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Synthesis of imidacloprid derivatives with a chiral alkylated imidazolidine ring and evaluation of their insecticidal activity and affinity to the nicotinic acetylcholine receptor

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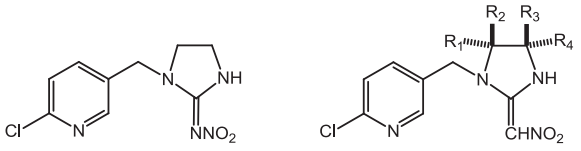
ABSTRACT

A series of imidacloprid (IMI) derivatives with an alkylated imidazolidine ring were asymmetrically synthesized to evaluate their insecticidal activity against adult female housefly, *Musca domestica*, and affinity to the nicotinic acetylcholine receptor of the flies. The bulkier the alkyl group, the lower was the receptor affinity, but the derivatives methylated and ethylated at the *R*-5-position of the imidazolidine ring were equipotent to the unsubstituted compound. Quantitative structure–activity relationship (QSAR) analysis of the receptor affinity demonstrated that the introduction of a substituent into the imidazolidine ring was fundamentally disadvantageous, but the introduction of a substituent at the *R*-5-position was permissible in the case of its small size. The binding model of the synthesized derivatives with the receptor supported the QSAR analysis, indicating the existence of space for a short alkyl group around the *R*-5-position in the ligand-binding site. In addition, positive correlation was observed between the insecticidal activity and receptor affinity, suggesting that the receptor affinity was the primary factor in influencing the insecticidal activity even if the imidazolidine ring was modified.

1. Introduction

Neonicotinoids, including imidacloprid (**1**, IMI, Fig. 1), are one of the widely used neuroactive insecticides. They specifically interact with the nicotinic acetylcholine receptor (nAChR) of insects, which results in superior selective toxicity to insects over vertebrates. A significant number of researches have been performed to explore the novel bioactive structure of neonicotinoids since the development and launch of IMI.[1–7] To clarify the structure–activity relationship (SAR) of neonicotinoids and to explore compounds with high potency, we also synthesized various neonicotinoid derivatives with the nitromethylene group. These include derivatives having a substituted phenyl ring instead of a pyridine ring,[8] various alkyl groups attached at the *N*3 atom of the imidazolidine ring,[9] and enlarged rings such as diazacyclohexane and diazacycloheptane with some modifications instead of the imidazolidine ring.[10]

Our previous SAR studies demonstrated that some steric and electrostatic factors around the pyridine and imidazolidine rings influence the receptor binding. However, most of the synthesized compounds in our studies were less potent than the basic nitromethylene derivative **2** (CH-IMI, Fig. 1). Recently, we asymmetrically synthesized four methylated imidacloprid derivatives to examine whether substitutions to the ethylene moiety of the imidazolidine ring influence bioactivity such as insecticidal activity and affinity



1, Imidacloprid (IMI) **2**, CH-IMI ($R_1 = R_2 = R_3 = R_4 = H$)

(*R*-5-; R_1 = alkyl, $R_2 = R_3 = R_4 = H$; alkyl = Me (**3**), Et (**7**), *n*-Pr (**11**), isopropyl (**15**), isobutyl (**19**))
(*S*-5-; $R_1 = H$, R_2 = alkyl, $R_3 = R_4 = H$; alkyl = Me (**4**), Et (**8**), *n*-Pr (**12**), isopropyl (**16**), isobutyl (**20**))
(*R*-4-; $R_1 = R_2 = H$, R_3 = alkyl, $R_4 = H$; alkyl = Me (**5**), Et (**9**), *n*-Pr (**13**), isopropyl (**17**), isobutyl (**21**))
(*S*-4-; $R_1 = R_2 = R_3 = H$, R_4 = alkyl; alkyl = Me (**6**), Et (**10**), *n*-Pr (**14**), isopropyl (**18**), isobutyl (**22**))

Figure 1. Chemical structures of neonicotinoid derivatives with an alkylated imidazolidine ring.

Abbreviations: CH-IMI, nitromethylene analogue of imidacloprid; IMI, imidacloprid; LBD, ligand binding domain; Ls, *Lymnaea stagnalis*; nAChR, nicotinic acetylcholine receptor; NIA, propargyl propyl phenylphosphonate (Niagara 16388); QSAR, quantitative structure–activity relationship; SAR, Structure–activity relationship.

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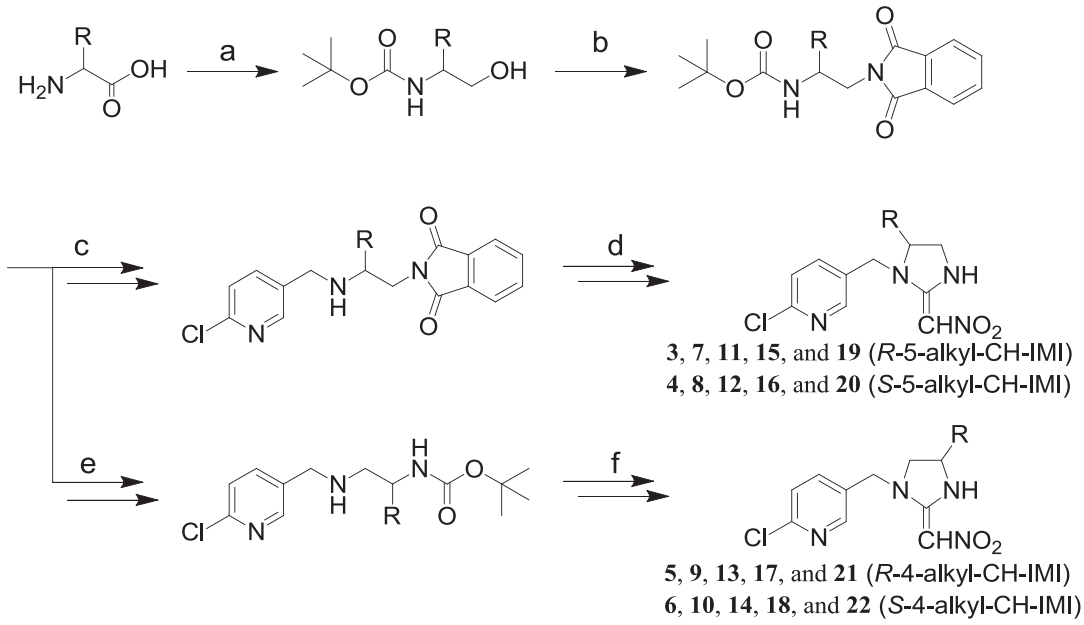

Figure 2. Synthetic route of alkylated imidacloprid derivatives. (a) Lithium aluminum hydride/anhydrous THF; $(Boc)_2O$/THF; (b) phthalimide, diethyl azodicarboxylate, triphenylphosphine/anhydrous THF; (c) concentrated HCl/THF; 2-chloro-5- (chloromethyl) pyridine, Et_3N/acetonitrile; (d) hydrazine hydrate/EtOH; 1,1-bis(methylthio)-2-nitroethylene, K_2CO_3/EtOH; (e) hydrazine hydrate/EtOH; 2-chloro-5- (chloromethyl) pyridine, Et_3N/acetonitrile; (f) concentrated HCl/THF; 1,1-bis(methylthio)-2-nitroethylene, K_2CO_3/EtOH.

to the nAChR (Figs. 1, **3–6**).[11] As a result, the compound with a methyl group at the 5-position of the imidazolidine ring (compound **3**) exhibited intrinsic activity comparable to the unsubstituted compound **2**. These findings motivated us to clarify the size of the substituent attached on the imidazolidine ring that would be considered permissible for its interaction with the receptor and to verify if these alkylated CH-IMI derivatives could exert high insecticidal activity.

In this study, a series of imidacloprid derivatives with various alkylated imidazolidine rings (Fig. 1, **7–22**) were synthesized to evaluate their receptor affinity. The SAR of the modified imidazolidine ring was then quantitatively analyzed using the Hansch–Fujita method, conventional quantitative SAR (QSAR) method, and comparative molecular field analysis (CoMFA) that is a three-dimensional QSAR technique. In addition, a ligand-binding model of the receptor combined with a test chemical was constructed to discuss the validation of results from the QSAR analyses. The insecticidal activities of the synthesized compounds were also evaluated to elucidate the relationship between their bioactivities.

2. Experimental

2.1. Insects

An insecticide-susceptible strain of the housefly (*Musca domestica* L., Takatsuki strain) was reared at 25 °C in our laboratory.

2.2. Chemicals

Compounds **7–22** (Fig. 1) were newly synthesized through the synthetic scheme reported earlier (Fig. 2).[11] In brief, the amino group of each 2-aminobutanol (for compounds **7–10**) or an appropriate amino alcohol (for others), which was prepared by the reduction of corresponding amino acid (norvaline for compounds **11–14**, valine for compounds **15–18**, and leucine for compounds **19–22**) with lithium aluminium hydride, was protected with the *t*-butoxycarbonyl group. The hydroxy group was changed to phthalimide using triphenyl phosphine, diethyl azodicarboxylate, and phthalimide. For synthesizing the compounds with the 5-position-modified imidazolidine ring, the *t*-butoxycarbonyl group was deprotected using concd HCl, whereas hydrazine monohydrate deprotected phthalimide to amino group for synthesizing the compound with the 4-position-modified imidazolidine ring. 2-Chloro-5- (chloromethyl) pyridine was then attached to the resultant amino group, followed by the deprotection of the other protected amino group. The target compounds were finally synthesized via cyclization using the resultant diamine and 1,1-bis(methylthio)-2-nitroethylene. Reagents used for the syntheses were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan), Nacalai Tesque, Inc (Kyoto, Japan), Tokyo Chemical Industry Co, Ltd (Tokyo, Japan), and Aldrich Chemical Co (Milwaukee, WI, USA). The metabolic inhibitor, NIA 16388 (NIA; propargyl propyl phenylphosphonate), was our stock sample.[11] The ^1H and ^{13}C NMR analyses were performed using a JEOL ECS-400 NMR spectrometer in deuterochloroform ($CDCl_3$) with tetramethylsilane as the internal standard. The authenticity of the final compounds was also confirmed by HRMS using Xevo Q-TOFMS (Waters, UK). Melting points of the compounds were measured with a Yanaco melting point apparatus (Kyoto, Japan) and were uncorrected. Optical rotation values were evaluated by using a P-2100 polarimeter (Jasco, Tokyo, Japan).

2.2.1. *R*-1-(6-Chloro-3-pyridylmethyl)-5-ethyl-2-nitromethyleneimidazolidine (7)

Mp 139–140. $[\alpha]_D^{25}$ +56 (*c* 0.02, $CHCl_3$). NMR δ_H ($CDCl_3$): 0.92 (3H, t, J = 7 Hz), 1.58 (1H, m), 1.78 (1H, m), 3.49 (1H, dd, J = 10 Hz, J = 8 Hz), 3.78 (1H, m), 3.89 (1H, t, J = 8 Hz), 4.27 (1H, d, J = 16 Hz), 4.37 (1H, d, J = 16 Hz), 6.56 (1H, s), 7.37 (1H, d, J = 8 Hz), 7.55 (1H, dd, J = 8 Hz, J = 2 Hz), 8.28 (1H, d, J = 2 Hz), 8.71 (1H, br). NMR δ_C ($CDCl_3$): 8.7, 24.8, 44.2, 47.3, 61.1, 96.5, 124.9, 129.5, 137.5, 148.3, 151.8, 159.4. ESIMS *m*/*z* $[M+H]^+$: calcd for $C_{12}H_{16}N_4O_2Cl$, 283.0962; found, 283.0967.

2.2.2. *S*-1-(6-Chloro-3-pyridylmethyl)-5-ethyl-2-nitromethyleneimidazolidine (8)

Mp 137–138. $[\alpha]_D^{25}$ −62 (*c* 0.03, $CHCl_3$). The NMR spectral data agreed with those of its enantiomer **7**. ESIMS *m*/*z* $[M+H]^+$: calcd for $C_{12}H_{16}N_4O_2Cl$, 283.0962; found, 283.0973.

2.2.3. *R*-1-(6-Chloro-3-pyridylmethyl)-4-ethyl-2-nitromethylene-imidazolidine (9)

Mp 138–139. $[\alpha]_D^{25}$ −21 (*c* 0.04, $CHCl_3$). NMR δ_H ($CDCl_3$): 0.97 (3H, t, *J* = 8 Hz), 1.67 (2H, m), 3.18 (1H, dd, *J* = 10 Hz, *J* = 7 Hz), 3.64 (1H, t, *J* = 10 Hz), 4.02 (1H, m), 4.30 (2H, s), 6.63 (1H, s), 7.38 (1H, d, *J* = 8 Hz), 7.55 (1H, dd, *J* = 8 Hz, *J* = 2 Hz), 8.29 (1H, d, *J* = 2 Hz), 8.82 (1H, br). NMR δ_C ($CDCl_3$): 9.5, 28.1, 46.5, 53.3, 56.2, 96.2, 124.9, 129.1, 137.9, 148.8, 152.0, 158.6. ESIMS *m*/*z* $[M+H]^+$: calcd for $C_{12}H_{16}N_4O_2Cl$, 283.0962; found, 283.0958.

2.2.4. *S*-1-(6-Chloro-3-pyridylmethyl)-4-ethyl-2-nitromethylene-imidazolidine (10)

Mp 138–139. $[\alpha]_D^{25}$ +20 (*c* 0.11, $CHCl_3$). The NMR spectral data agreed with those of its enantiomer **9**. ESIMS *m*/*z* $[M+H]^+$: calcd for $C_{12}H_{16}N_4O_2Cl$, 283.0962; found, 283.0968.

2.2.5. *R*-1-(6-Chloro-3-pyridylmethyl)-5-*n*-propyl-2-nitromethylene-imidazolidine (11)

Mp 123–125. $[\alpha]_D^{25}$ +98 (*c* 0.04, $CHCl_3$). NMR δ_H ($CDCl_3$): 0.95 (3H, d, *J* = 8 Hz), 1.30 (2H, m), 1.53 (1H, m), 1.71 (1H, m), 3.48 (1H, t, *J* = 9 Hz), 3.80 (1H, m), 3.89 (1H, t, *J* = 9 Hz), 4.28 (1H, d, *J* = 16 Hz), 4.36 (1H, d, *J* = 16 Hz), 6.55 (1H, s), 7.36 (1H, d, *J* = 8 Hz), 7.55 (1H, dd, *J* = 8 Hz, *J* = 2 Hz), 8.29 (1H, d, *J* = 2 Hz), 8.71 (1H, br). NMR δ_C ($CDCl_3$): 13.9, 18.1, 34.0, 44.3, 47.8, 60.1, 96.6, 124.9, 129.6, 137.5, 148.3, 151.7, 159.4. ESIMS *m*/*z* $[M+H]^+$: calcd for $C_{13}H_{18}N_4O_2Cl$, 297.1118; found, 297.1117.

2.2.6. *S*-1-(6-Chloro-3-pyridylmethyl)-5-*n*-propyl-2-nitromethylene-imidazolidine (12)

Mp 114–116. $[\alpha]_D^{25}$ −94 (*c* 0.02, $CHCl_3$). The NMR spectral data agreed with those of its enantiomer **11**. ESIMS *m*/*z* $[M+H]^+$: calcd for $C_{13}H_{18}N_4O_2Cl$, 297.1118; found, 297.1115.

2.2.7. *R*-1-(6-Chloro-3-pyridylmethyl)-4-*n*-propyl-2-nitromethylene-imidazolidine (13)

Mp 111–113. $[\alpha]_D^{25}$ −34 (*c* 0.04, $CHCl_3$). NMR δ_H ($CDCl_3$): 0.97 (3H, d, *J* = 8 Hz), 1.38 (2H, m), 1.57 (1H, m), 1.66 (1H, m), 3.17 (1H, dd, *J* = 8 Hz, *J* = 6 Hz), 3.65 (1H, t, *J* = 10 Hz), 4.06 (1H, m), 4.30 (2H, s), 6.63 (1H, s), 7.38 (1H, d, *J* = 8 Hz), 7.56 (1H, dd, *J* = 8 Hz, *J* = 2 Hz), 8.29 (1H, d, *J* = 2 Hz), 8.81 (1H, br). NMR δ_C ($CDCl_3$): 13.8, 18.7, 37.2, 46.5, 53.9, 54.9, 96.3, 124.9, 129.2, 137.9, 148.8, 151.9, 158.6. ESIMS *m*/*z* $[M+H]^+$: calcd for $C_{13}H_{18}N_4O_2Cl$, 297.1118; found, 297.1110.

2.2.8. *S*-1-(6-Chloro-3-pyridylmethyl)-4-*n*-propyl-2-nitromethylene-imidazolidine (14)

Mp 108–109. $[\alpha]_D^{25}$ +32 (*c* 0.15, $CHCl_3$). The NMR spectral data agreed with those of its enantiomer **13**. ESIMS *m*/*z* $[M+H]^+$: calcd for $C_{13}H_{18}N_4O_2Cl$, 297.1118; found, 297.1122.

2.2.9. *R*-1-(6-Chloro-3-pyridylmethyl)-5-isopropyl-2-nitromethylene-imidazolidine (15)

Mp 163–164. $[\alpha]_D^{25}$ +129 (*c* 0.04, $CHCl_3$). NMR δ_H ($CDCl_3$): 0.87 (3H, d, *J* = 7 Hz), 0.91 (3H, d, *J* = 7 Hz), 2.11 (1H, m), 3.58 (1H, dd, *J* = 10 Hz, *J* = 7 Hz), 3.75 (2H, m), 4.23 (1H, d, *J* = 16 Hz), 4.40 (1H, d, *J* = 16 Hz), 6.64 (1H, s), 7.38 (1H, d, *J* = 8 Hz), 7.57 (1H, dd, *J* = 8 Hz, *J* = 2 Hz), 8.28 (1H, d, *J* = 2 Hz), 8.72 (1H, br). NMR δ_C ($CDCl_3$): 14.4, 18.3, 27.7, 42.7, 44.0, 64.1, 96.4, 124.9, 129.5, 137.8, 148.5, 151.7, 159.4. ESIMS *m*/*z* $[M+H]^+$: calcd for $C_{13}H_{18}N_4O_2Cl$, 297.1118; found, 297.1125.

2.2.10. *S*-1-(6-Chloro-3-pyridylmethyl)-5-isopropyl-2-nitromethylene-imidazolidine (16)

Mp 165–166. $[\alpha]_D^{25}$ −108 (c 0.06, $CHCl_3$). The NMR spectral data agreed with those of its enantiomer **15**. ESIMS *m*/*z* $[M+H]^+$: calcd for $C_{13}H_{18}N_4O_2Cl$, 297.1118; found, 297.1110.

2.2.11. *R*-1-(6-Chloro-3-pyridylmethyl)-4-isopropyl-2-nitromethylene-imidazolidine (17)

Mp 114–115. $[\alpha]_D^{25}$ −47 (c 0.03, $CHCl_3$). NMR δ_H ($CDCl_3$): 0.91 (3H, d, *J* = 7 Hz), 0.97 (3H, d, *J* = 7 Hz), 1.82 (1H, m), 3.25 (1H, dd, *J* = 10 Hz, *J* = 8 Hz), 3.62 (1H, t, *J* = 6 Hz), 3.85 (1H, m), 4.32 (2H, s), 6.64 (1H, s), 7.38 (1H, d, *J* = 8 Hz), 7.57 (1H, dd, *J* = 8 Hz, *J* = 2 Hz), 8.29 (1H, d, *J* = 2 Hz), 8.84 (1H, br). NMR δ_C ($CDCl_3$): 17.8, 18.1, 32.5, 46.5, 51.7, 60.7, 96.1, 124.9, 129.1, 137.9, 148.8, 152.0, 158.7. ESIMS *m*/*z* $[M+H]^+$: calcd for $C_{13}H_{18}N_4O_2Cl$, 297.1118; found, 297.1114.

2.2.12. *S*-1-(6-Chloro-3-pyridylmethyl)-4-isopropyl-2-nitromethylene-imidazolidine (18)

Mp 115–116. $[\alpha]_D^{25}$ +51 (*c* 0.03, $CHCl_3$). The NMR spectral data agreed with those of its enantiomer **17**. ESIMS *m*/*z* $[M+H]^+$: calcd for $C_{13}H_{18}N_4O_2Cl$, 297.1118; found, 297.1119.

2.2.13. *R*-1-(6-Chloro-3-pyridylmethyl)-5-isobutyl-2-nitromethylene-imidazolidine (19)

Mp 131–133. $[\alpha]_D^{25}$ +75 (*c* 0.16, $CHCl_3$). NMR δ_H ($CDCl_3$): 0.87 (3H, d, *J* = 6 Hz), 0.96 (3H, d, *J* = 6 Hz), 1.55 (3H, m), 3.46 (1H, t, *J* = 8 Hz), 3.85 (1H, m), 3.91 (1H, t, *J* = 8 Hz), 4.28 (1H, d, *J* = 16 Hz), 4.35 (1H, d, *J* = 16 Hz), 6.53 (1H, s), 7.36 (1H, d, *J* = 8 Hz), 7.55 (1H, dd, *J* = 8 Hz, *J* = 2 Hz), 8.29 (1H, d, *J* = 2 Hz), 8.71 (1H, br). NMR δ_C ($CDCl_3$): 21.5, 23.8, 25.1, 41.1, 44.4, 48.3, 59.0, 96.7, 124.9, 129.5, 137.5, 148.3, 151.8, 159.3. ESIMS *m*/*z* $[M+H]^+$: calcd for $C_{14}H_{20}N_4O_2Cl$, 311.1275; found, 311.1286.

2.2.14. *S*-1-(6-Chloro-3-pyridylmethyl)-5-isobutyl-2-nitromethylene-imidazolidine (20)

Mp 133–135. $[\alpha]_D^{25}$ −71 (*c* 0.10, $CHCl_3$). The NMR spectral data agreed with those of its enantiomer **19**. ESIMS *m*/*z* $[M+H]^+$: calcd for $C_{14}H_{20}N_4O_2Cl$, 311.1275; found, 311.1275.

2.2.15. *R*-1-(6-Chloro-3-pyridylmethyl)-4-isobutyl-2-nitromethylene-imidazolidine (21)

Mp 100–103. $[\alpha]_D^{25}$ −35 (*c* 0.20, $CHCl_3$). NMR δ_H ($CDCl_3$): 0.95 (3H, d, *J* = 2 Hz), 0.97 (3H, d, *J* = 2 Hz), 1.43 (1H, m), 1.65 (2H, m), 3.13 (1H, dd, *J* = 9 Hz, *J* = 8 Hz), 3.65 (1H, t, *J* = 9 Hz), 4.12 (1H, m), 4.26 (1H, d, *J* = 16 Hz), 4.32 (1H, d, *J* = 16 Hz), 6.62 (1H, s), 7.38 (1H, d, *J* = 8 Hz), 7.56 (1H, dd, *J* = 8 Hz, *J* = 2 Hz), 8.29 (1H, d, *J* = 2 Hz), 8.81 (1H, br). NMR δ_C ($CDCl_3$): 22.2, 22.7, 25.2, 44.2, 46.5, 53.4, 54.4, 96.3, 124.9, 129.1, 137.9, 148.8, 152.0, 158.5. ESIMS *m*/*z* $[M+H]^+$: calcd for $C_{14}H_{20}N_4O_2Cl$, 311.1275; found, 311.1281.

2.2.16. *S*-1-(6-Chloro-3-pyridylmethyl)-4-isobutyl-2-nitromethylene-imidazolidine (22)

Mp 102–105. $[\alpha]_D^{25}$ +30 (*c* 0.20, $CHCl_3$). The NMR spectral data agreed with those of its enantiomer **21**. ESIMS *m*/*z* $[M+H]^+$: calcd for $C_{14}H_{20}N_4O_2Cl$, 311.1275; found, 311.1273.

2.3. Evaluation of receptor binding affinity

Assay method was essentially the same as in our previous reports.[11,12] In the competitive binding inhibition assay, the housefly head membrane fraction prepared according to our previous report[8] (3 mg protein ml^{-1}, 200 μl) was added to a plastic tube containing a dimethyl sulfoxide solution (2 μl) of various concentrations (10^{-4} to 10^{-13} M) of the test compound. After standing for 30 min, sodium phosphate buffer (50 μl, 10 mM, pH 7.4) containing sodium chloride (50 mM), Triton X-100 (1 g l^{-1}) and [^{3}H]imidacloprid (100 nM) was added to the reaction mixture and incubated for 1 h at ambient temperature. The reaction was stopped by filtering the mixture through a glass filter (GF/B; Whatman International Ltd, Maidstone, UK) that was pretreated with

Table 1
Insecticidal activity and receptor affinity of CH-IMI **2** and its alkylated derivatives **3**–**22**[a]

		K_i (nM)	ED_{50} (pmol/fly)
2[b]	H	0.0367	0.117
3[b]	*R*-5-Me	0.0428	0.0626
4[b]	*S*-5-Me	0.313	0.340
5[b]	*R*-4-Me	2.07	0.427
6[b]	*S*-4-Me	5.64	0.562
7	*R*-5-Et	0.0597 ± 0.0115	0.914 ± 0.155
8	*S*-5-Et	18.8 ± 3.40	93.1 ± 23.1
9	*R*-4-Et	31.0 ± 1.27	68.9 ± 31.0
10	*S*-4-Et	24.4 ± 8.53	24.2 ± 2.85
11	*R*-5-*n*-Pro	0.258 ± 0.137	0.253 ± 0.0813
12	*S*-5-*n*-Pro	25.2 ± 12.7	45.3 ± 18.3
13	*R*-4-*n*-Pro	211 ± 38.4	433 ± 130
14	*S*-4-*n*-Pro	73.7 ± 12.9	79.9 ± 5.74
15	*R*-5-Isopropyl	1.11 ± 0.29	33.1 ± 8.52
16	*S*-5-Isopropyl	2340 ± 561	>741 (22%)[c]
17	*R*-4-Isopropyl	590 ± 34.4	>891 (31%)[c]
18	*S*-4-Isopropyl	430 ± 117	33.7 ± 7.88
19	*R*-5-Isobutyl	4.54 ± 1.49	6.01 ± 1.62
20	*S*-5-Isobutyl	52.6 ± 3.57	603 ± 71.9
21	*R*-4-Isobutyl	2750 ± 1330	1129 ± 238
22	*S*-4-Isobutyl	396 ±70.3	356 ± 32.7

[a] The bioactivities of the compound **7–22** were represented as mean ± standard error of means ($n = 3$).
[b] The values of bioactivities of the compound were cited from Ref. 11.
[c] The value in the parentheses was mortality at the concentration presented.

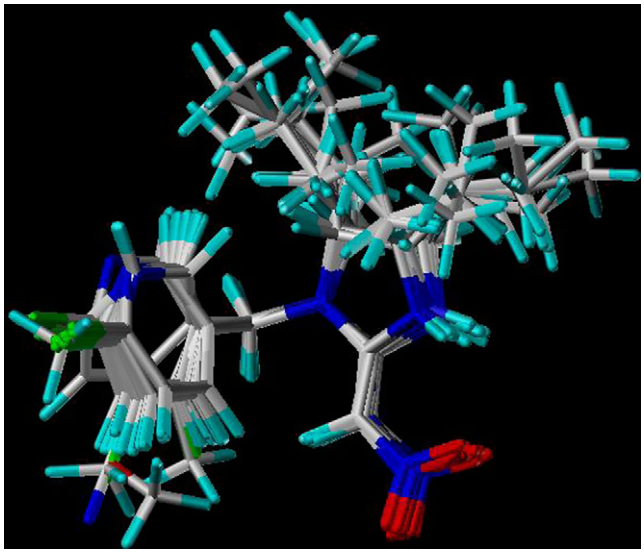

Figure 3. Superposed chemical structures of compounds **1**–**37**.

0.1% polyethyleneimine and mounted on a glass microanalysis holder (KGS-25; Advantec Toyo, Tokyo, Japan). Filtration was performed with the help of an air pump. The tube was rinsed with 1 ml of cold sodium phosphate buffer (10 mM, pH 7.4) containing sodium chloride (50 mM), and the washings were immediately filtered. The glass filter was washed four times with 2 ml of the same cold buffer, and each glass filter was then transferred to a scintillation vial. Radioactivity was measured using a liquid scintillation counter (ALOKA-1000; Aloka Co., Ltd., Tokyo, Japan) in 3 ml of Aquasol-2 (Packard Instrument Co, Meriden, CT). From the concentration–response curve, the molar concentration for 50% inhibition (IC_{50}) of [^{3}H]imidacloprid binding to the receptor was calculated. The K_i value was calculated according to the following equation using PRISM software:

$$K_i = IC_{50}/1 + ([L]/K_d)$$

in which [L] is the final concentration of the radioligand (20 nM) and K_d (2.65 nM) is the dissociation constant of [^{3}H]imidacloprid for the receptor fraction. K_i values of the test compounds were obtained from three separate assays performed in duplicate and are listed in Table 1.

2.4. QSAR analyses using Hansch–Fujita method and CoMFA

The relationship between the structure of the test compounds and the receptor affinity (presented as the reciprocal logarithm of K_i values (pK_i)) was quantitatively analyzed. For the Hansch–Fujita method, the steric parameter B_5 was used, which is the maximum width parameter of the Verloop's STERIMOL parameters,[13] and ΔB_5 was calculated by subtracting the B_5 value of hydrogen, 1, from the intact B_5 value of each substituent. The employed ΔB_5 values were as follows: 0 (H), 1.04 (Me), 2.17 (Et), 2.49 (*n*-Pr), 2.17 (isopropyl), and 3.45 (isobutyl). Superscript characters on the ΔB_5 term indicate the substituted positions. The I_{ipr} parameter was the indicator variable, being one for compounds **15**–**18** with an isopropyl group and zero for others. Linear regression analysis was performed using the QREG 2.05 software.[14]

For CoMFA, not only compounds **1**–**22** but also compounds **23**–**37** (Table 2), whose pK_i values have been reported previously,[12] were included in the data set. The calculations for the optimization and superposition of the test chemicals were performed using the molecular modeling software package SYBYL ver. 7.1 (Tripos Associates, Inc., St Louis, MO). For constructing the initial conformations of the compounds, the x-crystallographic data of imidacloprid was utilized, which binds to the acetylcholine binding protein of *Lymnaea stagnalis* (Ls-AChBP; PDB code, 2ZJU).[15] For all rotatable bonds, a systematic search in SYBYL module was performed. Structures were fully optimized by the semi-empirical molecular orbital method PM3 to give relatively stable conformations. For each optimized conformer, electrostatic potential charges were calculated using MNDO method (electron surface potential charge). The optimized conformer of IMI was used as the reference standard on which the other compounds were superposed. For the superposition of these compounds, four atoms were selected on the basis of our earlier studies (Fig. 3),[16,17] that is, the nitrogen atoms of the pyridine ring and at the 1-position of the imidazolidine ring, the carbon atom at the 2-position of the imidazolidine ring, and the bridge carbon atom between the aromatic and imidazolidine rings. For benzyl derivatives, their substituents were orientated to the opposite site of the nitrogen atom of the pyridine ring of IMI. All superposed conformers were placed in the lattice with 2 Å spaces, and the potential energy fields of each conformer were calculated at the lattice intersections. The electrostatic (Coulombic potential) and steric (Lennard-Jones potential) field energies were calculated at each lattice point using sp^3-carbon atom with a charge of +1.0 as a probe. The data for the receptor affinity of the compounds **1**–**37** were in correlation with these parameters by the partial least-squares method.

2.5. Construction of ligand-binding domain model of housefly nAChR combined with IMI

On the basis of the crystallographic data of the Ls-AChBP bound with IMI, the homology model of the ligand-binding domain (LBD) in houseflies nAChR was constructed using the homology modeling software PDFAMS Pro 2.0 (In-Silico Sciences, Inc., Tokyo, Japan) according to a previous report.[18] The amino acid sequence of the subunit 6 (isoform II) of *M. domestica* AChR (GenBank ID ABJ09669), which was most common among the six splicing variants in the subunit 6 of the housefly receptor,[19] was aligned with that of Ls-AChBP using PDFAMS, as shown in Figure 4. Using the

```
MdS6II   RLLNHLLSTYNTLERPV---ANESDPLEVKFGLTLQQIIDVDEKNQLLIT
Ls-AChBP LDRADILYNIRQTSRPDVIPTQRDRPVAVSVSLKFINILEVNEITNEVDV

MdS6II   NLWLSLEWNDYNLRWNDSEYGGVKDLRITPNKLWKPDVLMYNSADEGFDG
Ls-AChBP VFWQQTTWSDRTLAWD--SSHSPDQVSVPISSLWVPDLAAYNAISKPEV-

MdS6II   TYHTNIVVKHGGSCLYVPPGIFKSTCKMDITWFPFDDQHCEMKFGSWTYD
Ls-AChBP LTPQLARVVSDGEVLYMPSIRQRFSCDVSGVDTE-SGATCRIKIGSWTHH

MdS6II   GNQLDLVLSSEDGGDLSDFITNGEWYLIAMPGKKNTIVYACCPEPYVDVT
Ls-AChBP SREISVDPTTENSDDSEYFSQYSRFEILDVTQKKNSVTYSCCPEAYEDVE

MdS6II   FTIQIRRR
Ls-AChBP VSLNFRKK
```

Figure 4. Multiple sequence alignment of subunit 6 (subtype II) of nicotinic acetylcholine receptor of *Musca domestica* (MdS6II) with an acetylcholine binding protein of *Lymnaea stagnalis* (Ls-AChBP).

simulated annealing method,[20] the three-dimensional structure of the ligand-binding domain, which is formed with two adjacent subunits extracted from the homopentamer subunits, was constructed. The coordinate of IMI was fixed during the simulated annealing. The constructed model was then energy-minimized for 5000 iteration of conjugated gradients using the force field and partial charges of the molecular mechanics MMFF94.[21,22] A graphical image of the surface of the ligand-binding pocket was created using the multichannel surface tool of the MOLCAD module in the SYBYL software.

2.6. Evaluation of insecticidal activity

Assay method was essentially the same as in our previous report.[12] For evaluating the insecticidal activity, female houseflies anesthetized using carbon dioxide were topically treated with methanol containing synergists piperonyl butoxide and NIA16388 [0.2% (w/v)]. After 1 h, 0.22 μl of 50% ethanol solutions containing a test chemical at various concentrations were injected in the dorsal side of the thorax of reanesthetized flies. Insecticidal activity was evaluated 1 h after injection. The ED_{50} values (effective dose for inducing paralysis or death in 50% of the houseflies) were calculated using probit transformation and are listed in Table 1.

3. Results and discussion

3.1. Receptor affinity

The inhibition constant, K_i (nM), was evaluated as an indicator of the affinity to the receptor (Table 1). Among the ethylated compounds, *R*-5-Et compound **7** showed the equivalent potency to the unsubstituted **2** and *R*-5-Me compound **3**, whereas the potencies of compounds **8**–**10**, which were nearly similar, were 4- to 60-fold lower than those of the corresponding Me compounds **4**–**6**. The extension from ethyl to *n*-propyl group lowered the receptor affinity, but only to a little extent (1.3- to 6.8-fold lower than those of the corresponding compounds). In contrast, the change from ethyl to isopropyl group markedly decreased the affinity (compared to isopropyl with Et; 17-, 130-, 45- and 18-fold less potent at *R*-5, *S*-5, *R*-4 and *S*-4, respectively). By comparing the affinities of the compounds with the largest substituent, isobutyl group, with those of the compounds having a *n*-Pro group, more than 10-fold lower affinities were observed for *R*-position-substituted compounds (**11**, **13** vs **19**, **21**), whereas only 2- to 5-fold lower potencies were observed for *S*-position-substituted compounds (**12**, **14** vs **20**, **22**). These results demonstrated that the introduction of a substituent into the imidazolidine ring was fundamentally disadvantageous, but the introduction of a small substituent such as Me or Et groups to the *R*-5-position was permissible. This suggests that the position and type of substituent on the ethylene moiety of the imidazolidine ring should be recognized accurately by the receptor.

3.2. QSAR analyses

First, the relationship between the structure of the compounds and their receptor affinity was quantitatively analyzed using Hansch–Fujita method, a conventional QSAR method, shown as the following equation:

$$\mathrm{p}K_i = \underset{(0.33)}{-1.22}\Delta B_5^{S4} \underset{(0.33)}{-1.37}\Delta B_5^{R4} \underset{(0.33)}{-1.08}\Delta B_5^{S5} \underset{(0.33)}{-0.31}\Delta B_5^{R5} \underset{(0.63)}{-1.36}I_{ipr} + \underset{(0.66)}{10.31} \quad (1)$$

$$n = 21, \mathrm{SD} = 0.53, r^2 = 0.91, F(515) = 29.42 > 0.999$$

In this and following equations, n is the number of compounds, SD is the standard deviation and r is the correlation coefficient. F is the ratio between the regression and residual variances. The number in the parentheses was 95% confidence interval. In the multiple regression analyses, the number of variables reportedly influences the correlation efficient, known as 'chance correlation'.[23] Because the number of test compounds for generating Eq. 1 was a little smaller than that recommended by Topliss and Costello (approximately 30 compounds for 5 variables, whereas 21 compounds for 5 parameters in this study), Eq. 1 may be considered as the tentative result. However, the distinctive SAR characteristics possibly become clear by correlating the activity of these derivatives with the physicochemical parameters of their substituents. For example, the steric parameter, B_5, which is the maximum width parameter of the Verloop's STERIMOL parameters, was employed (ΔB_5 represents the value relative to that of the hydrogen atom). The equations formulated using other steric parameters such as molar refractivity and Taft's E_s showed inferior correlation coefficients to Eq. 1 (data not shown). All terms other than $\Delta B_5{}^{R5}$ in this equation were significant above the 99.9% level as examined by the t test, whereas the significance level was 92.9% for $\Delta B_5{}^{R5}$. The minus sign of the coefficient of each ΔB_5 term in Eq. (1) suggests that the introduction of the substituents at each position is unfavorable for receptor affinity, particularly unfavorable at the *R*-4-position. The smallest coefficient of $\Delta B_5{}^{R5}$ term suggests that the introduction of a substituent at the *R*-5-position could be less adverse for the receptor affinity in the case of the introduction of a substituent with a small size. The introduction of the I_{ipr} term improved the correlation coefficient, suggesting that the branched isopropyl group decreased the affinity drastically.

The statistical analysis of the receptor affinity for 37 compounds using CoMFA was shown in the following Eq. (2).

$$\mathrm{p}K_i = 6.47 + [\mathrm{CoMFA\ field\ terms}] \quad (2)$$

$$\mathrm{CN} = 6, n = 37, s = 0.32, r^2 = 0.97,$$

$$\mathrm{Cross\text{-}validated}[s_{cv} = 1.14, q^2 = 0.61],$$

$$\mathrm{RC}[\mathrm{Steric} = 63.6\%, \mathrm{Electrostatic} = 36.4\%]$$

In this equation, CN indicates the number of latent variables, and s_{cv} and q are the standard deviation and correlation coefficient obtained from the leave-one-out cross-validation, respectively. *RC* refers to the relative contribution of steric and electrostatic effects to variations in receptor affinity. The $\mathrm{p}K_i$ values calculated by Eq. 2 are shown in Table 2. In the preliminary analyses, the orientation of the substituents of benzyl derivatives to the same site of the nitrogen atom of the pyridine ring of IMI decreased the cross-validated s_{cv} and q^2 values (data not shown). The major steric and electrostatic potential contour maps with IMI were drawn according to Eq. 2 (Fig. 5). In Figure 5A, two large steric-favorable regions appeared around the chlorine atom attached at the pyridine ring and the *R*-5-positioned hydrogen atom of the imidazolidine ring, whereas the sterically hindered regions appeared around the nitrogen atom of the pyridine ring and the 4-positioned hydrogen atoms of the imidazolidine ring. Electrostatic interaction regions (Fig. 5B) did not appear around the imidazolidine ring, probably because such substituents were limited only to alkyl groups. CoM-

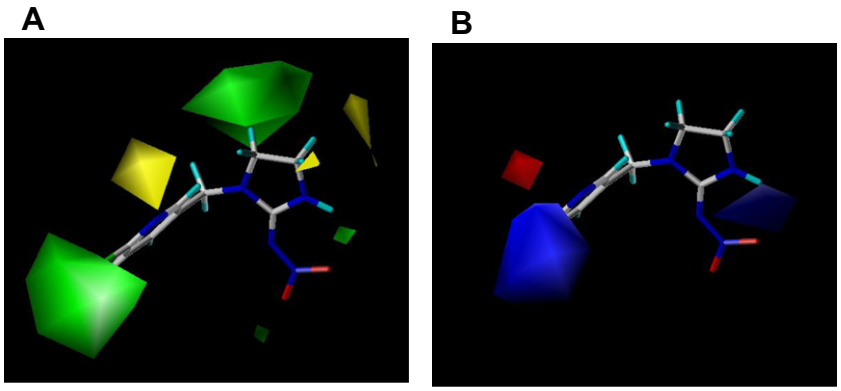

Figure 5. Contour diagrams of steric (A) and electrostatic (B) fields with imidacloprid according to Eq. 2. In (A), the green and yellow areas indicate the steric-permissible and unfavorable regions for the receptor affinity, respectively. In (B), the red and blue areas indicate the regions where the more negative and positive electrostatic interactions with the receptor binding site increase the receptor affinity, respectively.

FA as well as the analysis using Hansch–Fujita method suggests that the introduction of a substituent at the *R*-5-position could be less adverse.

Previously, various research groups, including ours, have performed 3D-QSAR analyses for neonicotinoids, particularly focusing on pharmacophores such as the nitroimino group and the pyridine ring.[16,17,24–26] The results showed various important physicochemical properties influencing the biological activities of the ligands; that is, an electron-withdrawing group such as the nitro, cyano, or trifluoromethyl group attached to the imino group and the chlorine atom attached to the pyridine ring were important. Because these analyses did not refer to the compounds with various asymmetrically modified ethylene moiety of the imidazolidine ring, the QSAR analysis for this part has not been elucidated in detail. Since the launch of acyclic neonicotinoids such as nitenpyram and acetamiprid as effective agrochemicals, the ethylene moiety has not been considered as an important pharmacophore. Nonetheless, our present study demonstrates that the ethylene moiety should also play an important role in the receptor affinity.

3.3. Construction of ligand binding domain model of housefly nAChR combined with IMI

The surface of the ligand-binding site of the receptor was drawn to examine whether the ligand used in this study could be acceptable in this pocket (Fig. 5A and B). Over the *R*-5-position of the imidazolidine ring of IMI, a vacant space to accept a small substituent was observed (Fig. 6A), and actually *R*-5-Me-CH-IMI was able to be packed in this space with some room directed over methyl group (Fig. 6B). Employing the sequences of α2 (GenBank ID ABD37617) or α5 (GenBank ID ABY40460) subunit instead of that of subunit 6 for construction of ligand binding domain model, almost the same vacant space appeared over the *R*-5-position (data not shown). The ethyl group was also accepted in this region, but *n*-propyl group was a little outside this region (data not shown). As the substituent-acceptable space was surrounded by the aromatic amino acid residues such as Trp and Tyr, the hydrogen atoms of the alkyl group could interact with the receptor surface *via* CH-π interaction. The binding model was consistent with the trend of the in vitro activity and the present QSAR analyses.

Crystal structures of mollusk AChBP interacting with imidacloprid have been resolved in 2008.[15,27] On the basis of these structures, it was pointed out that the structurally important parts of imidacloprid such as chlorine and nitrogen atoms of pyridine moiety and nitro group bind the receptor. Matsuda et al. also suggested that the hydrogen atoms of the imidazolidine ring (R_3 in Fig. 1) interact with the tryptophan residue of the receptor via CH–π interaction.[18] The substantial decrease in the binding affinity of *R*-4-substituted compounds is compatible with their suggestion. The introduction of an alkyl group at the 4-position might separate

Table 2
The reciprocal of the K_i values observed and calculated using the equation derived from the CoMFA analysis

No.	Compound	pK_i Obsd.	CoMFA		No.	Compound	pK_i Obsd.	CoMFA	
			pK_i Calcd	Δ				pK_i Calcd	Δ
1	Imidacloprid	8.43	8.46	−0.03	20	*S*-5-Isobutyl	7.28	6.90	0.38
2	H	10.44	10.60	−0.16	21	*R*-4-Isobutyl	5.56	5.60	−0.04
3	*R*-5-Me	10.37	10.31	0.06	22	*S*-4-Isobutyl	6.40	6.43	−0.03
4	*S*-5-Me	9.50	9.24	0.26	23	4,4-$(CH_3)_2$	5.81	6.27	−0.46
5	*R*-4-Me	8.70	8.12	0.58	24	5,5-$(CH_3)_2$	8.98	9.59	−0.61
6	*S*-4-Me	8.25	7.78	0.47	25	phenyl	5.24	5.54	−0.30
7	*R*-5-Et	10.22	10.07	0.15	26	*o*-F	5.43	5.39	0.04
8	*S*-5-Et	7.73	7.87	−0.14	27	*o*-Cl	5.26	5.26	0.00
9	*R*-4-Et	7.51	7.28	0.23	28	*o*-CH_3	5.05	5.14	−0.09
10	*S*-4-Et	7.61	7.41	0.20	29	*m*-F	6.52	6.65	−0.13
11	*R*-5-*n*-Pro	9.59	9.82	−0.23	30	*m*-Cl	6.30	5.50	0.80
12	*S*-5-*n*-Pro	7.60	7.54	0.06	31	*m*-CH_3	4.54	5.29	−0.75
13	*R*-4-*n*-Pro	6.68	6.83	−0.15	32	*m*-CH_3O	5.48	5.33	0.15
14	*S*-4-*n*-Pro	7.13	7.19	−0.06	33	*m*-CN	6.75	6.79	−0.04
15	*R*-5-Isopropyl	8.95	8.98	−0.03	34	*p*-F	4.30	4.20	0.10
16	*S*-5-Isopropyl	5.63	5.68	−0.05	35	*p*-Cl	6.76	6.79	−0.03
17	*R*-4-Isopropyl	6.23	6.31	−0.08	36	*p*-CH_3	6.38	6.34	0.04
18	*S*-4-Isopropyl	6.37	6.45	−0.08	37	Olefin	8.57	8.67	−0.10
19	*R*-5-Isobutyl	8.34	8.26	0.08					

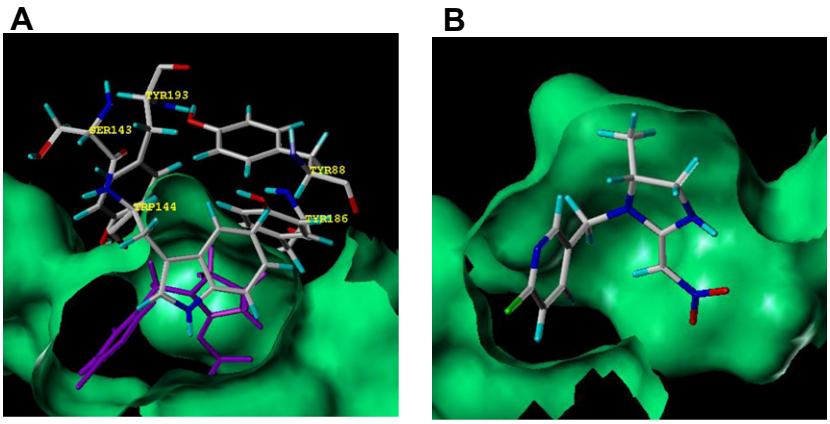

Figure 6. The molecular surface (green) of the ligand-binding site of the housefly receptor. (A) the binding site interacts with IMI (violet). Five amino acids (Y88, S143, W144, Y186 and Y193) constructing the vacant space over the *R*-5-position of the imidazolidine ring were also drawn together. (B) the binding model of the receptor with the *R*-5-methylated CH-IMI as a ligand.

the nitro group, one of the important moiety interacting with the receptor, from the interactive amino acid of the receptor.

Our binding model is consistent with the present QSAR analyses, but not for all compounds reported previously. For example, we cannot explain the high bioactivities of novel neonicotinoids with a bulky modification.[28] In relation to this, Tomizawa et al. recently demonstrated that two binding modes of imidacloprid are considerable in *Lymnaea* AChBP.[29,30] If the size and structure of ligands change, it should be fully considered that the binding mode would change. Because the ligand-binding site is orientated near the open entrance of the receptor, the bulkier moiety might be out of the ligand-binding region. The introduction of the alkyl group might influence the conformation of the receptor or the binding modes of the compounds, and we presently have no indication whether the binding mode of alkylated analogues with a nitromethylene group to the receptor would change. However, the *R*-5-position-specific bioactivity should be explained by the vacant space of our receptor model in this study.

3.4. Insecticidal activity and correlation between the biological activities

ED_{50} values of the derivatives are shown in Table 1. Compared with the unsubstituted CH-IMI **2**, the methylated compounds **4**–**6** showed 3 to 5-fold lower activity, whereas *R*-5-methylated compound **3** had 2-fold higher activity. The insecticidal activity of compounds with an ethyl or a *n*-propyl group was over 100-fold lower than that of the corresponding methylated compounds, except for the *S*-4-ethylated, *R*-5-ethylated and *n*-propylated compounds (**6** vs **10**; **3** vs **7** and **11**), whose activity fell only to 1/43, 1/14 and 1/4, respectively. The introduction of a wide substituent at any positions of the imidazolidine ring drastically decreased the activity (ethyl group vs isopropyl group, and *n*-propyl group vs isobutyl group). In particular, the ED_{50} values of the *R*-4- and *S*-5-isopropylated and isobutylated compounds **16**, **17**, **20** and **21** were more than 600 pmol/fly. Among the four 4- and 5-positions of the imidazolidine ring, the introduction of the substituent at the *R*-4-position was the most unfavorable for the insecticidal activity.

Positive correlation was observed between their insecticidal activity (presented as the reciprocal logarithm of ED_{50} values (pED_{50})) and receptor affinity (pK_i) (Fig. 7; $n = 19$, $r = 0.89$), suggesting that the receptor affinity was the main important factor influencing the insecticidal activity even if the imidazolidine ring was modified. In this study, we did not consider the metabolic effects in flies by a pre-application of the synergists. The ethylene moiety of the imidazolidine ring was reportedly metabolized oxidatively in houseflies.[31] Using methylated CH-IMI derivatives, we reported that the degree of the synergistic ratio changes according to the substituted position.[11] The modification of this part should prevent the moiety from being metabolized. If a compound with a modified imidazolidine ring has high receptor affinity and is not easily metabolized in insects, it is anticipated to exert the high insecticidal activity. In addition, we injected the test compounds into the flies to prevent the absorption of the compounds through the cuticle layer. Because the hydrophobicities of IMI and CH-IMI are reportedly low,[32] it is difficult for these neonicotinoids to exert the insecticidal activity by the topical application. The introduction of a hydrophobic substituent to the ethylene moiety is expected to improve the absorption potency of the compounds through the cuticle, resulting in the development of more effective insecticides.

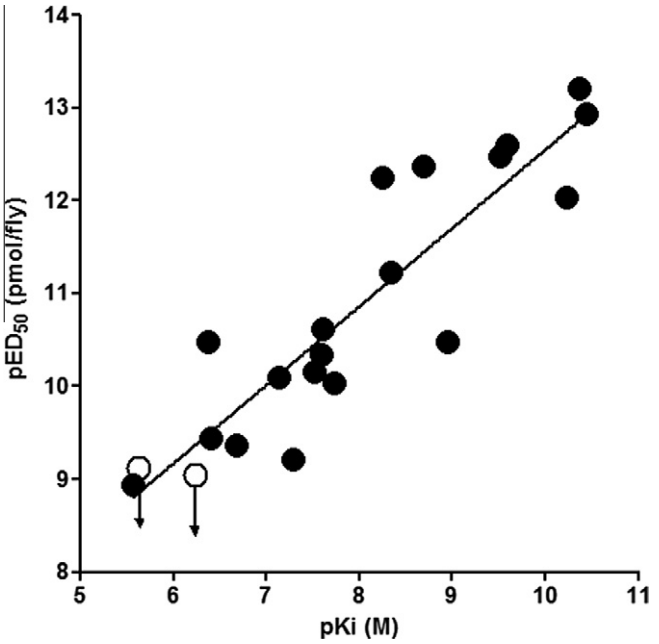

Figure 7. Correlation between the receptor affinity (pK_i) of the neonicotinoid derivatives and their insecticidal activity (pED_{50}). Open circles indicates compounds **16** and **17**, which were omitted from the correlation analysis.

4. Conclusion

We found the steric permissive region of the receptor over the ethylene moiety of imidacloprid, which was surrounded by

aromatic amino acid residues. Both electrostatic substituents and aromatic and unsaturated substituents could interact with this region to exert higher receptor affinity through electrostatic, van der Waals, π–π, and CH–π interactions. A more detailed SAR study of this moiety will be conducted in the near future. The present analyses are consistent with experimental results and may provide useful information for designing new insecticides.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.09.007. These data include MOL files and InChiKeys of the most important compounds described in this article.

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