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Neonicotinoid Substituents Forming a Water Bridge at the Nicotinic Acetylcholine Receptor

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Neonicotinoid insecticides are extensively used for crop protection. The chloropyridinyl or chlorothiazolyl nitrogen and tetrahydrofuryl oxygen atoms of neonicotinoids serve as hydrogen acceptors at the target site. This investigation designs and prepares neonicotinoid probes to understand the structure−activity relationships (SARs) at the target site focusing on the water-mediated ligand−protein interactions. 2-Nitroiminoimidazolidine analogues with hydrogen-acceptor *N*-$CH_2CH_2CH_2F$ and *N*-$CH_2CH_2C(O)CH_3$ substituents showed higher binding affinities to the *Drosophila melanogaster* nicotinic receptor than probes with different hydrogen-bonding points in location and capability, suggesting that the appropriately positioned fluorine or carbonyl oxygen plays an important role on hydrogen-bond formation. Their binding site interactions were predicted using a crystal structure of the acetylcholine binding protein. The fluorine or carbonyl oxygen forms a water bridge to Ile-118 (and/or Ile-106) at the binding domain, consistent with that of neonicotinoids with a chloropyridinylmethyl, chlorothiazolylmethyl, or tetrahydrofurylmethyl moiety. Therefore, the present SAR study on binding site interactions helps design potent neonicotinoids with novel substituents.

KEYWORDS: Acetylcholine binding protein; clothianidin; dinotefuran; imidacloprid; neonicotinoid insecticides; nicotinic acetylcholine receptor; water bridge

INTRODUCTION

Neonicotinoid insecticides are used throughout the world for crop protection and animal health care. The first commercial neonicotinoid imidacloprid (IMI) (**Figure 1**) with a chloropyridinyl moiety led to subsequent developments of clothianidin (CLO) and dinotefuran (DIN) bearing the isosteric chlorothiazolyl and tetrahydrofuryl substituents, respectively (*1*−*3*). The chloropyridinyl or chlorothiazolyl nitrogen and tetrahydrofuryl oxygen atoms are considered to serve as hydrogen acceptors at the target site. Neonicotinoid binding site interactions with the nicotinic acetylcholine receptor (nAChR) have been characterized with mollusk acetylcholine binding protein (AChBP), which is a structural surrogate of the extracellular ligand binding domain of the nAChR, by chemical and structural biology approaches with adequate resolution to understand the recognition properties of the ligand binding pocket (*4*). In crystal structures of the AChBPs with bound nicotinic ligands (nicotine, epibatidine, IMI, thiacloprid, and CLO), a water or solvent molecule is captured near the pyridine or thiazole nitrogen bridging to relevant amino acids in the drug binding pocket (*5*−*8*). Therefore, water-bridge formation is an important determinant for agonist binding interactions. However, the structure−activity relationship (SAR) regarding the hydrogen-bonding ability of neonicotinoid substituents associating with water is insufficiently understood. In the present investigation, we design (**Figure 1**) and prepare (**Scheme 1**) several types of neonicotinoid analogues with suitably positioned methylketone, trifluoromethylketone, fluoroalkyl, and epoxycyclopentylmethyl substituents as SAR probes to approach the mechanism in terms of binding affinity to the insect nAChR. Finally, we predict the binding site interactions with the AChBP structural template emphasizing the formation of a water bridge.

MATERIALS AND METHODS

Synthesis. Synthesis procedures for the new chemicals are summarized in **Scheme 1**, and the details and analytical results for structural confirmation are given in the Supporting Information. The preparation of compounds **1**−**6**, **17**, and **18** was published previously (*9*). All melting points (mp) are uncorrected. IR spectra were measured with a Perkin-Elmer FTIR 1600 spectrometer. ^1H, ^{13}C, and ^{19}F NMR spectra were recorded using a JEOL ECA-500 spectrometer at 500, 125, and 376.3 MHz, respectively. The chemical shifts were recorded in δ (ppm),

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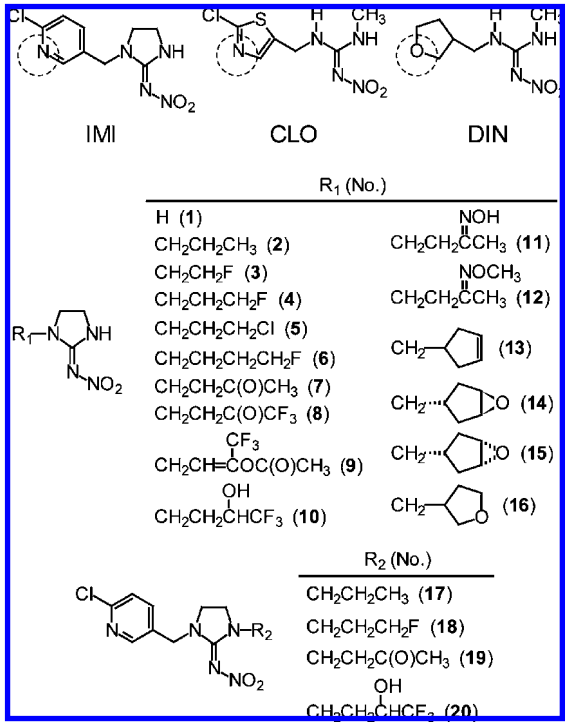


Figure 1. Chemical structures of neonicotinoid insecticides IMI, CLO, and DIN and the analogues examined in the present investigation.

and the coupling constants J_{H-H} were recorded in Hz unless otherwise stated. Mass spectra were recorded at 70 eV with the JEOL JMS-700 instrument.

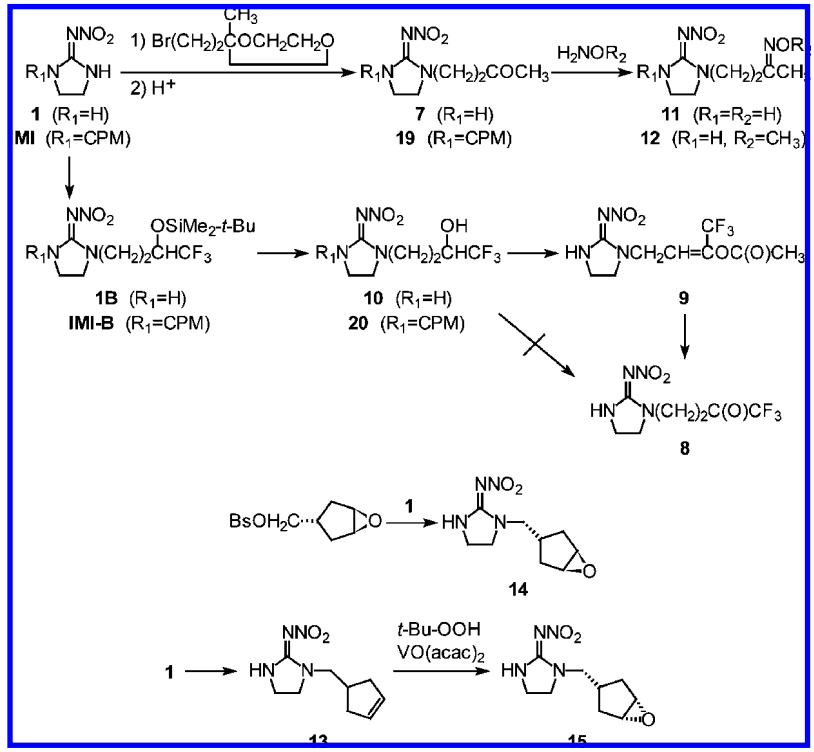
Biology. The potency of test compounds as inhibitors of [^{3}H]IMI binding to the native *Drosophila melanogaster* brain nAChR was assayed according to Tomizawa et al. (*10*). IC_{50} values (molar concentrations of test compounds necessary for 50% inhibition of specific [^{3}H]IMI binding) were determined by iterative nonlinear least-squares regression using Sigmaplot software (SPSS, Inc., Chicago, IL). IC_{50} values were finally converted to inhibition constants (K_i) using the equation by Cheng and Prusoff (*11*), i.e., $K_i = IC_{50}/(1 + [L]/K_d)$, where the concentration of radioligand [L] was 4 nM and the dissociation constant (K_d) of [^{3}H]IMI was 5.7 nM (*12*). To determine insecticidal activity, a solution of 2 mg of the test compound in 0.4 mL of acetone was diluted with 3.6 mL of water containing a spreader (Gramin-S at 1/4000, v/v). Three cucumber cotyledons were dipped into this sample solution of 500 ppm for about 5 s until the leaf surface was wet. After drying, the leaves were placed on a damp filter paper in a Petri dish (9 cm diameter) and 50 adult cotton aphids (*Aphis gossypii*) were released. The Petri dish was covered with a lid and kept at 25 °C. The mortality in percentage was observed after 72 h.

Modeling and Calculations. Chains A and E of the X-ray crystal structure for *Aplysia californica* AChBP with bound IMI (PDB code 3C79) (*7*) and one water (HOH-248) in the active site were partially minimized to remove any bad contacts. This energy minimization used the OPLS_2005 forcefield implemented in Macromodel 9.5 (*13, 14*) (Schrödinger, L.L.C., Portland, OR). In the initial minimization cycles, the backbone was held constant. In subsequent minimization cycles, the region within 15 Å of the binding pocket was free to move with progressive constraints on the remainder of the structure. Docking calculations were carried out using AutoDock 4 (*15, 16*). The receptor structure was treated as rigid, while flexible ligands were docked in a 15 Å cubic grid centered on the active site. In each case, a 100 step Lamarkian Genetic Algorithm search was performed.

RESULTS AND DISCUSSION

Synthesis. Several new neonicotinoids with methylketone, trifluoromethylketone, and epoxycyclopentylmethyl substituents were prepared to determine their contribution to forming a water bridge at the nAChR (**Scheme 1**). Butanoyl nitroiminoimidazolidine derivatives **7** and **19** were obtained from **1** and IMI. Butanoyl compound **7** was converted with the appropriate hydroxylamine derivatives to oxime **11** and oxime ether **12**. An attempt to prepare trifluoromethylketone derivative **8** by oxidation of alcohol **10** failed; therefore, it was prepared instead through ammonolysis of the enol acetate intermediate (**9**)

Scheme 1. Preparation of New Neonicotinoids[a]



[a] CPM = 6-chloropyridin-3-ylmethyl.

Table 1. Potencies of Neonicotinoids as Inhibitors of [^{3}H]IMI Binding to the *Drosophila* nAChR and as Insecticides against *Aphis*

compound number	binding affinity $K_i \pm$ SD (μM)	insecticidal activity mortality at 500 ppm (%)
1	>1000 (0%)[a]	0
2	12.8 ± 0.9	31
3	≥100 (44%)[a]	47
4	1.7 ± 0.2	55
5	3.6 ± 0.7	55
6	3.5 ± 0.7	27
7	1.2 ± 0.2	8
8	≥100 (43%)[a]	0
9	16.3 ± 1.1	ND[b]
10	18.1 ± 0.3	ND[b]
11	40.5 ± 1.7	10
12	61.2 ± 8.1	2
13	20.1 ± 1.7	20
14[c]	14.8 ± 1.5	18
15[d]	8.1 ± 0.32	0
16	0.11 ± 0.01[e]	100
17	0.89 ± 0.20	ND[b]
18	0.74 ± 0.06	ND[b]
19	6.1 ± 0.3	ND[b]
20	5.0 ± 0.5	ND[b]
IMI	0.003 ± 0.0003[e]	100
CLO	0.004 ± 0.0004[e]	100
(±)-DIN	0.076 ± 0.0035[e]	100

[a] Percent inhibition at the indicated concentrations. [b] Not determined. [c] *Trans*. [d] *Cis*/*trans* = 2:1 mixture. [e] Data from Honda et al. (*20*).

(obtained from silyloxy derivative **1B** via alcohol **10**) by Ohtake's one-pot procedure (*17*), i.e., oxidation of the alcohol followed by *in situ* enolacetalization using acetic anhydride and Me_2SO. Compound **8** was a ca. 1:3 *keto*/*enol* tautomeric mixture in $CDCl_3$ (on the basis of ^{19}F NMR). Compound **20** was obtained in a manner analogous to **10** from IMI via IMI-B.

Introduction of the *trans*-epoxycyclopentylmethyl moiety went smoothly using **1** and 3,4-*trans*-epoxycyclopentylmethylbrosylate to give **14**. However, *cis*-epoxycyclopentylmethylbrosylate (*18*) was not stable under the substitution conditions. We were able to obtain the desired isomer **15** from **13** by applying Sharpless's hydroxyl-directed epoxidation (*19*) to the imidazolidine system. Because the yield was very low, a mixture of *cis*/*trans* (2:1 based on ^{1}H NMR) was used for biological testing.

SAR (Table 1). N-Unsubstituted 2-nitroiminoimidazolidine (**1**) gave no binding even at 1 mM. Introduction of any substituent on the imidazolidine nitrogen generally enhanced the binding affinity to the *Drosophila* nAChR, confirming the importance of substitution on this position. We designed various probes replacing chloropyridinylmethyl, chlorothiazolylmethyl, and tetrahydrofurylmethyl with counterparts, which are different in position and capability, to serve as hydrogen acceptors. Introducing *n*-propyl (**2**) and fluoroethyl (**3**) groups conferred low potency, whereas their extended analogues with fluoropropyl (**4**), chloropropyl (**5**), and fluorobutyl (**6**) gave much higher potency, although with **5**, the chlorine atom may undergo hydrophobic contacts rather than hydrogen bonding. Compound **7** with a methylketone group also showed high potency, but in contrast, the trifluoromethylketone analogue (**8**) lost the activity. We attribute the affinity difference to the opposite tendency for a hydrogen bond. The carbonyl oxygen of alkyl ketones functions as a hydrogen acceptor, while the CH_2COCF_3 moiety is predicted to shift to the enol form [$CH{=}C(OH)CF_3$], with the strong electron-withdrawing property of CF_3 functioning as a hydrogen donor. Actually, NMR analysis showed that the 1:3 keto/enol equilibrium in $CDCl_3$ and the α-CH_2 protons were totally replaced with added D_2O. Compounds with branched CF_3 (**9**), OH (**10**), =NOH (**11**), and =$NOCH_3$ (**12**) moieties had reduced affinity. Compound **13** bearing the cyclopentene ring and the epoxide analogues with *trans* configuration (**14**) and *cis*/*trans* mixture (**15**) were also not potent, but the tetrahydrofuryl analogue (**16**) was highly active (*20*). IMI analogues with alkyl substituents on the N3 position of the imidazolidine ring (**17**−**20**) generally had reduced affinity relative to the parent IMI. Insecticidal activities of the present compounds were determined with aphids establishing that none of them reached the effectiveness of IMI, CLO, DIN, and closely related analogues. Therefore, the present SAR suggests that the

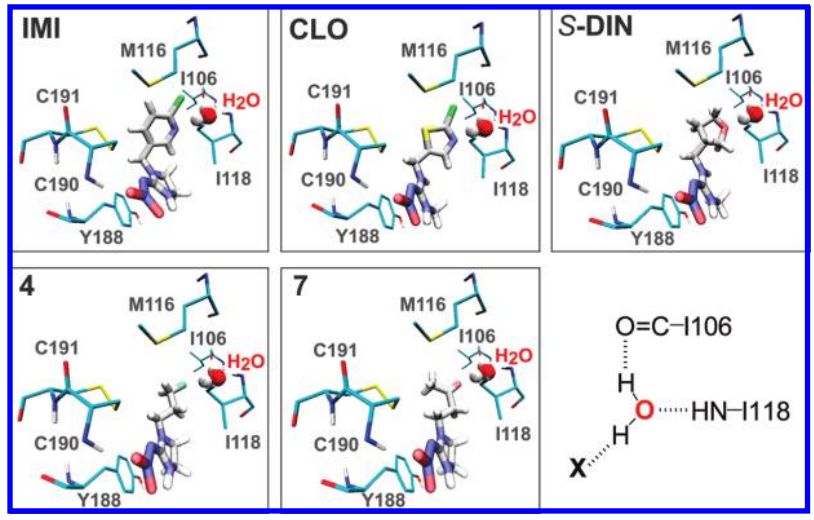


Figure 2. Predictive binding site interactions of compounds IMI, CLO, *S*-DIN, **4**, and **7** with the agonist binding pocket of the *Aplysia* AChBP, with an emphasis on the water-mediated ligand−protein interaction. The water (captured between chains A and E of the 3C79 crystal structure) forms a bridge between the I118 backbone NH and nitrogen of IMI or CLO, oxygen of *S*-DIN or **7**, or fluorine of **4**. The alternative water hydrogen undergoes hydrogen bonding to the I106 backbone carbonyl oxygen. Selected amino acids displayed are loop C Y188, C190, and C191 from the (+)-face or principal subunit and loop E I106, M116, and I118 from the (−)-face or complementary subunit. Other amino acids forming the binding pocket are omitted, so that they do not obscure important substituents or interactions. Schematic representation of the water-bridge formation is shown in the lower right panel, in which X refers to the 3-pyridinyl nitrogen of IMI or 1,3-thiazolyl nitrogen of CLO, the tetrahydrofuryl or carbonyl oxygen of DIN or **7**, and the fluorine of **4**.

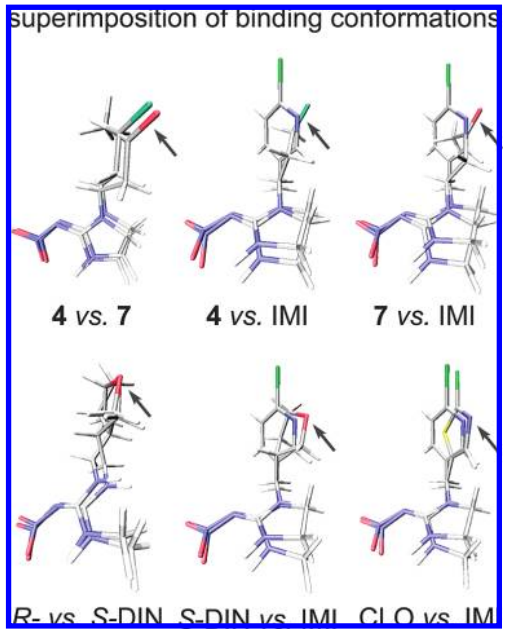


Figure 3. Conformational comparison of neonicotinoids and analogues as observed in the AChBP binding pocket. Each arrow shows the position of the water-bridge formation.

fluorine or carbonyl oxygen at a specific position plays an important role on hydrogen-bond formation presumably with a water molecule at the binding subsite.

Binding Site Interactions. *Aplysia* AChBP with high sensitivity to neonicotinoids serves as a structural homologue of the insect-type nAChR. The amino acids forming the binding pockets of the AChBP are structurally or functionally consistent with those of the insect nAChRs (*4*, *21*, *22*). The binding site interactions of representative compounds **4** and **7** were therefore simulated using a crystal structure of neonicotinoid-liganded AChBP (*7*) in comparison to those of IMI, CLO, and DIN (**Figure 2**). All ligands docked into the center of the binding pocket with binding energies of −7.72, −7.18, −6.40, −5.00, and −6.35 kcal/mol for IMI, CLO, DIN *S* isomer, **4**, and **7**, respectively. Interestingly, the DIN *R* isomer, with an energy value of −6.21 kcal/mol, was also docked in a similar manner to that of the *S* isomer. The nitro tip oxygen atom of all ligands undergoes hydrogen bonding to the backbone NH of loop C C190, and the nitro substituted-guanidine moiety π stacks with the loop C Y188 aromatic side chain in an off-centered geometry. Moreover, the fluorine of **4**, the C═O oxygen of **7**, or the tetrahydrofuryl oxygen of DIN hydrogen bonds with a water within a 1.8−2.2 Å distance; further, oxygen and the alternative hydrogen of the water also undergo hydrogen bonding with the backbone NH of loop E I118 and the carbonyl O of I106, respectively (1.8−1.9 Å). An identical interaction is found with the nitrogen atom of the IMI pyridine or CLO thiazole ring. The chlorine atom on the pyridine or thiazole ring of IMI or CLO van der Waals contacts the backbone carbonyl oxygen atoms of loop E I106 and M116, additionally enhancing the affinity. Superimposition of binding conformations as observed in the binding pocket for the five ligands is shown in **Figure 3**. The nitroimino-guanidine moieties of all ligands clearly fit in the same direction. The fluorine and C═O oxygen were superimposable onto each other and the pyridine N of IMI. Similarly, the pyridine N of IMI was overlaid onto the tetrahydrofuryl O of DIN and thiazole N of CLO. Interestingly, the tetrahydrofuryl O atoms of the *R* and *S* enantiomers matched perfectly, yet the *S* and *R* isomers of DIN have different potencies (*S* > *R*) (*23*, *24*).

Concluding Remarks. This SAR study considers the ability of neonicotinoid substituents to form a water bridge between the neonicotinoid and the relevant amino acid at the ligand binding pocket. The chloropyridinyl or chlorothiazolyl nitrogen and tetrahydrofuryl oxygen atoms can be potentially replaced by alkyl substituents with fluorine and carbonyl oxygen as a hydrogen-bonding acceptor. The position of the hydrogen-accepting point and orientation of the intervening water molecule are firmly restricted in the conserved directions. Accordingly, the present prediction of binding site interactions based on SAR findings may help design neonicotinoids with novel substituents to expand the insecticidal spectrum and circumvent the possible resistance caused by a modified target site.

ABBREVIATIONS USED

AChBP, acetylcholine binding protein; CLO, clothianidin; DIN, dinotefuran; IMI or [^{3}H]IMI, imidacloprid or its tritiated radioligand; nAChR, nicotinic acetylcholine receptor; SAR, structure−activity relationship.

Supporting Information Available: Synthesis procedures and analysis data for new products. This material is available free of charge via the Internet at http://pubs.acs.org.

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