Metabolism of Imidacloprid in Workers of *Reticulitermes flavipes* (Isoptera: Rhinotermitidae)

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Ann. Entomol. Soc. Am. 103(1): 84–95 (2010)

ABSTRACT The chloro-nicotinyl insecticide imidacloprid is used extensively as a soil treatment against subterranean termites. We conducted the first study of the metabolic fate of imidacloprid in termites, by exposing workers of the eastern subterranean termite, *Reticulitermes flavipes* (Kollar) (Isoptera: Rhinotermitidae) to radiolabeled imidacloprid through topical application and ingestion. Several days after topical application, we detected up to 11 radiolabeled compounds. The parent compound, IMI, and the following six metabolites were identified by liquid chromatography/mass spectrometry: olefin-imidacloprid (major metabolite), 4/5-OH imidacloprid, 4,5-di-OH imidacloprid, des-nitro olefin imidacloprid, des-nitro imidacloprid, and a glucuronide conjugate of des-nitro imidacloprid (des-nitro IMI-glu). Over time, detoxification of imidacloprid proceeded from less polar to more polar compounds, with des-nitro IMI-glu seeming to be the ultimate, major end product in surviving termites. Degradation of imidacloprid was limited to internal tissues of the termite. Workers fed wood treated with imidacloprid or provided with a treated substrate (sand) had metabolitic profiles similar to topically treated termites. Termites fed imidacloprid or exposed to it in soil excreted detectable amounts of all of the identified metabolites. Finally, we determined that imidacloprid metabolites were less toxic to termites than imidacloprid itself. Only the olefin-imidacloprid and 5-OH imidacloprid caused morbidity in termites exposed to sand treated with these compounds, but at concentrations ≈10–20-fold higher than the parent imidacloprid. Our results shed light on the metabolic pathway used by termites to detoxify imidacloprid and show how metabolism plays a key role in determining the availability of the active ingredient and its various metabolites for transfer among workers within the colony.

KEY WORDS imidacloprid, termite, metabolism, *Reticulitermes flavipes*, toxicity

Imidacloprid (IMI), the active ingredient in Premise, is a termiticide used to control subterranean termites in residential and commercial applications. At low concentrations, IMI is a slow-acting, nonrepellent insecticide, so termites that tunnel into a treated zone around a structure become exposed to the active ingredient ([AI]) either via ingestion when handling soil particles with their mouthparts or via penetration through the cuticle. Once internalized, IMI penetrates the insect central nervous system and selectively binds to postsynaptic nicotinic acetylcholine receptors (nAChRs) where it acts as an agonist to increase excitatory neurotransmission (Tomizawa and Casida 2003). Termites exposed to a sublethal dose exhibit a range of symptoms such as reduced mobility, feeding, grooming, and tunneling. However, when symptomatic termites are removed from the treatment and placed with untreated termites, they eventually recover and exhibit apparently normal behaviors (Thorne and Breisch 2001).

The distribution and metabolic fate of the toxicant on and in contaminated termites is of critical importance regarding the bioavailability of IMI to unexposed termites. For example, if most of the toxicant is carried on the cuticle in its active form, then significant transfer would occur via behaviors that involve the cuticle, such as incidental contact and grooming. However, if most of the toxicant is internalized by feeding or absorption through the cuticle, then IMI would be available to nestmates only via stomadeal or proctadeal trophallaxis, cannibalism, or coprophagy. Moreover, the transfer of the AI via these behaviors would be affected by the degree that metabolism either attenuates or intensifies its effects. Imidacloprid could be either detoxified, which would effectively attenuate its effects, or activated which would intensify its toxicity to termites.

Various metabolites of IMI have been characterized from soil, plants and mammals. The in vivo metabolic fate of IMI has been described in only a few insect species, including the honey bee (*Apis mellifera* L.) and the house fly (*Musca domestica* L.), but not in termites. Suchail et al. (2004) determined that honey bee workers metabolize IMI to five compounds: 4,5-diOH IMI, 4/5-OH IMI (4-OH or 5-OH IMI), olefin

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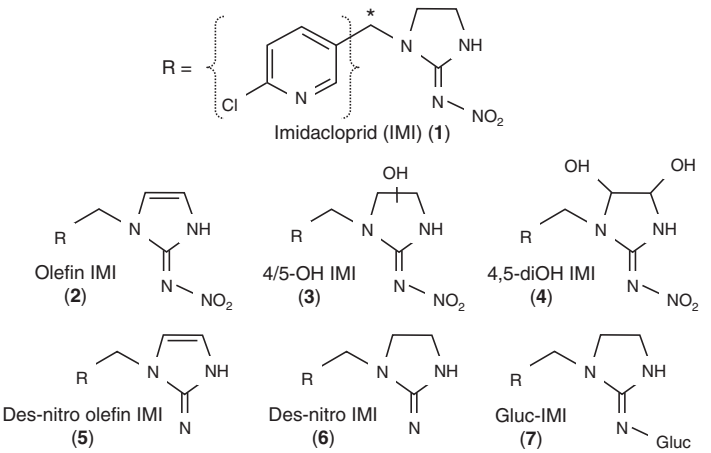

Fig. 1. Structures of IMI and some of its metabolites in *R. flavipes* workers. The asterisk indicates the position of the pyridinyl-^{14}C-methylene label.

IMI, urea IMI, and 6-chloronicotinic acid (6-CNA), of which the urea and 6-CNA were the major metabolites. They did not detect the guanidine (des-nitro) or conjugated derivatives. Nishiwaki et al. (2004) detected six metabolites by thin layer chromatography (TLC) in extracts of house flies injected with [^{14}C]IMI. The olefin IMI was identified as the major compound followed by the 4/5-OH IMI, 6-CNA, and 2-nitroimino-imidazolidine. Nishiwaki et al. (2004) were not able to characterize two other TLC spots and could not detect either the guanidine or urea derivatives. Interestingly, they also found that ≈70% of the radiolabel was found in the excreta 24 h after injection and that most of this was in the form of the olefin IMI.

In this article, we report on identification and biological activity of some of the major metabolites of IMI in the eastern subterranean termite, *Reticulitermes flavipes* (Kollar) (Isoptera: Rhinotermitidae), and discuss the role that these may play in the colony-level effects of IMI in termite control.

Materials and Methods

Chemicals. Pyridinyl-^{14}C-methylene]IMI (3.66 MBq, 99.0 μCi mg^{-1}) was provided by Bayer AG (Wuppertal, Germany). [^{14}C]IMI had a radiochemical purity of >98% by scanned silica gel thin-layer chromatography (TLC) and a chemical purity of >99% by high-performance liquid chromatography (HPLC). Unlabeled IMI (1, (1-[(6-chloro-3-pyridinyl)methyl]-*N*-nitro-2-imidazolidinimine) (Fig. 1) and the following metabolites were provided by Bayer CropScience (Stilwell, KS) and were all at least 97% pure: 1-[(6-chloro-3-pyridinyl)methyl]-1,3-dihydro-N-nitro-2*H*-imidazol-2-imine (olefin-IMI, 2), 1-[(6-chloro-3-pyridinyl)methyl]-2-(nitroimino)-5-imidazolidinol (5-OH IMI, 3), 1-[(6-chloro-3-pyridinyl) methyl]-2-(nitroimino)-4,5-imidazolidinediol (4,5-diOH IMI, 4), 1-[(6-chloro-3-pyridinyl)methyl]-1,3-dihydro-*N*-nitro-2*H*-imidazol-2-imine monohydrochloride (des-nitro olefin IMI, 5), and 1-[(6-chloro-3-pyridinyl)methyl]-2-imidazolidinimine monohydrochloride (des-nitro IMI, 6) (Fig. 1). HPLC-grade solvents were used for all chromatographic separations. Ultima Gold (PerkinElmer Life and Analytical Sciences, Boston, MA) was used as the scintillation cocktail. All other reagents were of the highest purity available from commercial sources.

Termites. *R. flavipes* workers were collected from below-ground traps placed at six different locations with four to six traps per location on the North Carolina State University campus, Raleigh, NC and used for all of the following experiments. Each trap consisted of a 10-cm-diameter by 30.5-cm-long section of polyvinyl chloride pipe into which we placed two 9-cm-diameter by 15-cm-long moistened, rolled corrugated cardboard cylinders; the trap was sunk upright 15 cm into the ground. Trapped termites were removed from the cardboard and placed into 150- by 15-mm petri dishes containing two sheets of 125-mm-diameter Whatman no. 1 filter paper moistened with distilled water. Species identity was determined by soldier morphology (Scheffrahn and Su 1994) and confirmed by polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) (Szalanski et al. 2003). All non-experimental termites and experimental arenas were housed in lidded 31-cm-long by 23-cm-wide by 10-cm-tall plastic containers (Pioneer Plastics, Eagan, MN) with moistened paper towels lining the bottom, and they were maintained in the dark at ambient temperature.

Metabolism Studies: Topical Application of Imidacloprid to Termites. We investigated the metabolic profile of IMI over time in topically treated *R. flavipes* workers that were maintained in isolation after treatment. Using topical application, we could more precisely control both the dose and time of exposure. Also, we minimized the role of intestinal microflora in IMI metabolism by treating the abdominal dorsum of termites to minimize self-grooming, and by placing termites in isolation after treatment to prevent interactions with other termites; thus grooming and inges-

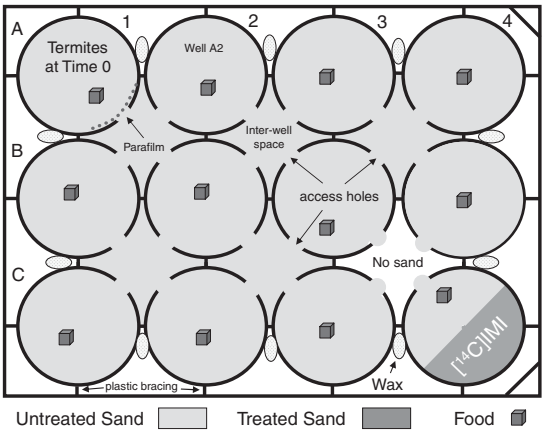

Fig. 2. Top view of the 12-well microtiter plate termite foraging arena used to examine the metabolic profile of IMI in termites exposed to [^{14}C]IMI-treated sand. Sixty termites were added to the well A1. Termites tunneled through wells of untreated sand connected with access holes in the sides of the wells. Termites had to traverse a section of the plate without sand to reach well C4, the distal half of which was treated with 50 μg of [^{14}C]IMI.

tion of IMI were minimized. Batches of ≈90 workers were placed in a 150- by 15-mm petri dish with a sheet of dry 125-mm-diameter Whatman no. 1 filter paper. Termites were anesthetized lightly with CO_2, to the extent that they stopped walking but remained standing upright and firmly gripping the paper. They were then treated on the dorsal abdomen with either 10 or 100 ng of [^{14}C]IMI in 200 nl of either 50 or 500 μg ml^{-1} [^{14}C]IMI in 50% ethanol with 0.01% Nile Blue A by using a digital microdispenser (21-169-20A, Thermo Fisher Scientific, Waltham, MA) fitted with a 10-μl Hamilton syringe. Immediately after the applications were completed (≈2–3 min), the CO_2 was dissipated by fanning fresh air over the termites. After the solution on the termites had dried (≈1–2 min), they were transferred to individual wells of a 48-well microtiter plate and incubated for various periods of time at ambient temperature, depending on the experiment (see below for descriptions of the different experiments). One termite was placed on the bottom of each well on top of a moistened filter paper disc.

Metabolism Studies: Exposure to Imidacloprid Treated Sand During Foraging. We examined the metabolic profile of IMI in termites that had been exposed to IMI under conditions that simulated foraging in the field. In this design, the termites could tunnel through 11 wells of untreated sand in a 12-well microtiter plate (Fig. 2). The sand in half of well C4, the well diagonally farthest from where termites were introduced in well A1, was treated with [^{14}C]IMI. Small access holes in the sides of the wells, even with the floor, allowed the termites to forage through all parts of the plate. A small piece of wax was placed on the ceiling of the plate between each of the outer wells to keep the termites from crawling out through the spaces between the wells. Approximately 1.5 ml of play-sand was placed in each well and in the interwell spaces and moistened with distilled water; the sand covered the access holes between the wells. The interwell spaces adjacent to the treated well remained free of sand to prevent tracking of radiolabeled IMI from well C4 on sand particles. One milliliter (1.6 g) of sand was placed in well C4 and then moistened with 400 μl of water. The sand in the distal half of this well was removed and replaced with dry sand that was then moistened with 250 ppm [^{14}C]IMI until just saturated (≈200 μl), giving 50 μg of [^{14}C]IMI g^{-1} of treated sand. A small piece (≈27 mm^3) of Summon termite bait (FMC, Philadelphia, PA) was placed in every well as food. A small piece of unstretched, two-layered Parafilm was placed over the access hole of well A1, and ≈60 workers were placed on the Parafilm and allowed to chew their way through the Parafilm to gain access to the rest of the plate. Because termites were observed to deposit sand up the sides of the wells and crawl out, the wells were covered with unstretched Parafilm that completely covered and overlapped the edges of the plate by ≈1 cm. A strip of basswood 2.0 mm thick by 7.6 cm wide by 11.4 cm long was placed on top of the Parafilm and gently pressed to seal the tops of the wells. With the basswood in place, the lid was placed on the plate with the Parafilm forming a seal between the wood and the top of the wells. These provisions restricted the termites to the wells and inter-well spaces during the course of the experiment.

Twenty-five termites were removed from each of two foraging arenas after 18 d, and analyzed for radiolabel. These termites exhibited moderate symptoms of intoxication, such as staggered walking and tremors, but their legs and antennae moved in a coordinated manner, and they righted themselves within 15 s of being placed on their backs.

Metabolism Studies: Imidacloprid Uptake From Treated Wood. The metabolic fate of IMI in termites that had access to IMI-treated wood was investigated to determine whether the metabolic profile after ingestion was different from the profile after topical application. We also investigated the effects of a recovery period after exposure to IMI on IMI metabolism. Approximately30 termites were placed into each of four wells of a 12-well microtiter plate that contained 2 ml of moistened play-sand and a small piece (≈27 mm^3) of Summon bait that had been soaked in an aqueous solution of 50 μg ml^{-1} [^{14}C]IMI. Termites were incubated in these wells for 6 d, after which all of the termites exhibited mild to severe symptoms of intoxication. Thirty-four live termites were extracted immediately with a cuticular acetone wash followed by homogenization and extraction of the internal radioactivity. Two other groups of ≈35 termites each were transferred to clean wells with moistened filter paper, incubated for a 5-d recovery period, and then 69 live termites were extracted for external and internal radiolabeled compounds. The wells and filter papers in which the termites were allowed to recover were rinsed with 4 by 1 ml methanol, the rinses were combined and then evaporated using a gentle stream of N_2 in a warm glass bead bath.

Extraction of Treated Termites. To extract the cuticular (external) radioactivity from termites, up to 40 live, treated termites were placed into a single 1.5-ml microcentrifuge tube and washed with 4 by 1 ml acetone for a total of 120 s. The internal radioactive compounds were then extracted by adding 1.2 ml of acetone to the washed termites, grinding the termites with a pestle, sonicating the homogenate for 10 s, and centrifuging at 12,000 rpm for 2 min in a microcentrifuge. The supernatant was removed, placed into a 7-ml scintillation vial, and the pellet resuspended in another 1.2 ml of acetone. This extraction sequence was repeated with 2 by 1.2 ml methanol. The supernatants were combined, the solvents evaporated using a gentle stream of N_2 in a warm glass bead bath, the residue was dissolved in 200 μl of methanol, the concentration of radiolabel determined by liquid scintillation spectrometry and the constituents were analyzed by silica gel TLC.

Chromatography and TLC Fractionation of Metabolites. Extracts were applied 2 cm from the bottom of precoated silica gel TLC plates (Whatman TLC 20 by 20 cm silica gel 60-coated glass plate with fluorescent indicator, 250 μm thick; 05-713-323, Theromo Fisher Scientific) that had been activated at 100°C for 60 min and cooled. The plates were developed in one dimension using either 80:15:5 or 75:20:5 chloroform/methanol/acetic acid, or 70:20:10 ethyl acetate/isopropanol/water. Plates were air-dried and then scanned for radioactivity using a TLC imaging scanner (Bioscan, Washington, DC) that was equipped with a 256 channel, low-resolution, high-sensitivity collimator, and capable of detecting the radiolabeled components in a single 1- by 20-cm lane. Each chromatogram consisted of the counts from each of 256 channels, and the signal from standard [^{14}C]IMI spotted on the origin peaked at channel 43. Unlabeled standard compounds were visualized using UV light.

Extracts of workers that had been topically treated with 100 ng of [^{14}C]IMI each and incubated for 5 d were fractionated by TLC before chemical analysis by liquid chromatography/mass spectrometry (LC/MS). Sample was spotted in a 4-cm narrow band 2 cm from the bottom of a 20- by 20-cm TLC plate, and chromatographed using 80:15:5 chloroform/methanol/acetic acid. The plates were scanned for radioactivity, and the peaks scraped from the plate in six fractions (Fig. 3). The scrapings were loaded into 2-ml Pasteur pipettes plugged with a small amount of silanized glass wool, and the compounds were eluted first using two by 1.5 ml of acetone and then two by 1.5 ml of methanol. The solvents were collected in 7-ml vials and then evaporated under a stream of N_2.

Analysis of Imidacloprid Metabolites by LC/MS and Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS). Identification of most of the principal [^{14}C]IMI metabolites represented by the TLC separation in Fig. 3 was done by liquid chromatography/electrospray ionization (ESI)/mass spectrometry to determine the pseudomolecular ion ($[M+1]^+$ or $[M-1]^-$) of the [^{14}C]IMI metabolites. Tandem mass spectrometry (LC/ESI/MS/MS) was also used

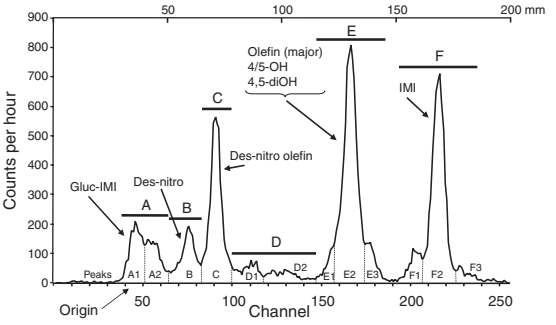

Fig. 3. Thin layer chromatogram showing radiolabeled compounds in *R. flavipes* workers after topical application of 100 ng of [^{14}C]IMI. Five days after topical application, termites were rinsed with acetone to remove IMI from the cuticle, and then extracted sequentially with acetone and methanol to recover the internal radiolabeled compounds. After silica gel TLC using 80:15:5 chloroform/methanol/acetic acid, each lane was radiometrically scanned for 60 min. The six horizontal solid black lines designate regions of silica gel corresponding to peaks of radioactivity (designated A–F) that were scraped from the plate, eluted, and submitted to LC-MS analysis. Peaks within each region were numbered if there were multiple peaks that could be resolved in that region.

to fragment the pseudomolecular ions into product ions to aid in confirming the identity of most of the metabolites.

A portion of the TLC sample from above, generally 20–100 μl, was chromatographed and analyzed. Either LC/ESI/MS (full scan) or LC/ESI/MS/MS (product ion) spectra were obtained for each [^{14}C]IMI metabolite depending upon the availability and purity of the sample. The spectra and retention times were then compared with authentic synthetic standards if available.

LC/ESI/MS or LC/ESI/MS/MS was done using a ThermoFinnigan Quantum Ultra AM equipped with an Ion Max source and a Surveyor MS LC pump and autosampler. HPLC separation of [^{14}C]IMI metabolites was achieved using a Zorbax SB-C_8 (75 by 4.6 mm, 3.5 μm) column with a mobile phase gradient consisting of water and acetonitrile (ACN), both containing 0.1% formic acid. The following step gradient (water/ACN @ 800 μl/min) was used: 98:2 for 15 min, 85:15 for 15 min, 5:95 for 4 min, and finally 98:2 for 6 min. The HPLC column effluent was split (4:1) to a Ramona Star radioactivity flow monitor to determine the retention time of the [^{14}C]IMI metabolites. All samples were initially analyzed in the full scan MS mode to survey for the presence of [^{14}C]imidacloprid metabolites. Once the identity of a metabolite was proposed, generally by observing corresponding peaks in both the MS and [^{14}C] chromatograms, MS/MS analysis was usually performed and the results compared with a synthetic standard (if available) for confirmation. Most work was done in positive ESI ($[M+1]^+$). However, when adequate sample or a discernible signal in positive ESI was not present, negative ESI ($[M-1]^-$) was attempted. Full scan (MS) data were collected by scanning from 50 to up to 750 amu at 1 s/scan. MS/MS (product ion spectra)

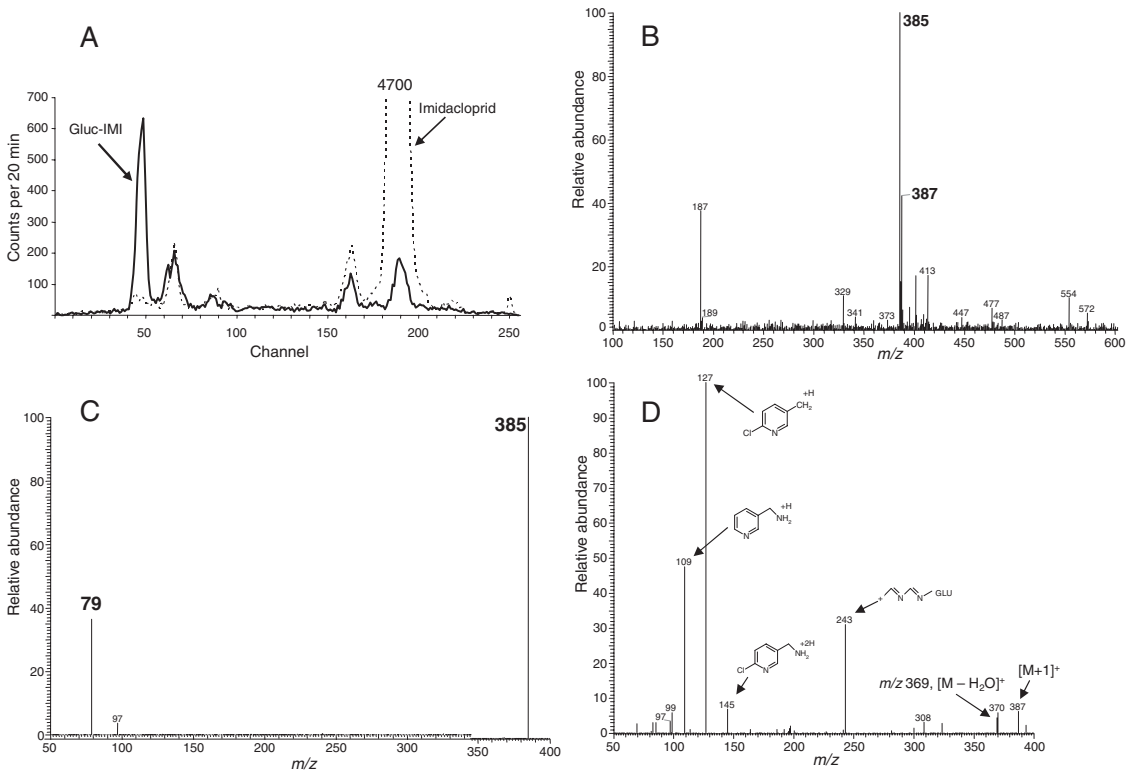

Fig. 4. Mass spectrometry of the most polar metabolic peak. (A) TLC of the cuticular wash (dashed line) and the corresponding internal extract (solid line) of termites 10 d after topical application of 10 ng of [^{14}C]imidacloprid per termite. The most polar peak (channel 43, peak A1) was isolated from the TLC plate, applied in a 4 cm narrow band on a new silica gel plate, and developed using 75:20:5 chloroform/methanol/acetic acid as the mobile phase. (B) Full scan negative electrospray spectrum of termite TLC metabolite peak A showing the proposed guanidine IMI glucuronide metabolite: m/z 385 is the $[M\text{-}1]^-$ ion and shows the presence of the single chlorine isotope pattern. (C) Product ion (MS/MS) negative ion spectra of m/z 385. The fragment at m/z 79 is proposed to represent the pyridine ring. (D) Product ion (MS/MS) positive ion spectra of m/z 387. The fragment at m/z 127 is proposed to be the protonated 4-chloro-1-methy pyridinium ion. The other fragments support the structure of the putatively identified des-nitro IMI-glu.

data were collected by scanning from 50 to 2 or 3 amu past the precursor ion, generally at 1 s/scan as well. Collision energy was adjusted to between 20–25 eV with a collision-induced dissociation pressure of ≈1.5 torr (argon).

Metabolite Toxicity to Termite Workers. We determined the activity of IMI metabolites relative to IMI and water (control) by exposing termites to treated sand. Dry play-sand (1.6 g) was placed in each well of 12-well microtiter plates and moistened with 400 μl of the appropriate aqueous solution of either IMI, olefin IMI, 5-OH IMI, 4,5-diOH IMI, des-nitro olefin IMI, des-nitro IMI, or water to give 0, 0.5, 5.0, 50, or 100 μg of compound per gram of treated sand. Twenty workers were placed into each well, and observed intermittently over 7 d for symptoms, tunneling, and walking activity.

Results

Metabolism of [^{14}C]IMI in Topically Treated Termites. Typically, ≈80% (range, 75–85%) of the total internal radioactivity was associated with compounds extracted using the sequential acetone-methanol extraction described above (data not shown). Up to 12 radiolabeled peaks were detected by silica gel TLC in an internal extract of acetone-rinsed termites that had been topically treated 5 d previously with 100 ng of [^{14}C]IMI (Fig. 3). Starting from the origin (channel 43), des-nitro IMI-glu (7) was tentatively identified as component A1 in fraction A. This polar metabolite was isolated from the TLC plate shown in Fig. 4A, which shows a separation of the internal extract of termites 10 d after topical application of 10 ng [^{14}C]IMI per termite. The polar peak (corresponding to channel 43, peak A1) was isolated and applied to a new silica gel plate and developed using a mobile phase of 75:20:5 chloroform/methanol/acetic acid. LC/MS of the polar metabolite isolated from the second TLC plate produced a full scan negative ESI spectrum (Fig. 4B) showing a definitive pseudomolecular ion with a single chlorine atom isotope pattern at m/z 385 $[M-1]^-$. The corresponding positive ESI spectrum (data not shown), although less definitive, seemed to produce a chlorine containing pseudomolecular ion at m/z 387 $[M+1]^+$. These data indicate the molecular weight of

the imidacloprid metabolite was 386 amu. MS/MS was performed using both negative and positive ESI (Fig. 4C and D). Product ions under negative ESI conditions were sparingly produced with only m/z 79 present in significant abundance; we propose that this fragment may represent the pyridine ring. However, product ions from positive ESI produced more definitive ions as observed in Fig. 4D, with the most abundant ion (m/z 127) being proposed as the protonated 4-chloro-1-methyl pyridinium ion. An authentic standard was not available for confirmation. Also, because the [^{14}C]activity was scraped from near the origin of the TLC plate, it is possible that other polar metabolites could have been present in the TLC peak that were not detected by [^{14}C]HPLC detection. However, it is probable that the proposed glucuronide of des-nitro IMI is a major component of the ^{14}C activity. Therefore given the data presented, this peak was tentatively identified as a glucuronide of des-nitro IMI.

The remaining [^{14}C]IMI metabolites were identified in the same manner as described for the des-nitro IMI-glu metabolite, i.e., a chlorine containing pseudomolecular ion was identified in the full scan spectrum corresponding to a ^{14}C peak followed by MS/MS for confirmation. Authentic standards were also available for most of the remaining metabolites for comparison. Generally, IMI and its metabolites produce positive pseudomolecular and fragment ions in more abundance, so most spectra were collected in the positive ESI mode. Peak A2 in Fig. 3 could not be characterized. Des-nitro IMI (6) was identified as the major component of fraction B, and des-nitro olefin IMI (5) was identified as the major component of fraction C. One or two unidentified components were observed as low broad peaks between channels 100 and 150 in fraction D. Between channels 150 and 190, there was a peak which included the olefin IMI (2) as the major compound in fraction E, and two other unresolved compounds in lesser abundance; the 4/5-OH IMI (3) and 4,5-diOH IMI (4). The parent compound, IMI (1), was identified as the major component in fraction F between channels 200 and 240 with one or two minor unidentified compounds. The unidentified minor components in fractions A, D, E, and F were neither abundant enough nor sufficiently resolved from the other components in this study to make a structural determination. In some chromatograms, the positions of the four polar peaks were shifted slightly or did not resolve into separate peaks. This was due, probably, to the large amount of unlabeled, highly polar contaminants extracted from the termite tissues that affected compound migration in silica gel, and reduced the resolution of radiolabeled metabolites which occurred within ≈50 channels of the origin.

To demonstrate the metabolic progression of IMI in termites, we topically applied a lower dose of IMI and extracted termites 10 d later, reasoning that termite morbidity would be lower, allowing for the accumulation of IMI metabolic end products. In cuticular extracts of treated termites, IMI was detected as a single, major component from two separate groups of

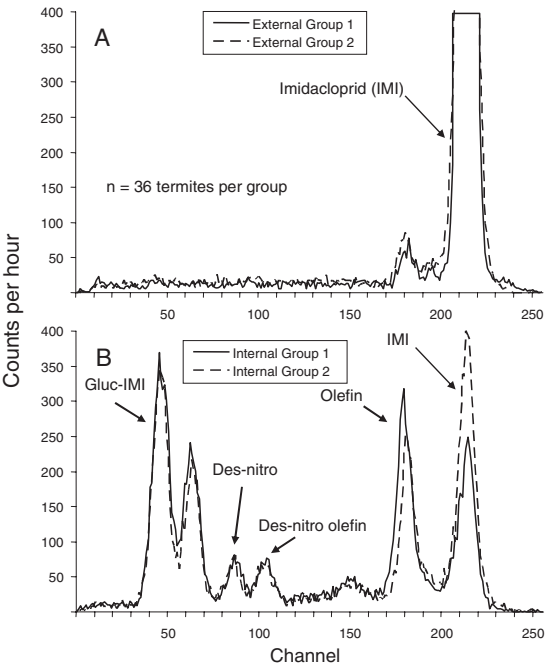

Fig. 5. Metabolite profile of radiolabeled compounds in *R. flavipes* workers 10 d after topical application of 10 ng of [^{14}C]IMI per termite. Thirty-six termites in each of two groups were extracted, extracts separated on a silica gel TLC plate (75:20:5 chloroform/methanol/acetic acid), and radioactivity analyzed. Group 1 termite workers were alive, their legs and antennae moved in a coordinated manner, they had a righting response, but exhibited staggered walking and tremors. Group 2 termites were alive also, but their legs or antennae only twitched or moved feebly, and they had no righting response. (A) The cuticular acetone wash of each group of termites was ≈20,000 counts after a 60-min scan, and almost all the radioactivity was associated with the parent IMI. (B) The internal extract of each group of termites contained ≈16,800 counts after a 60 min scan, and radioactivity was found in the IMI peak and distributed across five major metabolite fractions. The "olefin" fraction also contains 4/5-OH IMI and 4,5-di-OH IMI.

termites, indicating that IMI was stable on the cuticle during the 10 d of this experiment (Fig. 5A). The minor components in this rinse constituted <5% of the total radioactivity. Also, because the internal extracts (Fig. 5B) included peaks which were not present in the cuticular rinse, we reasoned that these compounds were produced primarily by termite metabolism and not by chemical decomposition.

TLC analysis of the internal extracts of termites 10 d after topical application of 10 ng [^{14}C]IMI to each termite produced a chromatogram (Fig. 5B) with a pattern qualitatively very similar to that observed for termites treated with 100 ng of [^{14}C]IMI and extracted 5 d later (Fig. 3). However, the lower dose of IMI and longer incubation time led to a greater abundance of more polar metabolites. Thus, the proposed des-nitro IMI-glu became the most abundant metabolite of the 10-ng dose, whereas des-nitro olefin IMI was the most abundant metabolite of the 100-ng dose. Also, the

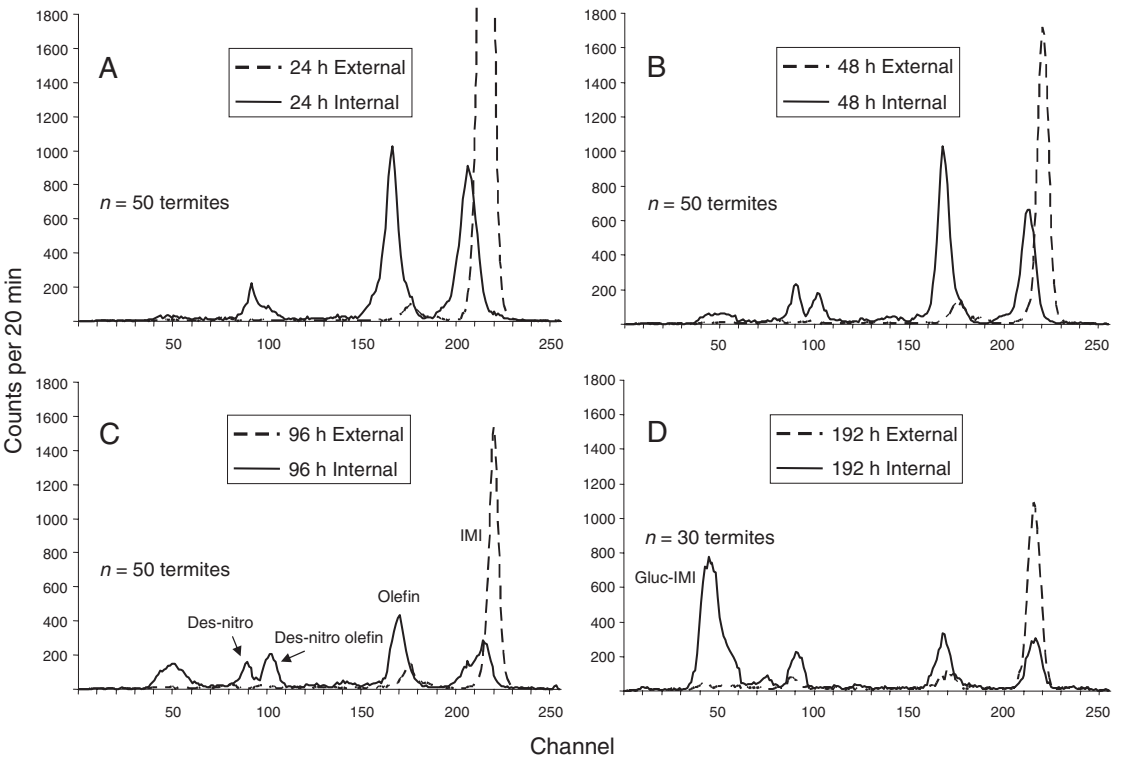

Fig. 6. Time course of IMI metabolism by *R. flavipes* workers after topical application of 10 ng of [^{14}C]IMI. Termites were processed for analysis at 24 h (A), 48 h (B), 96 h (C), and 192 h (D) after topical application. As in Fig. 5, the cuticular surface of termites was washed with acetone (dashed line), and the internal radioactivity was obtained from sequential extractions of homogenized termites with acetone and methanol. Metabolites were separated by TLC (80:15:5 chloroform/methanol/acetic acid) and scanned for radioactivity. The 192-h samples were prepared using only 30 termites, so the chromatogram was scaled-up by a factor of 1.6 to be equivalent to the 50 termites extracted at the other times. The "olefin" fraction also contains 4/5-OH IMI and 4,5-di-OH IMI.

olefin IMI (fraction E) was less abundant relative to IMI in the low dose-long incubation group (Fig. 5B) than in the higher dose-short incubation group (Fig. 3), indicating that the olefin probably was an intermediate in the IMI metabolic pathway. The olefin labeled peaks in Figs. 5–9 also contained small amounts of 4/5-OH IMI and 4/5-diOH IMI. Termites survived the lower dose better than the higher dose, which allowed for accumulation of higher amounts of more polar metabolite end products relative to IMI, and less metabolites of intermediate polarity. There were no obvious differences between the two replicate experiments in Fig. 5B: group 1 termite workers exhibited a righting response, but also staggered walking and tremors, whereas in group 2 termites the legs or antennae twitched and they had no righting response.

Further support for the metabolic progression of IMI to polar end products was provided by an analysis of topically treated termites. Over time, both total external and total internal IMI decreased (Fig. 6A–D) with a corresponding increase in the concentration of several compounds more polar than IMI. During the first 48 h (Fig. 6A and B), the internal concentration of the olefin IMI remained elevated, and little radioactivity was detected in more polar fractions. Between 48 h and 192 h after treatment, both IMI and olefin IMI declined, and des-nitro IMI-glu, near the origin of the TLC plate, became the most abundant internal metabolite. Possibly, this pattern occurred because the external amount of IMI was initially high, and therefore it penetrated the cuticle at maximum rate. Therefore, the internal IMI concentration was maintained such that the production of olefin IMI via the metabolism of IMI equaled the metabolism of olefin IMI to other products over this time. After 48 h, however, the olefin IMI decreased in parallel with IMI (Fig. 6B) probably because the internal concentration of IMI decreased to a level where the rate of olefin metabolism exceeded the rate of its production.

These results suggest that the olefin is a major intermediate product of IMI metabolism in this species. The internal amount of des-nitro IMI-glu (7) increased throughout the time-course investigation (Figs. 6 and 7), and at 192 h (Figs. 6D and 7) des-nitro IMI-glu was the most abundant metabolite peak, which again indicated that it was probably a major endpoint of IMI metabolism in *R. flavipes.*

Exposure to Treated Sand During Foraging. Just before extraction, most of the termites (≈50 out of 60)

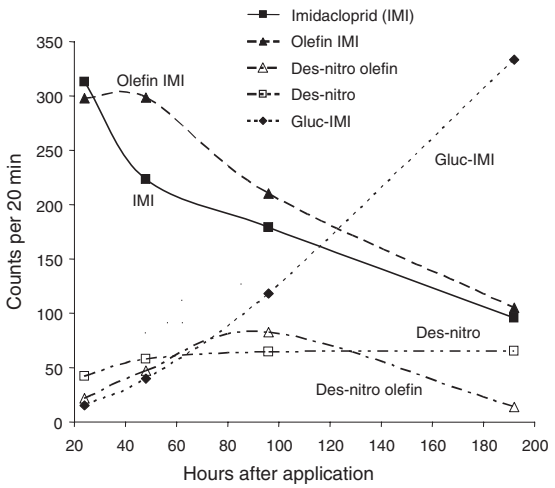

Fig. 7. Summary of changes in the internal amount of radiolabeled metabolites in *R. flavipes* workers 24, 48, 96, and 192 h after topical application of 10 ng of [^{14}C]IMI per termite. The data represent counts under each peak obtained from scanning the lanes of the TLC plates shown in Fig. 6. The amount shown is per termite. The olefin IMI fraction also contains small amounts of 4/5-OH IMI and 4,5-di-OH IMI.

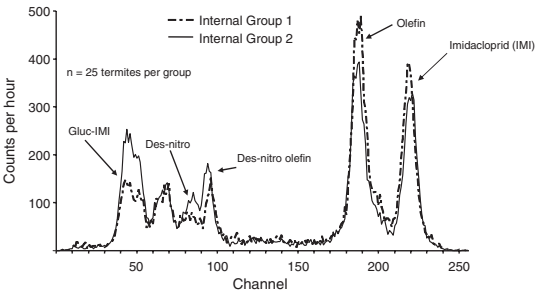

Fig. 8. Metabolism profile of IMI in *R. flavipes* workers after foraging for 18 d in a 12-well microtiter plate arena (see Fig. 2) that contained 50 μg g^{-1} sand [^{14}C]IMI in the distal well (C4). Only internal extracts of two replicates of termites are shown, because external acetone washes did not contain sufficient radioactivity to detect on TLC—175 dpm total, 7 dpm per termite. We applied 1,850 dpm for group 1 and 1,350 dpm for group 2 and developed the TLC plates in 75:20:5 chloroform/methanol/acetic acid. After a 60-min scan, the total counts for groups 1 and 2 were ≈20,600 and 16,000, respectively. The counts per channel for both groups were normalized to the average total, 18,300 counts per h. All termites were alive, their legs and antennae moved in a coordinated fashion, and they possessed a righting response, but exhibited staggered walking and tremors. The "olefin" fraction also contains 4/5-OH IMI and 4,5-di-OH IMI.

that were exposed to IMI by tunneling in the 12-well microtiter plate were mobile and could right themselves within 15 s of being placed on their backs, but they staggered while walking, and had tremors. The cuticular extracts from two groups of 25 termites, from each of two foraging arenas, averaged 32 ng per termite (29–35 ng, 95% CI), and this external rinse was not fractionated by TLC because insufficient radiolabel was present in this preparation. We recovered an average of 582 ng per termite (476–688 ng, 95% CI) from the internal extracts of acetone-rinsed termites (i.e., the total body dose averaged 614 ng per termite), and their internal metabolites profile (Fig. 8) was very similar qualitatively to that of topically treated termites (Figs. 3 and 5B), with 134 ng (23%) IMI, 198 ng (34%) olefin IMI (and 4/5-OH IMI and 4,5-diOH IMI), and 43% was a mixture of des-nitro IMI, des-nitro olefin IMI, and des-nitro IMI-glu.

Imidacloprid Uptake From Treated Wood. Termites in each well of a 12-well microtiter plate were allowed to feed for 6 d on wood that had been soaked in an aqueous solution of 50 μg ml^{-1} [^{14}C]IMI. The radioactivity in the internal extract of these termites was associated mostly with IMI and the metabolites olefin IMI, des-nitro IMI, and des-nitro IMI-glu (Fig. 9), similar to the profile of topically treated termites.

After a 5-d recovery, without exposure to treated wood, the symptoms of termites seemed to have abated considerably, and they regained mobility similar to untreated termites. The radioactivity in the internal extract of these termites was associated mostly with des-nitro IMI-glu (7) and low amounts of other polar compounds. Notably absent were less polar compounds, including IMI and its olefin (Fig. 9). The residue that accumulated in the well during the 5 d recovery contained similar metabolites, and at similar ratios, as in the internal extracts of termites immediately after a 6-d exposure to IMI, suggesting that termites effectively excreted IMI and nonpolar metabolites, and metabolized IMI to polar metabolites during recovery from IMI exposure.

Metabolite Toxicity in Termite Workers. The toxicity of IMI metabolites to termites was compared with that of IMI- and water-treated sand in dose–response

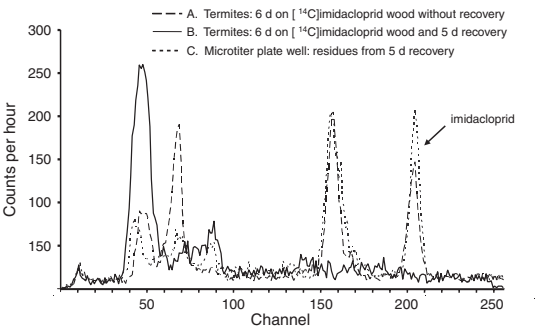

Fig. 9. Comparison of the metabolic profile of *R. flavipes* workers exposed for 6 d to wood that had been soaked in an aqueous solution of 50 μg ml^{-1} [^{14}C]IMI. The internal extract of termites that were not allowed to recover (A. 2,220 dpm; n = 34 termites) was compared with the extracts of termites after a 5-d recovery (B. 2,880 dpm; n = 69 termites) and the residues remaining in the well during the 5-d recovery (C. 2,000 dpm). The TLC plate was developed in 80:15:5 chloroform/methanol/acetic acid and 60 min TLC scans yielded total counts of 8,200, 8,900, and 9,200 counts per h, respectively for A, B, and C, so the total counts of all TLC lanes were normalized to 8,900.

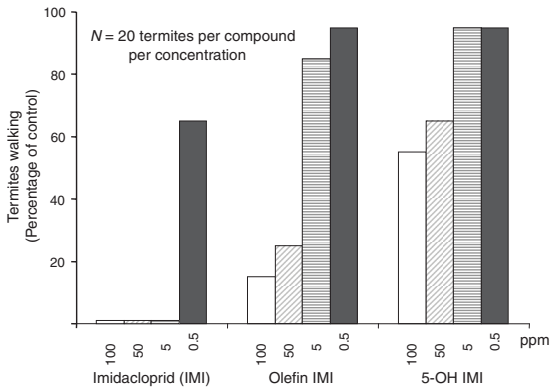

Fig. 10. Toxicity of IMI metabolites to *R. flavipes* workers after 7 d continuous exposure to sand treated with 100, 50, 5, or 0.5 ppm IMI, olefin IMI, or 5-OH IMI. Data from termites on IMI and metabolite-treated sand are presented as a percentage of termites on water-treated sand. Termite mobility on sand treated with either 4,5-diOH IMI, des-nitro olefin IMI, or des-nitro IMI was indistinguishable from that treated with water for all of the doses tested by this method of exposure (data not shown).

studies in 12-well microtiter plates (Fig. 10). After 7 d of continuous direct contact with sand treated with 5, 50, or 100 μg IMI g^{-1} sand, all termites were immobilized; only approximately half of the termites remained mobile at the lowest dose of 0.5 μg IMI g^{-1} sand. All five metabolites that we tested had lower toxicity than IMI to termites, and only the 50 and 100 μg g^{-1} doses of the olefin IMI and 5-OH IMI immobilized 20–60% of the termites. The activity of olefin IMI was about twice that of 5-OH IMI, and these were 10–100-fold less than IMI. Termite mobility on sand treated with either 4,5-diOH IMI, des-nitro olefin IMI, or des-nitro IMI was indistinguishable from that on sand treated with water for all of the doses tested by this method of exposure (data not shown).

Discussion

We report here that termites metabolized imidacloprid to its olefin, 4/5-OH, 4,5-diOH, des-nitro, des-nitro olefin, and des-nitro IMI-glu derivatives. Our most notable findings were the significant presence of guanidine (des-nitro) metabolites and the apparent formation of a phase II conjugate, tentatively identified as des-nitro IMI-glu, which, to our knowledge, is the first discovery of this IMI metabolite in insects. Other possible metabolites, such as 6-CNA and the urea derivative that have been found previously in other insects, were not identified in *R. flavipes* under our experimental conditions.

Because they share similar detoxification enzyme systems, we expected to find some of the same products of IMI metabolism in termites that have been found in honey bees and house flies. But a comparison of their IMI derivatives showed both similarities and differences. The olefin IMI, 4/5-OH IMI, and 4,5-di-OH IMI were found both in termites topically treated with [^{14}C]IMI and in honey bees fed a sucrose solution of [^{14}C]IMI. However, urea derivatives were present in honey bees but not in termites, and des-nitro IMI, des-nitro olefin IMI, and des-nitro IMI-glu were not recovered in honey bees. Furthermore, whereas the urea derivative and 6-CNA were the two main metabolites in bees, the olefin IMI and des-nitro IMI-glu were the major metabolites in termites. Likewise house flies injected with either [pyridinyl-^{14}C-methylene]IMI or [4,5-$^{14}C_2$-imidazolidine]IMI produced the olefin as the major metabolite, with small amounts of 6-CNA, 2-nitroimino-imidazolidine, and 4/5-OH IMI (Shelton and Grace 2003). However, the des-nitro, des-nitro olefin, and the urea derivatives of IMI were not detected in house flies.

All of the identified IMI metabolites in honey bees and house flies were produced by phase I enzymes, namely cytochrome P450 (Nauen et al. 2001, Nishiwaki et al. 2004), and to date, no conjugated IMI metabolites have been reported. The lack of Phase II products in these insects could be because they either were never formed in the first place, or were not present in detectable quantities due to either high turnover or production of only trace amounts. However, some differences in their metabolic profiles may indicate subtle, but distinct divergences in their detoxification mechanisms. For example, the des-nitro derivatives and des-nitro IMI-glu were found only in termites. In mammals, these compounds are intermediates in the pathway that reduces and cleaves the nitroimine group of IMI to form the nitrosoimine, des-nitro, and then the urea derivative (Schulz-Jander and Casida 2002, Caboni et al. 2003). Therefore, in termites, the metabolism may stop at des-nitro IMI-glu because the enzyme for the transformation to the urea is not present, and des-nitro IMI-glu is probably excreted efficiently so no further metabolism is needed. In honey bees, however, where urea is a major metabolite and the des-nitro derivatives are not detected, the des-nitro metabolite may be a short-lived intermediate and present in small, undetectable amounts. In flies, where neither the des-nitro nor the urea metabolite was detected, the enzyme that reduces the nitroimine to the nitrosoimine may be absent.

Further comparative studies of imidacloprid metabolism are needed to test the idea that in some insects (e.g., honey bee and house fly) oxidative detoxification may be the principal mode of metabolism and perhaps lead to more rapid mortality than in the termite, especially because some oxidative metabolites (olefin IMI and 4/5-OH IMI) exhibit toxicity as well. The termite and/or its gut symbionts may have (or exhibits greater) nitroreductase activity and thus reduces the pool of toxic metabolites faster than in other species, allowing time for more metabolism and the appearance of the des-nitro IMI-glu metabolite, possibly through a phase II conjugation.

Based on the metabolites and their appearance over time, we propose a metabolic pathway of IMI in termites, as depicted in Fig. 11. Our proposal that the olefin is the major metabolitic intermediate and des-nitro IMI-glu is the major metabolitic endpoint of IMI

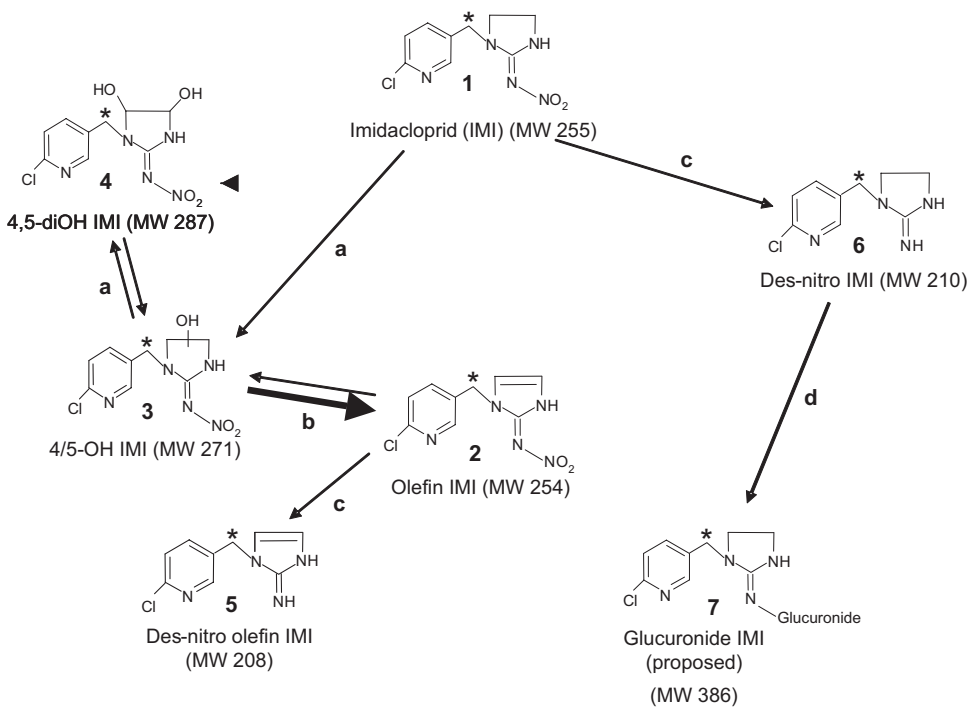

Fig. 11. Proposed metabolic pathway of imidacloprid in termites. Enzymatic hydroxylation (a) and desaturation (b) of the imidazolidine ring form the mono- and di-hydroxylated (3 and 4) and desaturated (2) metabolites. Reduction (c) of the nitroimino group would result in 5 and 6. We propose the addition of a carbohydrate (d) also takes place to form 7. The pathway between 4,5-diOH IMI (4) and olefin IMI (2) could be mediated by P450 and epoxide hydrolase. The asterisk indicates the position of the pyrindinyl-^{14}C-methylene label.

metabolism in termites is based primarily on the observed changes in the internal profile in topically treated termites over the course of 8 d (Figs. 6 and 7). The olefin IMI was the major metabolite early after IMI application and the amount of both IMI and its metabolite were approximately similar for up to 2 d after application. But four and 8 d after treatment, as the amount of IMI declined, a corresponding decrease in the olefin IMI was observed, and des-nitro IMI-glu accumulated as the major metabolite.

High levels of des-nitro IMI-glu relative to IMI and the olefin IMI were observed in termites 10 d after topical application of 10 ng of [^{14}C]IMI, and in termites given a 5-d recovery after 6-d exposure to treated food, indicating that low doses followed by a recovery period during which termites were not exposed to IMI result in metabolic accumulation of des-nitro IMI-glu. Appearance of this metabolite in termites, and not in honey bees and houseflies, suggests an intriguing hypothesis: It is possible that termites use uronic sugars, which are readily available components of hemicellulose, for xenobiotic conjugation. If so, glucuronide conjugates of IMI and of des-nitro IMI might be found primarily in xylanolytic insects.

Termite metabolism of IMI results in detoxification. We found, as others have (Thorne and Breisch 2001), that termites removed from IMI exposure are capable of recovering their normal behaviors. This recovery is due in large part to the degradation and elimination of the active molecule IMI, and the less active olefin IMI and 4/5-OH IMI. During a 5-d recovery period after 6 d of access to treated food, termites metabolized and excreted IMI and most of the active metabolites onto the substrate. The internal radioactivity was associated mostly with inactive, polar compounds and only trace amounts of radioactivity were associated with IMI and its olefin and hydroxylated derivatives. Des-nitro IMI-glu was the predominant metabolite in termites given time to recover, and accounted for most of the internal radiolabel. The internal radiolabel in termites not given a recovery period consisted of similar amounts of IMI, olefin IMI, and polar compounds near the origin. Although we could not test the insecticidal activity of the des-nitro IMI-glu component in termites, it is probably inactive because 1) it is present in recovered termites at a level similar to that of IMI and olefin IMI in intoxicated termites; 2) other polar metabolites, including 4,5-diOH IMI, des-nitro IMI, and des-nitro olefin IMI, were inactive in termites; and 3) des-nitro IMI (Suchail et al. 2004) was inactive in honey bees. Comparing the internal radiolabel profile of recovered termites with the radiolabel deposited in the well suggests that the recovering termites eliminated des-nitro IMI-glu more slowly than IMI, olefin and hydroxylated metabolites. It is possible that the slow elimination of glucuronic conjugates may be because termites store sugar conjugates for later metabolism or fermentation, consistent

with the hypothesis that uronic sugars are used to form glucuronic conjugates in xylophagous insects.

Some of the IMI metabolites have been found to have insecticidal activity equal to or even greater than that of IMI in certain insects. For example, the activities of both the olefin and 5-OH IMI are similar to that of IMI in honey bees, whereas the 4,5-diOH IMI is less active than IMI, and the urea IMI and 6-CNA are inactive (Nauen et al. 2001). Investigating the effects of plant metabolites of IMI on pest homopterans, Nauen et al. (1998, 1999) found that some of these are significantly more active than the parent IMI. The olefin IMI is ≈10-fold more active than IMI in *Aphis gossypii* Glover, the cotton whitefly (Nauen et al. 1999), and ≈16-fold more active than IMI in both the green peach aphid, *Myzus persicae* (Sulzer), and cotton aphid aphids by oral ingestion (Nauen et al. 1998).

We did not detect any substantial qualitative differences among the internal metabolic profiles derived from the internal extracts of termites that acquired IMI by various routes, including topical application, foraging through treated sand, and feeding on treated wood. Therefore, these data indicate that either the gut microflora metabolized IMI to the same compounds as did termite tissues, or they did not produce microbe-specific compounds in enough quantity to be resolved and detected by TLC in our study.

Interestingly, after 18 d of tunneling, foraging and feeding in the 12-well arena containing a 50 ppm [^{14}C]IMI treatment zone, 95% of the termites' total body radiolabel was internal, and a small amount of IMI remained on the cuticle. Moreover, approximately half of this was composed of insecticidal compounds, including IMI, olefin IMI, and 4/5-OH IMI. Furthermore, >80% of the termites retained relatively coordinated mobility, although most were somewhat impaired. This would imply that termites could transport IMI and toxic metabolites some distance away from the treatment zone, and transfer them to nestmates either by grooming or cannibalism. Transfer by oral or proctadeal trophallaxis would depend on the location of these compounds in the termite. IMI and IMI metabolites in the foregut would be available for oral trophallaxis and, if present in the hindgut, would be available for proctadeal trophallaxis or deposition onto the soil to be picked-up by other passing termites by contact. However, transfer by oral trophallaxis may be hampered because the termite's mouthparts seem to become paralyzed after IMI intoxication (M.T., unpublished data).

In the course of foraging and feeding, termites acquire imidacloprid from treated substrates and become intoxicated, but then metabolize it via oxidative and reductive pathways that reduce its toxic effects. The increased appearance of reductive metabolites in *R. flavipes* implies that the termite and/or its symbionts have detoxification pathways or enzymes that are not as active or possibly not present in other species. If termites are removed from the treatment after becoming intoxicated, they are able to eliminate the acutely active compounds from their bodies and, in the process, recover their mobility, and excrete IMI and active metabolites onto the substrate. As long as these termites have access to the treatment zone, they are capable of re-entering treated areas (Thorne and Breisch 2001) and potentially repeating this cycle. Moreover, Boucias et al. (1996) reported that imidacloprid intoxication disrupts colony hygienic behavior and can lead to increased pathogen susceptibility in *R. flavipes.* We envision that, over time and with thousands of termites, this would result in the dissemination and gradual accumulation of active compounds in areas the termites frequent. During this accumulation of toxins, termites would gradually be exposed to increasing concentrations of active compounds and finally become immobile and ultimately die.

The impact of imidacloprid metabolism on the complex dynamics between termites and imidacloprid in the treatment zone, and between contaminated and uncontaminated nestmates in the context of social interactions in tunnels and the colony region, has not been well studied. We suspect that metabolism plays an important role, not only in the availability of this AI and its potentially termiticidal metabolites for transfer between foragers and nestmates, but also for transport to and deposition of active material in colony areas away from the treatment zone, and in enabling contaminated termites to engage in these activities before they are totally and irreversibly incapacitated. Thus, metabolism may actually be responsible for imidacloprid's effect on the colony level by slowing its action and thereby allowing contaminated individuals to maintain enough of their normal behavior for an extended period so they can transport and transfer the AI and its metabolites to nestmates via grooming or trophallaxis. Importantly, it is possible that these subtleties may be overlooked in laboratory assays involving prolonged exposures to high concentrations of imidacloprid.

Acknowledgments

[Pyridinyl-^{14}C-methylene]IMI was graciously provided by Bayer AG (Wuppertal, Germany). Unlabeled IMI and IMI metabolite standards were provided by Bayer CropScience (Stilwell, KS). We thank Gary Brookhart for valuable and insightful discussions about the manuscript. This work was funded in part by a grant from Bayer Environmental Science (to E.L.V.) and the Blanton J. Whitmire Endowment at North Carolina State University.

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Received 23 June 2008; accepted 11 March 2009.