14. M. F. Piercey, K. Folkers, L. A. Schroeder, F. J. Einspahr, J.-C. Xu, J. Horig, in *Seventh American Peptide Symposium*, D. Rich, Ed. (Pierce Chemical Company, Rockford, Ill., in press).
15. D. J. Finney, *Statistical Methods in Biological Assay* (Hafner, New York, 1952), p. 524.
16. P. J. K. Dobry, M. F. Piercey, L. A. Schroeder [*Neuropharmacology* **20**, 267 (1981)] showed that somatostatin and kainic acid elicited a weak one-sided scratching syndrome similar to that evoked by very low doses of SP. Other biogenic amines, neuropeptides, and central nervous system drugs and analgesics did not, however, evoke any scratching syndrome.
17. R. Werman, *Comp. Biochem. Physiol.* **18**, 745 (1966).
18. R. W. Ryall and G. Belcher, *Brain Res.* **137**, 376 (1977); W. A. Krivoy, J. R. Couch, J. L. Henry, J. M. Stewart, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 2346 (1979); W. A. Krivoy, J. R. Couch, J. M. Stewart, E. Zimmermann, *Brain Res.* **202**, 365 (1980).
19. R. C. A. Frederickson, V. Burgis, C. E. Harrell, and J. D. Edwards [*Science* **199**, 1359 (1978)] and P. Oehme, H. Hilse, E. Morgenstern, and E. Göres [*ibid.* **208**, 305 (1980)] suggest that SP can produce both analgesic and hyperalgesic modulation of pain thresholds. This modulation did not occur (*14, 16*) during the time period of our observations; rather, we only saw scratching and biting after injecting SP directly into the central nervous system.
20. P. G. Guyenet, E. A. Mroz, G. K. Aghajanian, and S. E. Leeman [*Neuropharmacology* **18**, 553 (1979)] as well as M. F. Piercey and F. J. Einspahr (unpublished data) have shown that the slow neuronal response to iontophoretic SP is likely to be a technical artifact.
21. M. Otsuka and M. Yanagisawa, in *Proceedings of the 7th International Congress of Pharmacology*, P. Simon, Ed. (Pergamon, Oxford, 1978), vol. 2, p. 181; W. Zieglgansberger and I. F. Tulloch, *Brain Res.* **166**, 273 (1979).
22. T. Hokfelt, O. Johansson, J.-O. Kellerth, L. Ljungdahl, G. Nilsson, A. Nygards, B. Pernow, *Nobel Symp.* **37**, 117 (1977).
23. J. J. G. Boivie and E. R. Perl, in *Neurophysiology*, C. C. Hunt, Ed. (Butterworths, London, 1975), p. 303.
24. H. H. Dale, *Proc. R. Soc. London Ser. B* **28**, 319 (1935).
25. F. Lembeck and P. Holzer, *Naunyn-Schmiedebergs Arch. Pharmakol.* **310**, 175 (1979); S. Rossell *et al.*, *Acta Physiol. Scand.* **111**, 381 (1981).
26. Supported in part by the Robert A. Welch Foundation.

21 October 1981

## Chemical Self-Defense by Termite Workers: Prevention of Autotoxication in Two Rhinotermitids

Abstract. *Soldiers of the lower termites* Prorhinotermes simplex *and* Schedorhinotermes lamanianus *(Isoptera, Rhinotermitidae) have electrophilic contact poisons used in colony defense. Workers of these termites die when exposed to the defense secretion of the other species, but survive when exposed to chemicals from conspecific soldiers. Detoxication occurs by an initial substrate-specific reduction of the electron-deficient double bond of the nitroalkene (*Prorhinotermes simplex*) or vinyl ketone (*Schedorhinotermes lamanianus*) followed by complete catabolism to acetate.*

Chemical defense has evolved numerous times in the termite families Rhinotermitidae and Termitidae (*1*). Three defensive strategies are employed by chemically armed soldier termites (*2*): (i) biting, with the addition of an oily, toxic or irritating secretion from the frontal gland reservoir (Termitinae), (ii) brushing a copious amount of a hydrophobic contact poison onto the cuticle of an attacker (Rhinotermitidae), or (iii) ejection of an irritating glue-like secretion (Nasutitermitinae). Each of these modes of chemical defense requires a strategy for chemical self-defense (*3*), that is, a mechanism for conspecifics to avoid intoxication. Termite workers of the species *Prorhinotermes simplex* (Prorhinotermitinae) survive exposure to vapors and liquid forms of a volatile, toxic nitroalkene (*4*) secreted by conspecific soldiers during an attack on the colony. Similarly, *Schedorhinotermes lamanianus* (Rhinotermitinae) workers survive exposure to volatile vinyl ketones (*5*) produced by their soldiers in response to disturbance. We now report that the termite workers of these two species have substrate-specific alkene reductases which, in the presence of a reduced nucleotide cofactor, catalyze the reduction of the electron-deficient double bond of the unsaturated electrophilic group. The saturated compounds are subsequently recycled in vivo by way of catabolism to acetate. This represents a rare example of an electrophilic insecticidal compound that is detoxified by initial reduction rather than by oxidation or conjugation (or both) (*6, 7*).

Radioactively labeled defense secretion compounds were synthesized (Fig. 1) (*8*). Condensation of [1-$^{14}$C]tetradecanal (*8*) with nitromethane in methanolic sodium methoxide (*4*) followed by stirring the nitroalcohol with acetic anhydride-pyridine gave (*E*)-1-nitro-1-[2-$^{14}$C]pentadecene (**1**) (0.05 mCi/mmole) in 37 percent chemical yield after chromatography and recrystallization from cold hexane. The vinyl ketone was prepared (*5, 6*) by reaction of 11-[1-$^{14}$C]dodecenal (*8*) with vinylmagnesium bromide followed by oxidation of the allylic alcohol with manganese dioxide to give 1,13-[3-$^{14}$C]tetradecadien-3-one (**3**) (0.32 mCi/mmole) in 53 percent yield after chromatography. Unlabeled nitroalkene **1** and vinyl ketone **3** were also prepared for comparative toxicity studies and for monitoring detoxication by gas chromatography.

Twenty worker termites of *Schedorhinotermes lamanianus* (*9*) were treated with the labeled vinyl ketone [3-$^{14}$C]**3** by topical application of 100 μg per termite and were held in sealed dishes for 24 hours at 27°C. The termites were then homogenized in methanol, the extract was centrifuged, and the supernatant was subjected to reverse-phase high-performance liquid chromatography (*10*). Three peaks of radioactivity could be observed during gradient elution from water to methanol. Peak 1 (36 percent of soluble radioactivity) eluted at the solvent front and was shown to contain [$^{14}$C]acetate by purification of the *p*-bromophenacyl derivative (*8*). Peak 2 (21 percent) cochromatographed with the glutathione conjugate of **3** (*8, 11*) under these high-performance liquid chromatography conditions; however, a higher resolution separation (*8*) revealed the absence of glutathione or cysteine conjugates, and the identity of this peak is uncertain. Peak 3 (43 percent) cochromatographed with starting vinyl ketone **3**, but lacked the ultraviolet absorption at

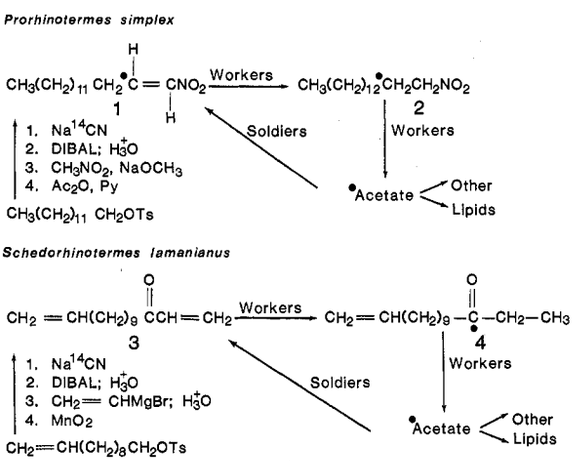


Fig. 1. Synthesis and detoxication of radioactively labeled soldier defense secretions. (Top) Nitroalkene **1** is converted first to nitroalkane **2** and then to acetate. (Bottom) Vinyl ketone **3** is converted first to ethyl ketone **4** and then to acetate. Biosynthesis of the nitroalkene **1** from acetate by soldiers has also been demonstrated (*8*).


0036-8075/81/1218-1363$01.00/0 

Table 1. Interspecific toxicity ($LD_{50}$, median lethal dose) of defense substances to three species of rhinotermitid workers. Twenty-five workers were exposed to pure synthetic compounds impregnated in moistened cellulose pads in sealed dishes (*8*).

| Substance tested | $LD_{50}$ (milligrams per dish at 48 hours) | | |
|---|---|---|---|
| | *R. flavipes* | *P. simplex* | *S. lamanianus* |
| Nitroalkene **1** | 0.45 | > 10 | 1.2 |
| Nitroalkane **2** | 0.75 | > 10 | 1.5 |
| Vinyl ketone **3** | 0.05 | 0.11 | 0.60 |
| Ethyl ketone **4** | 0.65 | 0.65 | > 5 |

219 nm. Peak 3 was more abundant after the reaction mixture was incubated for 4 hours (peaks 1, 2, and 3—16:26:58) and was virtually absent after 48 hours (peaks 1, 2, and 3—75:25:0).

The identity of the first-produced detoxication product contained in peak 3 was established by capillary gas chromatography (*12*) and by synthesis (*8*) as the saturated ketone **4**. The time course of production of **4** was established by incubation of **3** with tissue homogenates containing different cofactors and then monitoring the appearance of **4** and disappearance of **3** by quantitative gas chromatography analysis (Fig. 2). Furthermore, reduction of the unconjugated 13,14 double bond was not detected during those incubations, although this product has now been detected (*12*) in the soldier secretion. The initial reductive detoxication of vinyl ketone **3** in vitro was independent of added glutathione but dependent on the presence of a reduced nucleotide cofactor. Moreover, the half-life of **3** in vitro was less than 1 hour in the presence of a tenfold excess of NADH or NADPH (nicotinamide adenine dinucleotide reduced or nicotinamide adenine dinucleotide phosphate reduced) and the more electrophilic conjugated alkene was reduced selectively.

Topical application of 100 μg of the labeled nitroalkene [2-$^{14}$C]**1** to each of 20 workers of *Prorhinotermes simplex* (*9*) was followed by incubation (24 hours), homogenization, and analysis by high-performance liquid chromatography (*10*) and gas chromatography (*12*) as described above. Radioactivity peaks 1 (72 percent), 2 (4 percent), and 3 (24 percent) were observed in the liquid chromatogram, corresponding to labeled acetate, the glutathione conjugate of **1** and reduced starting material, respectively. Gas chromatographic analysis of peak 3 confirmed that only the saturated nitroalkane **2** was present after 24 hours of incubation.

The interspecific toxicities of the two defense secretions and their reduced forms were determined by exposing workers of *P. simplex*, *S. lamanianus*, and the eastern subterranean termite *Reticulitermes flavipes* to filter pads containing unlabeled **1**, **2**, **3**, or **4**. Mortality after 48 hours for these two compounds is shown in Table 1. Exposure to the vinyl ketone **3** (*S. lamanianus* soldier secretion) results in rapid mortality of *R. flavipes* even at low doses, whereas *S. lamanianus* is killed slowly at 2 mg per dish. An intermediate ability to detoxify this "unfamiliar" electrophilic compound is shown by *P. simplex*. Exposure to the nitroalkene **1** (*P. simplex* soldier secretion) also causes mortality in *R. flavipes*, whereas *P. simplex* is unaffected even at high doses. *Schedorhinotermes lamanianus* possesses an intermediate ability to survive this electrophilic toxin. The vinyl ketone **3** is more toxic than **1** by an order of magnitude. The saturated detoxication products **2** and **4** showed lower toxicity for each of the three species tested.

The reductive detoxication of electrophiles by an insect is unprecedented and surprising. We had begun this project with the expectation that either mercapturic acids or glutathione conjugates (*13*, *14*) would be isolated as the primary detoxication products, in which addition of nucleophilic sulfhydryl groups to the unsaturated systems would provide more water-soluble, less reactive compounds. Glutathione *S*-transferase activity has been found in flies (*15*), caterpillars (*16*), cockroaches (*17*), and other invertebrates (*18*), and is implicated in resistance to chloronitrobenzenes, diazinon, and various organophosphorus insecticides (*15*). Although these two termite species do have glutathione *S*-transferase activity (*8*), this is clearly not the primary mode of detoxication.

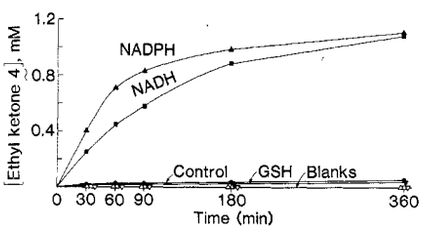


Fig. 2. Cofactor requirements and time course of reductive detoxication of vinyl ketone **3** by tissue homogenates of *Schedorhinotermes lamanianus* workers. Workers (100) were homogenized in 50 m*M* phosphate buffer (*p*H 7.2) containing 25 m*M* $MgCl_2$, and incubations were performed with 1.3 m*M* vinyl ketone **3** and added cofactors (closed symbols) at the following concentrations: NADPH (triangles), 10 m*M*; NADH (squares), 10 m*M*; reduced glutathione (inverted triangles), 4 m*M*; and with no cofactors (circles). Blank determinations on cofactor solutions lacking tissue homogenates are shown as open symbols. The natural detoxication product, saturated ketone **4**, was determined by quantitative gas liquid chromatography of ethyl acetate extracts of the incubation mixtures (*12*).

Soldiers of the advanced rhinotermitines have evolved as unusual ability to biosynthesize (*8*) lipid-soluble contact poisons on the basis of the modification of fatty acids into nitroalkenes, vinyl ketones, and β-ketoaldehydes (*19*). Each minor soldier of *S. lamanianus* contains 300 μg of a mixture of vinyl ketones, sufficient to kill hundreds of biochemically unprotected arthropods (*5*). The rapid reduction of endogenous electrophilic compounds may be the result of evolutionary pressures on termites to develop detoxication pathways which are nitrogen-conserving. The low dietary nitrogen intake of termites has resulted in a variety of nitrogen conservation strategies (*20*). Indeed, detoxication of a defense substance by reduction of the cytotoxic moiety followed by the recycling of its carbons by oxidative catabolism represents an adaptation for the avoidance of nitrogen loss inherent in the excretion of glutathione or cysteine conjugates of insecticides (*21*).

S. G. SPANTON
G. D. PRESTWICH
*Department of Chemistry,*
*State University of New York,*
*Stony Brook 11794*

**References and Notes**

1. G. D. Prestwich, *J. Chem. Ecol.* **5**, 459 (1979); *Sociobiology* **4**, 127 (1979).
2. A. Quennedey, *Recherche* **6**, 274 (1975).
3. P. Howse, *Proc. IUSSI Symp.* (Dijon) (1975), p. 23.
4. J. Vrkoč and K. Ubik, *Tetrahedron Lett.* (1974), p. 1463.
5. A. Quennedey, G. Brule, J. Rigaud, P. Dubois, R. Brossut, *Insect Biochem.* **3**, 67 (1973); G. D. Prestwich, M. Kaib, W. F. Wood, J. Meinwald, *Tetrahedron Lett.* (1975), p. 4701.
6. Detoxication of endogenously produced insecticides has been demonstrated for a polydesmid millipede that produces HCN and phenol [S. S. Duffey and M. S. Blum, *Insect Biochem.* **7**, 57 (1977)].
7. T. Nakatsugawa and M. A. Morelli, in *Insecticide Biochemistry and Physiology*, C. F. Wilkinson, Ed. (Plenum, New York, 1976), p. 61; R. S. H. Yang, *in ibid.*, p. 177.
8. All synthetic compounds were homogeneous as judged by gas chromatography, thin-layer chromatography, and/or high-performance liquid chromatography, and their structures were consistent with all spectral data [S. G. Spanton and G. D. Prestwich, in *Tetrahedron Symposium-in-Print on Animal Chemical Defenses*, J. Meinwald, Ed. (Pergamon, Oxford, in press)].
9. *Prorhinotermes simplex* (Hagen) colonies were obtained (December 1978) from rotten pine in Miami, Fla., by M. E. Melcer and P. Luykx. *Schedorhinotermes lamanianus* (Sjöstedt) was obtained by G.D.P. and B. L. Bentley (March

1980) from a carton nest in a fallen tree (Jadini Forest, Diani, Kenya). Both have been maintained in the laboratory at 27°C and 85 percent relative humidity since their arrival at Stony Brook. *Reticulitermes flavipes* (Kollar) colonies were obtained on campus and held under similar laboratory conditions.
10. After incubation, 20 termites were homogenized in 1 ml of methanol in a Microflex vial, and insoluble material was removed by centrifugation (2000 rev/min) followed by filtration through a Millipore HPLC sample clarification kit. Radioactivity was determined in samples of soluble and tissue-digested insoluble fractions. Of the recovered radioactivity (62 percent of total applied), 82 percent was in the soluble fraction, 6 percent was in the insoluble fraction, and 12 percent remained in the petri dish. The methanol was removed at reduced pressure, and the residue was dissolved in 2 ml of water buffered to *p*H 5.6 with 0.1 percent acetic acid and ammonium hydroxide, and a portion was injected onto a Whatman PXS 10/25 ODS reverse-phase column and eluted at 1.5 ml/min following a linear gradient to 100 percent methanol. Eluent was monitored continuously at 254 and 219 nm. Samples collected at 2-minute intervals were counted with the use of a Packard TriCarb, with quench corrections by automatic external standarization.
11. Glutathione and cysteine adducts were prepared by modifications (*8*) of the thiosilyl ether method [J. Rokach *et al.*, *Tetrahedron Lett.* **21**, 1485 (1980)].
12. Gas chromatography was performed on a Varian 3700 equipped with a 50-m glass capillary coated with Carbowax 20M (160°C) and a Vista CDS-401 data reduction system. An internal standard (*n*-hexadecane) was added to incubations to correct for injection variability and an external standard (*n*-pentadecane) was added to samples to check total recovery.
13. E. Boyland and L. F. Chasseaud, *Adv. Enzymol.* **32**, 173 (1969); L. F. Chasseaud, in *Glutathione: Metabolism and Function*, I. M. Arias and W. B. Jakoby, Eds. (Raven, New York, 1976), p. 77.
14. W. Jakoby, *Adv. Enzymol.* **46**, 383 (1978).
15. N. Motoyama and W. C. Dauterman, *Pestic. Biochem. Physiol.* **5**, 489 (1975); F. J. Oppenoorth, L. J. T. van der Pas, N. W. X. Houx, *ibid.* **11**, 176 (1979).
16. F. Gould and E. Hodgson, *ibid.* **13**, 34 (1980).
17. K. Usui, J. Fukami, T. Shishido, *ibid.* **7**, 249 (1977).
18. A. G. Clark, J. N. Smith, T. W. Speir, *Biochem. J.* **135**, 385 (1973); J. N. Smith, in *Drug Metabolism—from Microbe to Man*, D. V. Parke and R. L. Smith, Eds. (Taylor and Francis, London, 1977), p. 219.
19. G. D. Prestwich and M. S. Collins, *J. Chem. Ecol.*, in press.
20. G. D. Prestwich, B. L. Bentley, E. J. Carpenter, *Oecologia* **46**, 397 (1980) and references therein.
21. We thank the NSF for partial financial support (DEB-7823257 and CHE-7925081), and the Alfred P. Sloan Foundation for a fellowship to G.D.P. (1981–1983).

22 May 1981; revised 6 July 1981

## Side-Effect Reduction by Use of Drugs That Bind to Separate but Equivalent Binding Sites

Abstract. *If two drugs cause the same molecular effect by binding to separate noncompetitive binding sites, therapy with a mixture of the two drugs can provide a given desired effect with a lower level of side effects than therapy with either drug alone.*

We recently demonstrated that batrachotoxin-activated sodium channels have at least two binding sites for local anesthetics and that binding of a local anesthetic at either or both sites prevents ions from passing through the channel. When we simultaneously applied two local anesthetics, each with a specificity for a separate site, we observed that the drugs acted synergistically to block the sodium channels (*1*). Synergism of two local anesthetics was also observed by Mrose and Ritchie (*2*). We have suggested the possible clinical usefulness of this effect (*1*).

This type of synergism is quite general, and corresponds to the case of cooperative noncompetitive inhibitors in enzyme kinetics (*3*). It occurs whenever (i) there are two receptor sites, binding to either or both of which causes the same molecular effect; (ii) drugs are available that have a specificity for each site; and (iii) the dissociation constant for the binding of each drug to its receptor does not depend on whether the other site is occupied.

Local anesthetics that are more potent in terms of desired effects also have stronger side effects (*4*). We considered the case in which both this generalization and cooperative noncompetitive inhibition apply and calculated the relative side effects for single-drug therapy and for two-drug therapy with additive side effects. We found that two-drug therapy results in weaker side effects. The ratio of side effects for two-drug therapy to side effects for single-drug therapy can be expressed as a function of the therapeutic ratio and the fraction of channels (or other molecules) that must be inhibited.

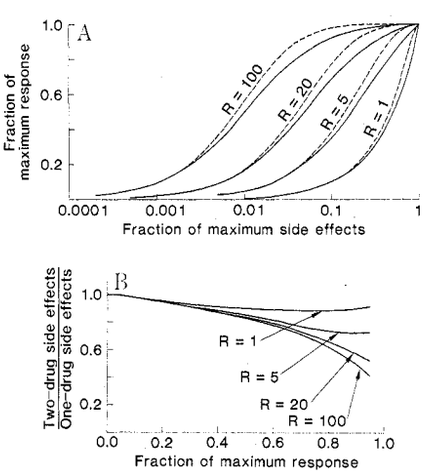


Fig. 1. (A) Side effect–response curves. The parameter $R$ is the ratio of side-effect dissociation constant to desired-effect dissociation constant. For each $R$, the solid curve corresponds to one drug and the dashed curve corresponds to mixtures of two drugs that bind to separate but equivalent sites. (B) Side-effect ratio as a function of $R$ and the fraction of the maximum response.

Although mixed-drug therapy with the intent of reducing side effects while maintaining maximum desired effect has been common medical practice for some time, it generally has not been employed for the case in which the side effects are additive. In this report we explicitly consider this case.

For two cooperative noncompetitive inhibitors, the fraction of states that have at least one drug bound is (*3*)

$$F_2 = \frac{(D_1/K_1) + (D_2/K_2) + (D_1D_2/K_1K_2)}{1 + (D_1/K_1) + (D_2/K_2) + (D_1D_2/K_1K_2)} \quad (1)$$

where $D_1$ and $D_2$ are the drug concentrations at the receptor sites (which we will refer to as dosage) and $K_1$ and $K_2$ are the respective dissociation constants.

Mathematically, the synergism between the two drugs occurs because the cross-term in Eq. 1 increases $F_2$. Using the example of drugs that block sodium channels, we can give a more physical explanation for the synergism. After application of the first drug, addition of a second drug not only blocks additional channels (as would increased dosage of the first drug), it doubly blocks some channels that would otherwise be singly blocked. Since there is a lower probability for a channel blocked by two drugs to open than there is for a channel blocked by one drug, the equilibrium is driven toward more closed channels. At low dosages of both drugs, the fraction of channels with both sites occupied is small, so there is relatively little synergism. As the dosages increase, the relative fraction of doubly blocked channels increases, as does the synergism.

Equation 1 represents the dose-response curve for two drugs. If only one drug is present, $D_2$ is zero and Eq. 1 reduces to the dose-response curve for one drug binding to a single site:

$$F_1 = D_1/(K_1 + D_1) \quad (2)$$

Comparison of Eqs. 1 and 2 indicates that only when the two dissociation constants are approximately equal does administration of a mixture of two drugs result in a lower total dosage for a given desired effect. Furthermore, reduction of dosage per se is only important for expensive drugs. A more important advantage of synergism is the reduction of


0036-8075/81/1218-1365$01.00/0  