functional groups common to their monovalent counterparts. It is rational that monovalent and divalent derivatives share the recognition site on the nicotinic acetylcholine receptor as the primary action arena. Based on the latest binding study with bis-IMI compounds, we have predicted the presence of a second binding pocket for the neonicotinoid pharmacophore.[16] The latent niche resides in a loop F domain around a distance that fits the heptamethylene length from the primary cavity, and each of the two pharmacophores of a bis-IMI may interact with each of the niches on the receptor. Supplemental accommodation by the second cavity will contribute to the conspicuous biological potencies of C6–C9 IMIs compared with other homologues. However, granting that the interaction mode at the target site for divalent molecules varies partly from the monovalent counterparts, such deviations would not markedly alter the preferable structural indices for bioavailability.

The Lipinski rule and other enforcing virtual screening strategies will be useful tools for selecting promising molecules among large numbers of randomly prepared compounds because these rules are based on the structural characteristics of the existing prototypes. However, the explanation of the biological properties of novel structures, such as alkylene-tethered divalent insecticides, deviating from the prevailing prototypes, may require other molecular descriptors besides the standards defined by the Lipinski rule. Recently, Vistoli *et al.*[31] emphasized the importance of molecular flexibility to assess "drug-likeness". Considering that the conformational space is crucially controlled by the number of rotatable bonds, the biological behavior of molecules, such as the presented bis-IMI derivatives tethered with a long alkylene chain, could not be estimated solely by using the parameters defined for the frozen state of a molecule.

Further investigations into the detailed mechanisms of the plant-systemic property of such unprecedented agents and the intriguing binding site interactions should be conducted to prompt the discovery of novel insecticidal compounds.

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