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ORIGINAL PAPER

N-demethylation of neonicotinoid insecticide acetamiprid by bacterium *Stenotrophomonas maltophilia* CGMCC 1.1788

Ting Chen · Yi-Jun Dai · Juan-Fang Ding · Sheng Yuan · Jue-Ping Ni

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Abstract Our previous study found that *Stenotrophomonas maltophilia* CGMCC 1.1788 could hydroxylate imidacloprid (IMI) to 5-hydroxy IMI. Here we first report that *S. maltophilia* CGMCC 1.1788 can demethylate acetamiprid (AAP) to form IM 2-1 that was characterized by HPLC-MS/MS and NMR. IM 2-1 retained only 10.5% contact activity and 13.1% oral activity of AAP against horsebean aphid. Time course of biotransformation under existing of sucrose revealed that 58.9% of AAP disappeared, but only 16.7% of reduced AAP was transformed to IM 2-1, after 8 days. Both demethylation and degradation of AAP contribute to the weak bioefficacy of AAP in soil application. The differences in metabolism and detoxification pathways between AAP and IMI are probably originated from the structural differences of these insecticides.

Keywords Acetamiprid · Hydroxylation · Imidacloprid · N-demethylatylation · *Stenotrophomonas maltophilia*

Chen Ting and Yi-Jun Dai are co-first authors.

T. Chen · Y.-J. Dai · J.-F. Ding · S. Yuan (✉) · J.-P. Ni
Nanjing Engineering and Biotechnology Research Center for Microbiology, Jiangsu Key Lab for Biodiversity and Biotechnology, College of Life Science, Nanjing Normal University, Nanjing 210046, P.R. China
e-mail: shengyuan@email.njnu.edu.cn

J.-P. Ni
Jiangsu Pesticide Research Institute, Nanjing 210036, P.R. China

Introduction

Neonicotinoid insecticides, imidacloprid (IMI) and acetamiprid (AAP) (Fig. 1), belong to the chloronicotinyl class of chemistry and possess similar physicochemical properties. They represent the fastest-growing class of insecticides introduced to the market because they are active against numerous sucking and biting pest insects, including aphids, whiteflies, thrips, leaf miners, beetles, and some lepidopteran species. Their superior physicochemical properties render them to be applicable for a wide range of techniques, including foliar, seed treatment, soil drench, and stem application (Nauen et al. 2003; Rouchaud et al. 1994; Tokieda et al. 1997). Previously we reported that some strains of bacterium *Stenotrophomonas maltophilia* isolated from soils and screened from stock culture were able to hydroxylate IMI to form 5-hydroxy IMI that was easily converted to olefin IMI under acid condition. The insecticidal activity of olefin IMI was 19 times higher than that of IMI against horsebean aphid (Dai et al. 2006). These results lead a new investigation on the microbial transformation to understand why longer time and better control are achieved when IMI is applied as a soil drench application than the other methods. In the present study, we analyzed different neonicotinoid insecticides for biotransformation substrates, and discovered that the same *S. maltophilia* strain CGMCC 1.1788, which had the highest hydroxylation activity of IMI, could demethylate

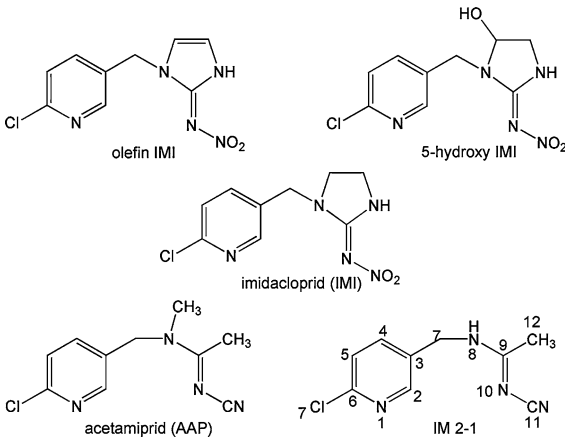

Fig. 1 Molecular structure of imidacloprid, acetamiprid and its metabolites

AAP to form IM 2-1. Bioassay results showed that IM 2-1 exhibited comparative bioefficacy against horsebean aphid in contact and oral test; however its activity was about 10 fold lower than the parent compound AAP. The demethylation of APP was first reported from pure microorganisms. The effect of soil microbial demethylation on the application method of insecticides and the mechanism are discussed in this study.

Materials and methods

Chemicals

AAP and IMI samples (greater than 97% purity) were provided by Nanjing Pesticides Factory, Nanjing, China. Acetonitrile (HPLC grade) was used for high performance liquid chromatography (HPLC) analysis; other solvents and reagents were analytical grade from commercial sources.

Microorganism, medium and cultivation

Bacterium *S. maltophilia* CGMCC 1.1788 was purchased from China General Microbiological Culture Collection Center. Cultivations were carried out in a 100-ml flask containing 30 ml of Luria-Bertani (LB) broth containing peptone 10 g l^{-1}, yeast extract 5 g l^{-1} and NaCl 10 g l^{-1} (pH 7.2), and incubated at 30°C in a rotary shaker with 220 rpm for 24 h. The culture broth (0.1 ml) was then inoculated in a 100-ml flask containing 10 ml of LB broth, and incubated at the same conditions.

Large-scale preparation of metabolites of AAP

The biotransformation process of AAP in a 5-L fermenter was described in a previous study (Dai et al. 2006). In the end of the transformation, cells were removed by centrifugation at 6,000*g* for 20 min. The collected supernatant was extracted with equal volume of ethyl acetate. The organic phase was then dehydrated with anhydrous sodium sulfate, and concentrated to about 1/20 of original volume in a vacuum rotary evaporator. The resulting solution was filtered by a 0.22-μm pore size ultrafiltration membrane, and the filtrate solution was further concentrated for the identification of biotransformation metabolites.

The metabolites were purified using thin-layer chromatography (TLC) by concentrating the extract on a pre-coated silica-gel TLC plate (silica G, 20 × 20 cm, 0.25 mm thickness) with chloroform-methanol solution (90:10 by volume). The Rf value of a major metabolite and AAP were 0.64 and 0.83, respectively. The collected metabolites was dissolved in acetonitrile and centrifuged at 10,000*g* to remove the silica. The organic solvents were then removed and the residue was dried under vacuum condition.

Identification of the metabolite of AAP

Mass spectrum was conducted using an Agilent 1100 LC-MSD mass spectrometer equipped with an electrospray interface that was operated in the positive ion mode. ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectra of the biotransformation product were obtained in DMSO-d_6 using Bruker AV-400 spectrometer (Switzerland) operating at 100 and 400 MHz, respectively. Chemical shifts were referenced against internal TMS. Several kinds of NMR techniques were used, including ^{1}H, ^{13}C NMR, DEPT, ^{1}H–^{1}H, ^{1}H–^{13}C COSY to assign chemical shifts to proton and carbon atom (δ).

Bioassays of metabolite of AAP

The feeding and contact bioassay against horsebean aphid *Aphis craccivora* for metabolites of AAP was tested by the method described by Nauen et al. (1999), and was conducted at National Pesticide Research & Development South Centre, Nanjing.

Biotransformation of IMI and AAP

The bioconversion of AAP and IMI were conducted by adding 0.5 g l^{-1} AAP and IMI (w/v) and 5% sucrose into LB broth, in which the transformation was carried out in standard cultivation conditions for indicated time. For the control, sucrose was excluded from the broth. Before sampling, double-distilled water was added to the transformation broth up to the initial weight. The samples were then centrifuged at 10,000*g* for 10 min to remove cell residues. The supernatant was collected and diluted to an appropriate volume for the analysis of substrates and transformed products.

Inhibition of N-demethylation of AAP by piperonyl butoxide (PBO)

The inhibition of AAP demethylation activity by PBO was examined using the method of Matsuzaki and Wariishi (2004). The detail procedures have been described in our previous report (Dai et al. 2007).

HPLC analysis

Agilent 1100 HPLC system equipped with a Zorbax ODS C18 column (4.6 × 250 mm, 5 μm) was employed for the analysis of AAP and its metabolites. Elution was carried out at a flow rate of 1 ml min^{-1} with mobile phase containing in 65% (volume) water and 35% (volume) acetonitrile as well as 0.01% acetic acid. The signal was monitored at 236 nm with an Agilent G1314A UV detector. For the analysis of IMI and 5-hydroxy IMI, the mobile phase was 75% water and 25% acetonitrile, and the monitored wavelength was 269 nm.

Results

Microbial conversion of AAP and the identification of metabolites

In addition to the transformation from IMI to 5-hydroxy IMI, *S. maltophilia* could transform AAP to a polar metabolite (retention time 5.8 min) (Fig. 2). HPLC-MS/MS analysis exhibited that the ion mass of metabolite was m/z 209 (M + H) (Fig. 3a). Compared with the substrate AAP (223, M + H), the molecular weight of the metabolite was reduced by 14, suggesting that the metabolite might be a derivate of AAP after demethylation. The ion masses of the fragments of the metabolite (Fig. 3b) were m/z 194 (M + H-CH_3), 167 (M-CH_3-CN) and 126 (M-$NHCCH_3NCN$). The mass spectrography of the metabolite crystals was consistent with the results of HPLC-MS/MS analysis. Further analysis by NMR indicated that the metabolite possesses nine hydrogen and nine carbon atoms (Table 1). Therefore the metabolite was formed by reducing one carbon and two hydrogen atoms from the substrate AAP. Furthermore, a new proton that was non-coupled with carbon atom in ^{13}C–^{1}H COSY spectrum could be explained by the proton attachment to nitrogen atom (N8). Therefore, it can be concluded that the metabolite was an N-demthylated metabolite of AAP. This compound is usually named as IM 2-1.

Bioassay of IM 2-1 against aphid *Aphis craccivora*

Nitroguanidine and cyanoimine are the most important pharmacophore for neonicotinoid insecticide; metabolites that do not carry these pharmacophores are not toxic (Suchail et al. 2004). Because IM 2-1 retains cyanoimine moiety, its bioassay exhibited a comparative activity against aphid *A. craccivora*, and its oral ingestion appeared to be much more superior to the contact test. However, because of the loss of the N-methyl group, IM 2-1 had only 10.5% contact activity and 13.1% oral activity of those of AAP against aphid (Table 2).

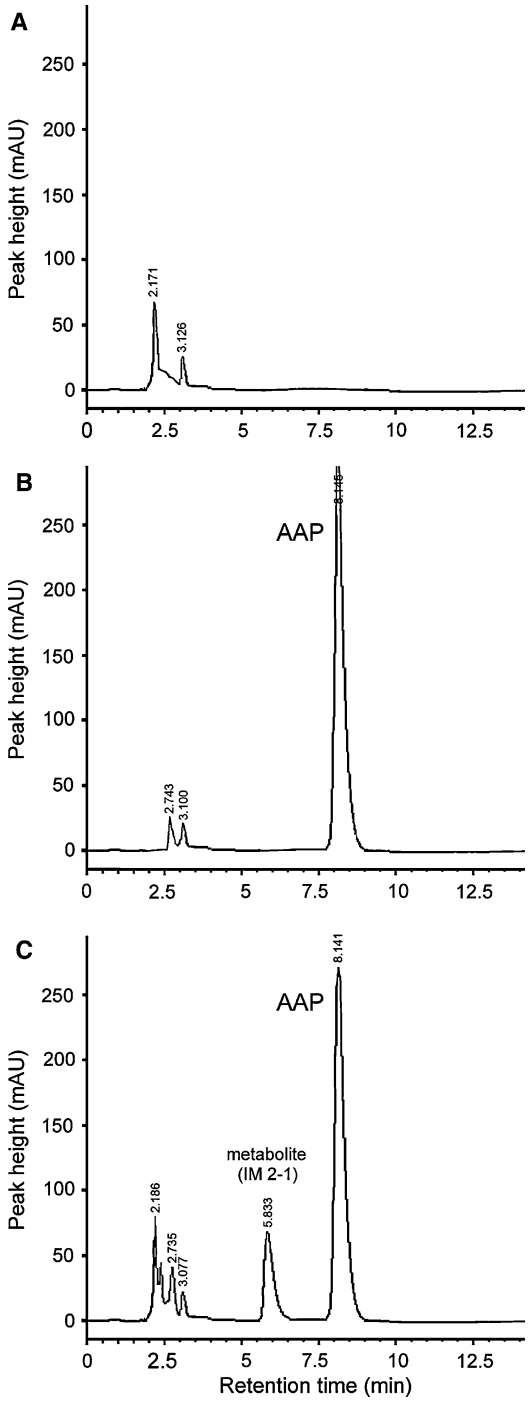

Fig. 2 HPLC spectrum of metabolism of AAP by *S. maltophilia.* (**a**) the broth containing *S. maltophilia* CGMCC 1.1788 alone for control; (**b**) the broth containing substrate AAP alone for control; (**c**) the broth containing AAP and bacterium. HPLC analysis was conducted with Agilent HPLC system; the retention time at 5.8 and 8.1 min are the peaks of metabolite IM 2-1 and AAP, respectively

Time course of microbial conversion of AAP

The time course of bioconversion of AAP to IM 2-1 (Fig. 4a) in LB broth with addition of 5% sucrose showed that the metabolism rate was almost equal to the rate from IMI to 5-hydroxy IMI (Fig. 4b). After bioconversion for 8 days, 58.9% of AAP (initial amount 2.55 mmol l^{-1}) and 66.3% (initial amount 2.35 mmol l^{-1}) IMI were disappeared, the reduced amounts of AAP and IMI were 1.50 and 1.56 mmol l^{-1}, respectively under existing of sucrose. However, the produced amount of IM 2-1 and 5-hydroxy IMI were 0.25 and 1.26 mmol l^{-1} respectively, that is, only 16.7% of reduced APP was transformed to IM 2-1, whereas 80.8% of reduced IMI was transformed to 5-hydroxy IMI. Compared to hydroxylation IMI, most of the reduced AAP was apparently degraded during biotransformation, rather than deposited as IM 2-1.

In previous study, we reported that carbohydrates could prompt the IMI hydroxylation activity of *S. maltophilia* CGMCC 1.1788, and sucrose is the best promoter (Dai et al. 2006). In growing culture transformation, sucrose enhanced the biomass and improved the hydroxylation activity of IMI (Dai et al. 2007). In this study, sucrose was selected to examine if it also accelerate the demethylation of AAP. From Fig 4a, the reduced amount of AAP was 1.50 mmol l^{-1} in LB broth with addition of sucrose, however it was only 0.85 mmol l^{-1} in that without sucrose after biodegradation for 8 days. Meanwhile the amount of formed IM 2-1 in LB broth with addition of sucrose was four times than that without sucrose (0.25–0.06 mmol l^{-1}). Therefore sucrose could accelerate the biodegradation of AAP and enhanced the demethylation of AAP by *S. maltophilia* CGMCC 1.1788, which may be due to increase of biomass by addition of sucrose into LB broth.

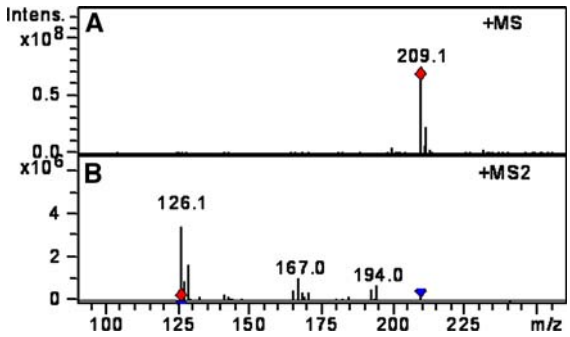

Fig. 3 Mass spectrography of the metabolite of AAP

Table 1 ^{13}C-NMR and ^{1}H-NMR chemical shift assignments for the demethylated product of AAP in DMSO-d_6

Position	^{13}C NMR spectrum	^{1}H NMR spectrum
C2	149.7	8.34(d) 1H, J = 2.3 Hz
C3	133.6	–
C4	139.7	7.77(dd) 1H, J = 8.2 Hz, J = 2.3 Hz
C5	124.5	7.51(d) 1H, J = 8.2 Hz
C6	149.6	–
C7	41.9	4.41(s) 2H
N8	–	9.29(bs) 1H
C9	172.4	–
C11	118.0	–
C12	20.9	2.24(s) 3H

Inhibition of PBO on the N-demethylation of AAP

PBO is a common inhibitor of cytochrome P450, and is often used for demonstrating whether the reaction is catalyzed by cytochrome P450 enzymes (Sato et al. 2002). Previously we hypothesized that cytochrome P450 was involved in hydroxylation of IMI by PBO inhibition (Dai et al. 2007). In this study, we demonstrated that the N-demethylation of AAP was also inhibited by PBO, and the relative activity was reduced by increasing PBO concentration (Fig. 5).

Discussion

Study on the metabolism of AAP by plant, rat and honeybee showed that, besides producing metabolites such as IM 1-2, IM 1-4, IM 1-5 and IC-0, the N-demthylation of AAP to yield IM 2-1 was the main pathway (Brunet et al. 2005). However, metabolic products of AAP in soils under aerobic conditions were IM 1-4, IM 1-5 and IC-0, while the metabolic products of AAP in an aerobic sediment–water system were IM 1-2, IM 1-4 and IC-0. Transformation of AAP to IM 2-1 in the pure cultures of bacteria or in soils has not been observed so far (Tokieda et al. 1997; 1999a, b). Our results indicated that some soil bacteria such as *S. maltophilia* could transform AAP to IM 2-1, hinting that soil containing these degrading bacteria might be able to metabolize AAP to form IM 2-1.

In the process of biotransformation of AAP in medium with addition of sucrose, the amount of product IM 2-1 was increased in initial four days, after that it was no longer increased and kept at a constant level. However the substrate AAP was continuously reduced. In honeybee and plant, the further degradation of IM 2-1 yields the end products namely, IC-O (6-chloro-nicotinic acid) (Brunet et al. 2005). However beside the product IM 2-1, no other metabolite in transformation broth was observed under UV wavelength by HPLC analysis in this study. It is possible that when the amount of IM 2-1 reaches up a threshold value, over IM 2-1 above threshold further come from AAP was converted to other metabolites without UV absorbance or degraded completely.

Table 2 Efficacy of IM 2–1 against aphid *Aphis craccivora* in oral ingestion and contact test bioassays (48 h)

Mode of activity	Compounds	*LC* 50[a] (mg/l)	*FL* 95%[b]	Slope
Contact (Dip Test)	IM 2–1	2.314	1.619–3.307	0.98
	AAP	0.244	0.212–0.280	2.18
Oral ingestion	IM 2-1	0.367	0.305–0.443	1.28
	AAP	0.048	0.039–0.060	2.12

[a] *LC* 50 is 50% lethal concentration

[b] *FL* 95% is 95% fiducial limits

Bioassay was replicated three times, the number of pest exceeded 30 in each dependant experiment, and the total number of pest was more than 100

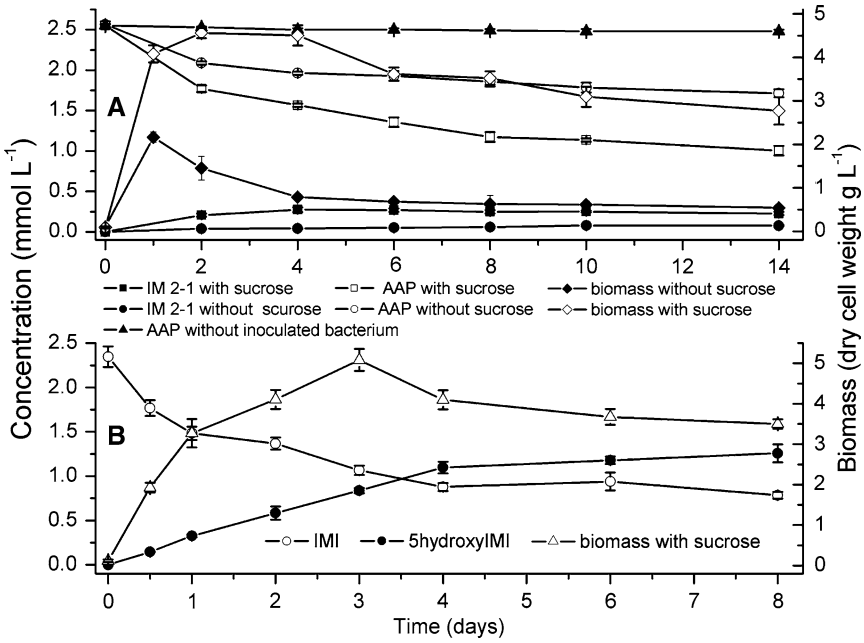

Fig. 4 Time course of metabolism of AAP (**a**) and IMI (**b**) by *S. maltophilia*. The volume of growing culture was 10 ml medium in 100-ml flask. Other cultivation and reaction conditions were the same as described in the section of Materials and Methods. Experiments were replicated three times, data are expressed as mean ± SD ($n = 9$)

It is noticed that *S. maltophilia* mainly transform IMI to 5-hydroxyl IMI, which can be further transformed to high toxic olefin IMI at a higher molar ratio. However, *S. maltophilia* mainly degrades AAP and only transforms a part of AAP to low toxic metabolite IM 2-1 at a low molar ratio. It had been reported that AAP was superior after foliar application, whereas IMI soil application demonstrated a much greater systemic activity (Palumbo et al. 1999; Buchholz and Nauen 2001) although the metabolic mechanism in soil was not clear. Based on our results, the low efficacy of AAP in soil application can be attributed to the fact that AAP could be easily degraded and the partial degraded intermetabolite—demethylated IM 2-1 remained less bioactivity. In contrast, IMI in soil application can be transformed to highly active metabolites such as olefin IMI, which exhibits 10 and 16 fold more active against whitefly and aphid, respectively. Nauen et al. (1998) speculated that, in soil application, a few of biologically active metabolites arising from the parent compound were acting in concert with the remaining parent compound imidacloprid, and this provided a good control and long-lasting residual activity against plant-sucking pests in certain crops. As leaf diping of IMI did not produce olefin metabolite, it can be concluded that microbial action in soil is vital to the efficacy of this insecticide.

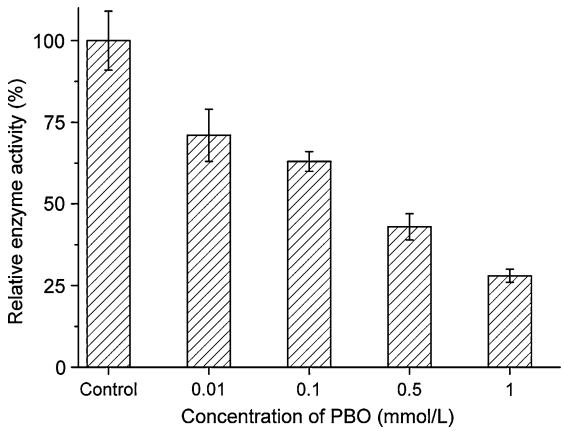

Fig. 5 Effect of PBO on N-demethylation activity by resting cells transformation. The biomass in transformation broth was 0.35 mg ml^{-1} dry cell weight. Reaction was conducted in a 100-ml flask containing 10 ml transformation broth. Experiments were replicated three times, data are expressed as mean ± SD ($n = 9$)

Interestingly, the same *S. maltophilia* CGMCC 1.1788 can hydroxylize IMI, but demethylize AAP in this study. Our previous study indicated that cytochrome P450 was involved in the hydroxylation of IMI by the inhibition of PBO (Dai et al. 2007). In this study, we proved that N-demethylation of AAP was also inhibited by PBO, and the relative activity was inversely affected by the increase of PBO

concentration. Therefore, the N-demethylation of AAP by *S. maltophilia* strain CGMCC 1.1788 might proceed through a system coupled with cytochrome P450. The demethylation activity of AAP by cell-free extracts of *S. maltophilia* CGMCC 1.1788 was further examined, but was not detected (data not shown). It is known that P450-enzyme-systems require an electron transport chain that consists of two enzymes (a reductase and ferredoxin) and NADH, and these complexes are often membrane-associated (Urlacher et al. 2004). Therefore the lost the AAP demethylation activity in cell free extract of *S. maltophilia* CGMCC 1.1788 may be contributed to lost of protein components associated with cell membranes during extraction, which is favor for involvement of cytochrome P450 system in demethylation of AAP.

The involvement of P450 in the N-demethylation of some drugs has been reported in human cells. For example, specific cytochrome P450 isoforms CYP3A4 in lymphoblastoid cell microsomes was found to catalyze the N-demethylation of diltiazem (Sutton et al. 1997) and CYP3A4 in human liver microsomes reportedly mediated the N-demethylation of adinazolam (Venkatakrishnan et al. 1998). Iwasa et al. (2004) found that insecticidal activity of the metabolites of AAP differed from that of IMI in the honey bee. They postulated that the variation of metabolism and detoxification pathways might be due to insect species and that the variation could eventually affect insect susceptibility to neonicotinoids. However, our data in the present study suggest that the variation of metabolism and detoxification pathways may be originated from the structural difference of the insecticides. This hypothesis is supported by the facts that the biotransformation system is the same, but substrate structures are different.

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