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Novel substrates for the kinetic assay of esterases associated with insecticide resistance

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Abstract. Naphthalene thioesters were synthesized as substrates for a continuous, non-disruptive kinetic assay of general carboxylesterase activity. The continuous nature of the assay is based on the production of a soluble dianion chromophore from the reaction of naphthalene thio with 5,5′-dithiobis (2-nitrobenzoic acid). Applications with 1- and 2-naphthalene thioacetates demonstrated their use in a fast, accurate kinetic microassay of esterase activity, using porcine carboxylesterase as a model. These novel esters proved to be useful as substrates for the spectrophotometric assay of insecticide-resistance in two aphid species and may be applicable to other esterase-based diagnostic procedures.
Key words. Esterase; carboxylesterase; hydrolase; *Myzus nicotianae*; tobacco aphid; *Myzus persicae*; green peach aphid.

The determination of the structure of field populations of arthropod pests, with respect to the distribution and relative abundance of different insecticide-resistant genotypes, is a key factor in the management of insecticide resistance. During the past decade interest has focused on the development of biochemical detection methods for resistance[1–3]. These methods are more specific than the traditional bioassay tests and are capable of investigating individuals in a population. The advantage of this approach is that it provides critical information on the microevolution of resistance.

Since the introduction of organophosphorus (OP) and carbamic acid esters for insect control, great interest has been focused on insect hydrolases not only as target enzymes for insecticidal action but also because of their role in insecticide detoxification. The detoxifying enzymes are capable of hydrolyzing insecticidal esters, thus conferring resistance in some insect species. The discovery of insensitive acetylcholinesterase as a biochemical mechanism of OP- and carbamate-resistance in several dipterans, heteropterans and hemipterans[4–8] has enabled scientists to use microassay techniques based on the Ellman's DTNB reagent, 5,5′-dithiobis (2-nitrobenzoic acid)[9,10], and acetylthiocholine to detect different acetylcholinesterase genotypes in insect populations[11–16]. In addition, researchers[17] found lower rates of methyl butyrate hydrolysis in OP-resistant compared to susceptible housefly strains. It was theorized that the genetic modification of a particular esterase in OP-resistant flies increased its hydrolytic activity towards OPs and reduced its capacity to hydrolyze methyl butyrate as well as other non-insecticidal esters. It is noteworthy that OP-resistance could be monitored by measuring esterase activity alone instead of using toxicity tests.

An extremely sensitive colorimetric technique is available to assay for esterase activity in mammalian[18] and insect[19] tissues. The assay is based on the estimation of naphthol produced from the hydrolysis of naphtholic esters. There are two major components of this colorimetric procedure: 1) an azo dye which couples to naphthol to produce a colored conjugate; and 2) a surfactant (usually dodecyl sulfate sodium salt, SDS) which solubilizes the naphtholic azo dye conjugate. Unfortunately, SDS is a protein-denaturing agent, known to inactivate the hydrolytic activity of esterases; therefore, the application of naphtholic esters is limited to end-point assays only. For unknown reasons, naphtholic esters but not nitrophenolic and other esters[19–21] detect esterase activity, that is negatively correlated with OP-resistance in houseflies[19] and is positively correlated with OP-resistance in aphids[20–22]. The introduction of this simple and sensitive colorimetric assay for esterase activity, even with its limitations, has marked a breakthrough in our ability to monitor and study insect insecticide resistance.

With all the advantages of the naphthol-based assays, there are also limitations[23,24]. For example, SDS, commonly used to solubilize the naphtholic azo dyes and terminate enzymatic reactions, does not always inactivate esterase activity[23]. Furthermore, SDS retards the reaction between many diazonium salts and naphthols. Adding Fast Blue at the beginning of enzyme reactions, to make the assay continuous in the absence of SDS, also results in difficulties. For example, the diazonium salt can effect enzyme, catalytic activity. The addition of Fast Garnet GBC salt to mosquito homogenates in the presence of Triton X-100 immediately inactivated esterase activity[24]. The excessive insolubility of the naph-

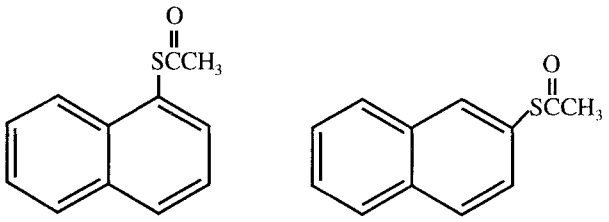

Figure 1. 1-Naphtyl thioacetate (left) and 2-naphthyl thioacetate (right) synthesized by the reaction of the corresponding naphthalene thiol with acetic anhydride in the presence of sulfuric acid as a catalyst.

tholic diazo dye complex in an aqueous reaction mixture[18] interferes with the measurement of the absorbance, especially at high concentrations. The formation of Schlieren lines adds to the error, especially when cuvettes are washed and used repeatedly.

In the present study, new esterase substrates, chemically related to the conventional naphthyl acetates but able to generate an ionizable chromophore with high solubility in an aqueous reaction mixture, were developed to circumvent the problems associated with traditional naphtholic esters. This approach excluded the use of solubilizing agents like SDS, enabling enzymatic reactions to be monitored continuously. 1- and 2-naphthyl thioacetates (fig. 1) were synthesized by the reaction of the corresponding naphthalene thiol with acetic anhydride in the presence of sulfuric acid as a catalyst[25]. DTNB[9,10] could then be used as a chromogen for the estimation of the free thiol, the product of ester hydrolysis. The instantaneous reaction of free thiol with DTNB produces 2-nitro-5-thiobenzoic acid, a dianion chromophore, which is highly soluble in water and readily quantified at 412 nm (fig. 2). The absorbance spectra (fig. 2) appeared to be identical to that obtained from the reaction of DTNB with other thiols[9,10]. The 1-naphthyl thioacetate substrate was found to be more useful than its 2-isomer, because of its higher water solubility and resistance to oxidation to the disulfide. The reaction of DTNB with naphthalene thiols was found to be essentially instantaneous while at least 10 min were needed for the completion of reactions with 1 naphthol. The naphthalene thiolic azo dyes demonstrated maximum absorbance for the 1- and 2-isomers at 431.4 and 425.1 nm, respectively (fig. 2). Figure 2 includes the absorbance spectra of the naphtholic azo dye complexes for comparison.

Naphthalene thioacetates were evaluated as esterase substrates in both an end point and a kinetic microassay using porcine carboxylesterase[26]. The table includes some kinetic parameters for this reaction using 1- and 2-naphthyl thioacetates as well as 1-naphthyl acetate. As expected, both the kinetic and end point assays of porcine carboxylesterase activity gave similar K_m and V_{max} values for reactions with 1-naphthyl thioacetate. The V_{max} values are almost the same for all the tested

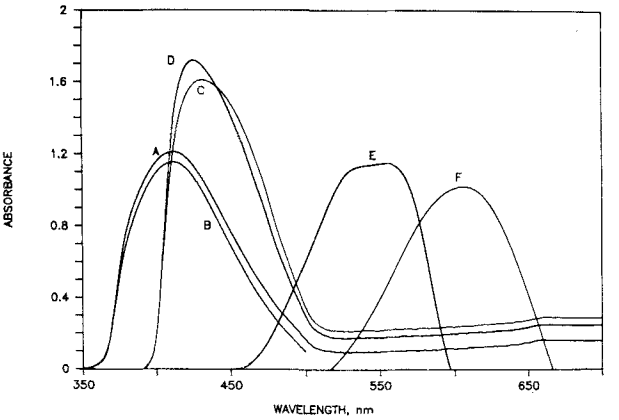

Figure 2. Spectrophotometric scanning of the product of the reaction of 89.5 nmol/ml of *A* 2- and *B* 1-naphthalene thiol with 525 nmol DTNB/ml. Scans of the conjugates resulting from coupling the same concentrations of *C* 1- and *D* 2-naphthalene thiol and *E* 2- and *F* 1-naphthol with Fast Blue B salt (0.084%, w/v) in the presence of SDS (0.357%, w/v) are also shown. All the chromophores were run in sodium phosphate buffer (I = 0.2 M, pH 7.4) at 30 °C, using an SLM Aminco DW-2000 Spectrophotometer (Urbana, IL USA) interfaced with an IBM computer. The optimal wavelength for the above scans are 411.1, 411.6, 431.4, 425.1, 553.6 and 604.2 nm, respectively.

substrates irrespective of the structure of the leaving group and the assay procedure. It should be noted that V_{max} under steady state kinetics is the product of the catalytic turnover number and the molar equivalency of the tested enzyme. Since the activity is calculated per mg protein, the second term is identical for all substrates. Consequently, the catalytic turnover number, i.e., the first order rate constant of deacylation, was identical irrespective of the structure of the leaving group. The slightly larger values for the end point assay may indicate that SDS did not completely stop the enzymatic reaction in the time period between adding the SDS-Fast Blue mixture and the absorbance reading. In summary, our results demonstrate that naphthalene thioesters provide reproducible and accurate measurements of esterase activity consistent with previous assay methods. These novel substrates are applicable to a continuous microassay for general carboxylesterase activity using microtiter plate technology (described in the footnote of the table) and should all but negate the need for the traditional naphthol-based, end-point carboxylesterase assay. An example of the application of this new technology follows.

The intensive use of insecticides, as well as the introduction of cultivars resistant to the infestation of aphids, has resulted in many new biotypes. Insecticide-resistant aphids have now become common, including those which reproduce entirely by parthenogenesis. The green peach aphid (GPA), *Myzus persicae* (Sulzer), has received great attention due to its vast economic importance as an agricultural pest and vector of a multitude of viral diseases. Insecticide resistance in this species has

Table. Michaelis constant (K_m) and maximum velocity (V_{max}) of the catalytic reaction of porcine carboxylesterase with selected naphtholic esters.

Ester[a]	Assay	K_m, μM	V_{max} μmol/min-mg protein
2-NTA	Kinetic	82	56.5
1-NTA	Kinetic	134	57.0
	End point	155	62.7
1-NA	End Point	76	68.6

[a]1- and 2-NTA are the position isomers of naphthalene thioacetate and 1-NA is 1-naphthyl acetate. The steady state kinetics were run with at least 9 concentrations from 9.38 to 600 μM. The specific activity was the average of at least six replicates conducted on two separate occasions. The standard deviations were always less than 5% of the mean. A microplate assay in a reaction mixture of 200 μl was performed at 30 °C in a Thermomax Microplate Reader interfaced with version 2.01 of SOFTmax from Molecular Devices Corp., Palo Alto, CA (USA). For the kinetic assay, 25 μl of 5 mM DTNB and 75 μl of the enzyme solution were added to each well. The enzymatic reaction was started by adding the substrate in 100 μl of sodium phosphate buffer (I = 0.2 M, pH 7.4). The absorbance at 405 nm was monitored automatically at 10 s intervals and the activity calculated in the linear range. Only the concentrations (at least 6) which fell in the middle of the rectangular hyperbola generated from the plot of incubation time versus absorbance, were used to generate double reciprocal plots for the calculation of K_m and V_{max}. In the end point assay, the substrate was added to the enzyme and the reaction was incubated for 10 min. At the end of the incubation period, 25 μl of 3.4% SDS and 0.8% Fast Blue B salt in water was added. 10 min later, the absorbance was read at 450 and 595 nm, for 1-NTA and 1-NA, respectively.

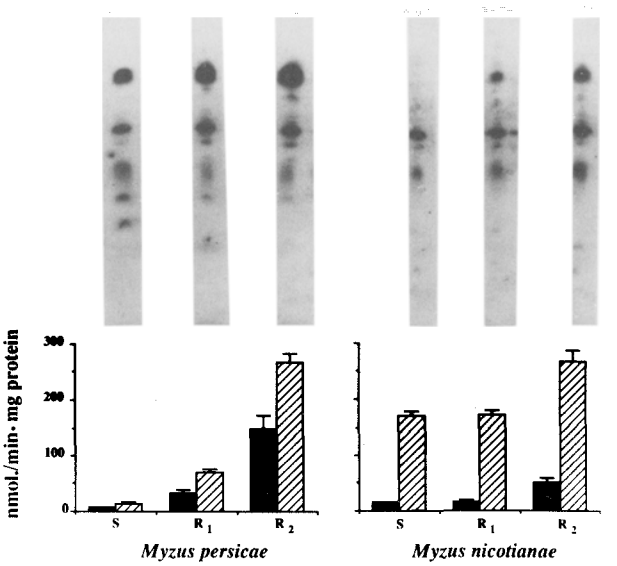

Figure 3. Activity of aphid esterases towards 1-naphthyl thioacetate (solid bars) and 2-naphthyl thioacetate (hatched bars). S represents the reference susceptible strains while R_1 and R_2 represent the OP-resistant strains. The resistance factors for malathion in R_1 and R_2 were 3.5- and 3.8-fold, respectively, for the tobacco aphid; and 3.5- and 148-fold, respectively, for the green peach aphid. The lanes above the histograms are esterase banding patterns[33] resolved by wide range isoelectric focusing (IEF, pH 3.5–9.5). The anode is at the top. Note that the most acidic band in resistant tobacco aphids (a resistance associated esterase) is absent in the most OP-susceptible strain of the same species. It is not clear from this IEF analysis whether the same esterase is found in the green peach aphid. However, we have shown that the most anodic resistance associated esterase of the tobacco aphid exhibited some degree of cross reactivity with IgG for esterase 4 from the British green peach aphid.

now been taken from bioassay to biotechnology[27]. The milestone in the advancement of our knowledge of insecticide resistance in the GPA has come from the early finding[22] that resistance is linked to high esterase activity towards 1-naphthyl acetate. The tobacco aphid (TA) has been recently identified as a new species, *Myzus nicotianae* Blackman, closely related to the GPA, but with its own morphometric characteristics[28]. This new species, although often parthenogenetic[28,19], has also evolved resistance to most of the registered organophosphorus insecticides in the southeastern United States. Both red and green colored TAs demonstrate this resistance[30].

The above described thioacetates were tested as potential diagnostic substrates for the detection of insecticide resistance in both the GPA and TA[31]. The esterase activity for susceptible and OP-resistant strains of these two species is shown in figure 3 and is in close agreement with malathion toxicity[32]; high esterase activity was always correlated to low susceptibility (high LC_{50} values), independent of the species tested.

Esterases from resistant and susceptible strains of the GPA and TA were examined[33] using isoelectric focusing (IEF). The IEF-resolved esterases (fig. 3) correlated favorably with the general esterase activity in aphid homogenates, confirming the association between insecticide resistance and high esterase activity. The esterase banding pattern was identical for susceptible and resistant GPAs; however, the intensity of some bands was stronger in resistant as compared to susceptible aphids. The TA showed some qualitative differences in that one of the resolved esterases was apparently missing in the susceptible strain.

Conventional susceptibility tests (bioassays) monitor the rate of development of resistance, and multiple discriminating dosages can roughly identify the proportion of resistant phenotypes, regardless of the mechanism of resistance. However, knowledge of the mechanism of resistance is certainly among the most important factors which guide decisions for the implementation of rational resistance management strategies. It was previously reported that genetic and biological factors proceed independent of management strategies during the step-wise microevolution of resistance[34]. However, we believe that understanding the mechanism of resistance and its distribution among field populations will help in the judicious use of insecticides. This management strategy will indirectly control both the genetic and biological factors responsible for resistance. Detection methods for insecticide resistance which are directly related to genetic factors in individual insects

will be the method of choice for understanding the genotypic architecture of populations and responses to pesticide use.

In several arthropod species from different orders[17,35], organophosphate- and carbamate-resistance is somehow linked to esterase activity toward surrogate substrates. End point assays, using 1-naphthyl acetate as an esterase substrate, have been used most frequently to monitor this resistance. However, this approach only discriminates between resistant and susceptible allozymes based on activity[36,37]. The novel naphthalene thioesters reported here make available a continuous microassay for carboxylesterases without changing significantly the basic premise of the traditional 1-naphthyl acetate substrate. Accuracy is improved because of the ability of multiple measurements at different time intervals; only a single measurement is made in an end-point measurement. A continuous assay also allows for the measurement of the initial turnover rate, and the calculation of K_m and V_{max} from the same reaction. End-point assays can introduce errors in quantification since rates can inadvertently be measured outside of the linear range for the assay.

In summary, resistance to insecticidal esters, especially OPs, has been associated with higher esterase activity in several insect species[1-3,20-22,38-41]. In most of the cases, the elevated esterase activity, even towards non-insecticidal esters, is due to specific genes that sort with resistance in insect populations. The naphthalene thioesters described here provide a new detection methodology for measuring elevated esterase activity in insects like that of aphids. The monitoring of resistance-associated esterases in insect populations should be a useful tool in measuring not only the level of activity but also the catalytic features of these enzymes so that this information can be incorporated into management strategies for insecticide resistance[42]. In addition, there are a number of applications for these new substrates in other fields. Esterases can be specific markers of T-cells in humans and other animals[43] and act as models for the study of regulatory mechanisms for protein synthesis, expression and metabolism[44]. Besides providing increased sensitivity in the kinetic assay of esterases in solution, the use of these substrates under native conditions coupled with the production of insoluble conjugates, make these compounds powerful and versatile research tools for the characterization of esterases in animal or plant tissues and on solid support matrices. The latter feature may allow us to restructure the traditional esterase assay methodology for new applications in research and applied agriculture and medicine.

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31 The OP-susceptible strain of the GPA was from a clone maintained with insecticidal pressure in the laboratory of Dr. Susan Halbert, University of Idaho, Parma, ID. Two OP-resistant strains of this species, maintained under weekly insecticidal treatments, were collected from greenhouses at North Carolina State University, Raleight, NC (USA). Two OP-resistant (red and green color forms) and an OP-susceptible (green color form) clonal culture of the TA were maintained in the laboratory by E. P. Lampert at North Carolina State University.
32 The concentration of malathion required to kill 50% of the tested population (LC_{50}) in a slide dip test[20,21,30] were 91, 323 and 13,445 ppm for susceptible (S), resistant (R_1), and exttremely resistant (R_2) strains of the GPA. The LC_{50} values for the TA were 23.9, 83.8 and 91.7 ppm for the susceptible (S), resistant green (R_1) and resistant red (R_2) clonal cultures, respectively[30]. A linear relationship was observed between the malathion LC_{50} ratio using the most susceptible TA culture as a reference, and esterase activity towards 1-naphthyl valerate in six clones of the TA and GPA[45,46].
33 Apterous adults from each strain were homogenized in distilled water containing 0.1% Triton X-100 at 20 mg of aphid fresh weight/ml and the homogenate was then centrifuged at 5,000 g for 5 min. The supernatant was applied to LKB ampholine PAGplates (pH range 3.5–9.5) at 5 µl/lane and the gel was focused at 5 °C with a constant power of 25 watts for 1.5 h. After electrofocusing, the gel was incubated in 0.02% (w/v) 1-naphthyl acetate in sodium phosphate buffer (pH 7.4, I = 0.2 M) for 30 min. The gel was then gently washed with distilled water and incubated for 20 min in 0.13% (w/v) Fast Blue B salt in distilled water. The stained gel was subsequently washed and stored in water at 4 °C for analysis.
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