Chemical Defenses of Insects: A Rich Resource for Chemical Biology in the Tropics

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Abstract Insects make up the largest and most diverse group of organisms on earth, contributing to as much as 80–90% of the world's biodiversity. Approximately 950,000 species of insects have been described; some estimate there are 4,000,000+ species in total. Over 70% of drugs on the market are derived from natural compounds. However, insects are one of the least explored groups in drug discovery. The world adds about 70 million people each year. In this chapter you will find: (1) an introduction to the topic of arthropod chemical biodiversity and chemical defense; (2) a brief discussion on various uses of insect chemistry by various cultures; (3) an overview of insect venoms and other chemical defense studies, with a case study on methods utilized to analyze ant venoms; (4) a short discussion on the importance of preserving tropical habitats for bioprospecting; (5) a review of research on stick insect (Order Phasmatodea) chemical defenses, stick insects as a model for biosynthesis studies and my personal experiences with the editors of this book and 2008 PASI workshop in Peru which resulted in this chapter; (6) an overview of examples from the literature of insect-derived substances with medicinally relevant biological properties such as toxins and antibiotics; (7) a brief description of the importance of studying biosynthetic pathways in insects and other organisms from whence valuable natural products are identified and (8) a list of recommended literature which I expect would be of particular interest to the readers of this chapter.

1 Introduction

Up the trunk of a giant Brazil nut tree crawled an intriguing and majestic creature: a walkingstick insect. As it made its way across, another fellow citizen of the jungle approaches – a lizard. This is not good news for the insect, as most lizards will make

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a meal or light snack of an insect such as this without batting an eye. The lizard approaches stealthily and attacks with the utmost precision. With the insect nearly within the munching distance of the lizard's mouth, suddenly the mission is aborted! In an instant the lizard retreats as if it had encountered some sort of invisible force field. Capitalizing on this opportunity the insect makes its escape: quickly falls to the ground, spreads its bright yellow wings, and takes flight into the deeply shaded darkness of the Peruvian rainforest of the Tambopata National Reserve.

Stories like this are played out many times every day in the wild (Fig. 1). This masterful escape by the walkingstick insect is just one of thousands of examples in the insect world. The lizard was an unfortunate victim of that insect's most potent defense mechanism, its chemical weaponry. Many species of walkingsticks, as well as hundreds of thousands of other insect species (beetles, true bugs, ants, etc.), produce defensive sprays, secretions, or venoms that help protect them from predation.

Fig. 1 *Anisomorpha buprestoides* deploying its chemical spray and repelling a brown Cuban Anole (*Anolis sagrei sagrei*). (**a**) Black and white Ocala National Forest color form of *A. buprestoides* mating pair (females are larger than males) deploying its chemical defense spray. (**b**) Same type of insect as in (**a**) successfully repelling a brown Cuban Anole (*Anolis sagrei sagrei*), a non-native invasive species in Florida. Photograph by Rod C. Clarke and Adam Scott, British Broadcasting Corporation (BBC) Natural History Unit. Still image taken from high-speed video for the future natural history series "Life" (Fall 2009)

2 Background

It is impossible to cover every published example of insect chemical defenses in a single chapter, or even in a single book. However, I will provide some classic and intriguing examples of insects and the chemical compounds they produce to protect themselves from attack. To provide a sense of the vastness of the chemical biodiversity which exists among insect defense mechanisms, one can look at Pherobase (http://www.pherobase.net), a resource aimed at cataloging insect semiochemicals. Semiochemicals are chemical substances used by organisms for communication (pheromones, kairomones, etc.) or defense, which is arguably a form of communication. This database consists of species, taxonomic designations, literature references, and most importantly chemical structures linked to the species which produce them and their function. In Pherobase alone, there are a total of 623 chemical substances identified as "defense substances" (as of July 22, 2008). This number includes other invertebrate taxa, but the vast majority of compounds represented in this database come from insects. Even as large and impressive a database as Pherobase fails to encompass the entire diversity of reported chemical defense substances from insects.

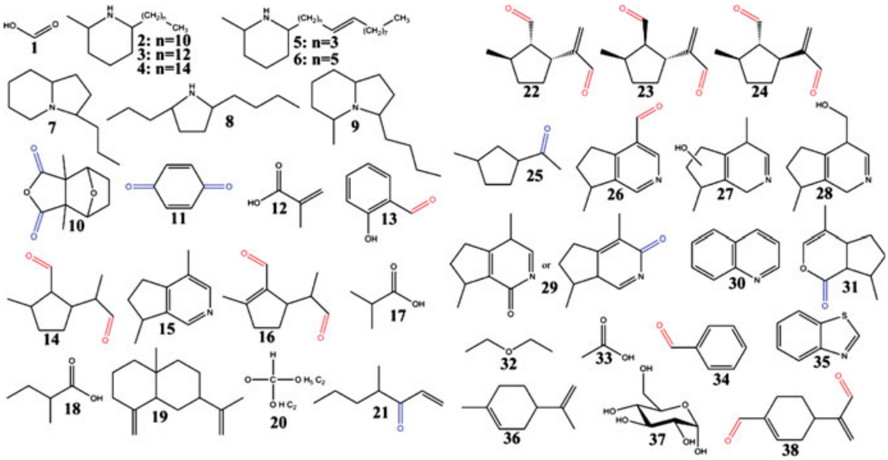
Insects make up 80–90% of the total biodiversity on earth (Hellmann and Sanders 2007), and there are approximately 950,000 described species of insects (Berenbaum and Eisner 2008). It is estimated that 4,000,000 insect species exist on earth (May 2000), but the vast majority is yet to be discovered or described. In addition to their abundance and diversity, insects are of critical importance to mankind. They perform such vital functions as decomposition of dead plant matter and animal waste, they add nutrients to the soil, keep the populations of plants and other animals in check, pollinate at least 177 crop species worldwide, and provide food for many other animals and humans (Hellmann and Sanders 2007). Beyond these examples, there is a vast amount of scientific and technological discovery yet to be explored in insects. In fact little is known about the biosynthetic mechanisms or greater ecological significance of the vast majority of insect defensive chemicals and there remains a vast wealth of chemistry, biochemistry, and chemical ecology yet to be explored. Thus, it is imperative that we preserve and protect the natural environment upon which we depend for our existence, especially the insects with which we share it.

In this chapter, I will separate arthropod chemical defenses into two groups (1) *venoms*, which will be defined as chemicals injected directly into the body tissue of the recipient and (2) *sprays and secretions*, which includes substances sprayed, secreted, oozed, bled, or otherwise exuded from their respective arthropod sources – which encompasses nearly all nonvenom chemical defense strategies utilized by arthropods. In chapter "Defensive Behaviors in Leaf Beetles: From the Unusual to the Weird", another fascinating defensive strategy, fecal shields, used by insects of a particular family of beetles, the Chrysomelidae (leaf beetles), will be described. It is as yet uncertain, however, if chemistry plays a significant role in that particular case of insect self-defense.

3 Chemical Weapons in Insects: History and Examples

3.1 Ethnoentomology

A large portion of animals on earth, particularly insects, utilize chemicals as their primary tools of warfare and defense (Scheme 1). Their study has been the topic of many publications over the past few decades (Blum 1981; Eisner 2003; Eisner et al.



Scheme 1 Molecular structures for chemical compound used by various insect species for defense. *Lines* indicate chemical bonds, ends of lines and intersections are carbon atoms unless otherwise noted by “O” (oxygen atom), “H” (hydrogen atom), or “N” (nitrogen atom). Characteristic ketone moieties are *blue* and aldehyde groups are *red*. (**1**) Formic acid, (**2**) solenopsin A, (**3**) solenopsin B, (**4**) solenopsin C, (**5**) dehydrosolenopsin B, (**6**) dehydrosolenopsin C [**2–6** are from venom of the ant *Solenopsis invicta* (Lofgren et al. 1975; MacConnell et al. 1971)], (**7**) 3-propylindolizidine, (**8**) 2-butyl-5-propylpyrrolidine, and (**9**) 3-butyl-5-methylindolizidine (**7–9** are from the ant *Myrmicaria melanogaster* (Jones et al. 2007), (**10**) cantharidin from blister beetles (Family Meloidae), (**11**) benzoquinone from darkling beetles (Order Coleoptera, Family Tenebrionidae), millipedes (Class Diplopoda), and various other arthropods, (**12**) methacrylic acid, (**13**) salicylaldehyde (compounds **12–13** from beetles in the genus *Calosoma* (Family Carabidae) (McCullough 1969), (**14**) iridodial from various insect species and some plants, (**15**) actinidine from various insects and some plants, (**16**) chrysomelidial from the larvae of leaf beetles (Family Chrysomelidae) (Meinwald et al. 1977), (**17**) isobutyric acid, (**18**) 2-methylbutyric acid, and (**19**) selinene (**17–19** from butterfly caterpillars in the Family Papilionidae), (**20**) compound hypothesized from *Agathemera crassa* in 1934 (Schneider 1934), (**21**) 4-methyl-1-hepten-3-one produced by *Agathemera elegans* (Schmeda-Hirschmann 2006), (**22**) dolichodial, (**23**) anisomorphal, (**24**) peruphasmal [compounds **22–24** produced by *Anisomorpha buprestoides* and *Peruphasma schultei* (Dossey et al. 2006, 2008)], (**25**) 1-acetyl-3-methylcyclopentane from *Megacrania* sp., (**26–29**) analogs of actinidine (compound **17**) produced by *Megacrania* sp. (Ho and Chow 1993), (**30**) quinoline produced by *Oreophoetes peruana* (Eisner et al. 1997), (**31**) nepetalactone, produced by the walkingstick species *Graeffea crouani* (along with iridodial, Scheme 1, Compound **14**) (Smith et al. 1979) and other insects and plants, (**32**) diethyl ether, (**33**) acetic acid, (**34**) benzaldehyde, (**35**) benzothiazole, (**36**) limonene (compounds **32–36** from *Sipyloidea sipylus* (Bouchard et al. 1997), (**37**) glucose, and (**38**) parectadial from *Parectatosoma mocquerysi* (Dossey et al. 2007)

2005), aided tremendously by modern technologies in analytical chemistry (Dossey et al. 2006; Brey et al. 2006). However, various properties of insect chemical defenses have been known to mankind for much of our history.

In one case, the Satere-Mawe people from the Amazonian basin of Brazil have used bullet ants (*Paraponera clavata*) in rite of passage rituals for manhood and social status (Haddad et al. 2005; Bailey et al. 2007; Tremaine 2004). In this gruesome ritual, a hundred or so of these bullet ants are first mixed into an herbal brew until they are fully anesthetized. Next, the ants are woven into a glove made of palm leaves with the stingers on their abdomens pointing toward the inside. This glove is then placed inside a more ornate ceremonial glove. Young men in the tribe must place their hands into the glove for a full 10 min and do this a total of 20 times in order to be respected as men by their elders and to be eligible to hold leadership positions. The excruciating pain from just one of these stings can last several hours.

Another example of ancient use of insect defense chemicals by man is the defensive secretion from blister beetles (Family Meloidae). Cantharidin (Scheme 1, compound **10**), the active substance in blister beetle defensive secretions, causes irritation of the urinary tract that gives a false impression of sexual stimulation (Sandroni 2001). This substance was used by both the ancient Chinese and Greeks for a wide variety of medicinal purposes, from removing warts to enhancing sexual libido (Moed et al. 2001). Examples of its use by European royalty include Livia Caesar of Rome (wife of Augustus Ceasar) (James and Thorpe 1994) and King Henry IV of England. However, the use of this chemical, typically referred to as "Spanish Fly", is illegal in the USA except for the prescribed treatment of warts or for use in animal husbandry (Gottlieb 1993).

The famous poison dart frog toxins used by natives of South America are also insect-derived chemicals that are passed to the frogs through diet. Poison dart frogs are used by South American natives to create poison darts and spears used for hunting. These frogs, when kept in captivity, tend to lose their toxicity over time (Daly et al. 2005). In nature, frogs largely feed on arthropods and it has been shown in recent years that their alkaloid toxins are largely sequestered from arthropods, particularly insects and millipedes, that they ingest (Clark et al. 2005; Daly et al. 2005). Since many insect chemical weapons are intended for use against vertebrate assailants, it is also clear that insect chemical defenses represent a large reservoir of potentially medically relevant substances. Indeed some insect defensive substances have been studied for their potential use as medicines.

3.2 Venoms

Probably the best known examples of arthropod chemical defenses are the venoms used in the bites and stings of such creatures as spiders (Class Arachnida), scorpions (Class Arachnida), ants, bees, and wasps (Class Insecta, Order Hymenoptera). The chemical weapon payloads of these creatures are directly injected into the victim. Many of these venoms contain proteins and peptides as their active components.

The most famous example of proteinaceous insect venom is mellitin found in the sting of the European honeybee (Api's mellifera). The venom of the previously mentioned bullet ants contains a neurotoxic protein called poneratoxin refs.

Ants are also known to use a variety of small molecules such as formic acid (Scheme 1, compound **1**) and alkaloids to defend themselves. Some examples of these compounds are given in Scheme 1. One of the better known alkaloid venoms in the USA comes from the tropical red imported fire ant, *Solenopsis invicta*, and other ants in this genus. This invasive species (not native to the USA) was introduced into the USA (Mobile, AL, or Pensacola, FL) from cargo ships coming from Brazil between 1933 and 1945 and has spread into at least 18 states since (Collins and Scheffrahn 2001–2008). The venoms of these ants are mostly made of piperidine alkaloid compounds called solenopsins (Scheme 1, compounds **2–6**) (Lofgren et al. 1975; MacConnell et al. 1970, 1971). Later in this chapter, I will use this example to give a basic explanation of some of the tools and methods used by chemists to study such natural substances as venom chemicals.

Fire ants characteristically attack in numbers by congregating onto a victim, possibly an unfortunate person standing in a fire ant mound (Fig. 2), and, in response to an alarm pheromone they produce, will all basically sting at the same time. To inject their venom, they bite the victim with their mandibles (jaws) and then pierce his or her skin with the stinger found at the end of their abdomen (back end). The solinopsins in their venom cause a small area of necrosis (cell dying) about 1–2 mm in diameter over a few days. In many cases, swelling and itching will also occur, but this response is actually due to proteins found in the venom (Tankersley 2008).

Besides the solenopsins from fire ants, other species of ants make a variety of alkaloids. A good and recently published example of the variety of alkaloids produced by ants comes from the ant species *Myrmicaria melanogaster* of Brunei (Jones et al. 2007). This single species of ant was found to contain 14 different alkaloids – three generic structures of several of those shown in Scheme 1 (compounds **7–9**). In addition to alkaloids, ants have been shown to use other organic compounds as chemical defense such as iridodial (Scheme 1, compound **14**), dolichodial (Scheme 1, compound **22**), and actinidine (Scheme 1, compound **15**), among others.

3.2.1 Studying Venoms

The venom of fire ants is a classic and well-studied example of insect chemical defense. Thus, they make a very useful model with which to explain some of the tools and methods used in insect chemical ecology. To start any analysis of venom, it is necessary to have or develop an efficient and robust method of collecting the desired chemical components. Such a method should be performed as to be sure that the substances analyzed are indeed from the venom glands of the organism and not from some other part of the body. Often ant venom compounds are analyzed from samples of ants simply rinsed in methanol. While this is a very efficient method, one cannot be sure that the compounds identified are all venom components. Other

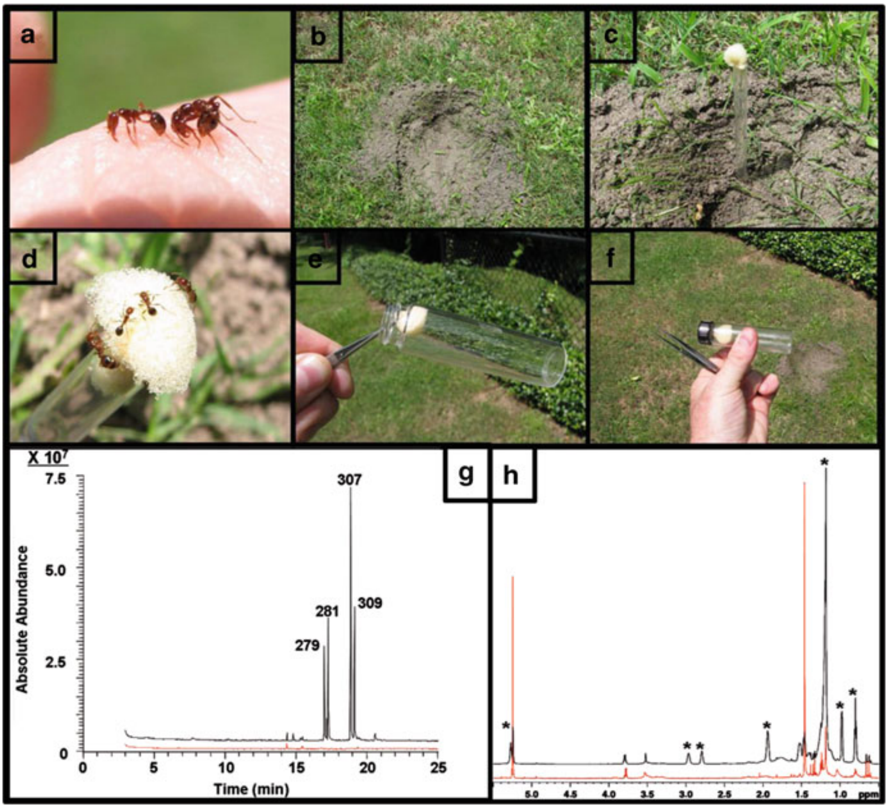


Fig. 2 Stinging behavior of and venom collection from the red imported fire ant: *Solenopsis invicta*. (**a**) Major and minor workers stinging Dr. Aaron T. Dossey's hand in their typical stinging posture, (**b**) ant mound with a footprint to disturb the ants, (**c**) closeup of glass pipette stuck in mound in (**b**), (**d**) ants stinging foam in glass pipette, (**e**) foam being placed into glass vial with clean forceps for venom extraction, and (**f**) glass vial and forceps in (**e**) with vial shown capped with a Teflon® coated cap, (**g**) one-dimensional ^{1}H NMR spectrum of venom alkaloids collected from *S. invicta*. The foam is extracted with CH_2Cl_2, dried by blowing nitrogen gas over it, then re-dissolved in about 15 μL of deuterated methylene chloride (CD_2Cl_2) for NMR. The black spectrum is the venom alkaloid extract and the red spectrum is a blank. *Asterisks* show the peaks which correspond to those previously reported for *S. invicta* venom alkaloids (MacConnell et al., 1970), (**h**) gas chromatograph of *S. invicta* venom alkaloids. The *black trace* is the venom alkaloid extract and the *red trace* is a blank. Four peaks are noted with the masses of the compounds they represent. These masses, qualitative relative retention times, and mass spectra (data not shown) correspond to those of published data for known *S. invicta* venom alkaloids (MacConnell et al., 1971). Photographs by Dr. Aaron T. Dossey

researchers have developed a variety of ways to isolate ant venom by (1) electrocuting the ants so that they eject their venom components onto some inert surface, (2) dissecting the venom glands and removing the contents with a glass capillary, or even getting the ants to sting an inert porous material for further extraction (Piek 1986). To do this is rather simple, but this requires a sufficiently aggressive ant species and a good number of ants. Venoms are certainly a diverse and rich source of chemical substances with very clear hypothesis-generating biological activities. However, it is often challenging to obtain sufficient quantities for analysis.

Once a crude natural substance is collected, the chemicals of interest must be separated and subjected to various analytical chemistry techniques to determine what the substance is made of. Various methods of extraction and types of chromatography are used to separate the different substances. The chemist will then use a variety of spectroscopic and spectrometric techniques (such as NMR and mass spectrometry), to determine the type, connectivity, and arrangement of atoms in those molecules – these being important aspects of their molecular structure.

Nuclear magnetic resonance (NMR) utilizes giant superconducting magnets with magnetic fields typically 200,000–400,000 times that of the earth to study the molecular structure of chemical compounds. NMR uses some convenient properties of atoms to achieve this (1) in the presence of a magnetic field, atomic nuclei precess (like a toy top tilting as it spins) at frequencies depending on what element it is (hydrogen, carbon, etc.) and (2) the rate (or frequency) of this precession is affected by the amount of electrons around those atoms. In NMR, radio frequencies are sent into a chemical sample placed inside the large magnetic field. The atoms in the sample will then give off a characteristic set of radio frequencies of their own. These frequencies and how those nuclei interact with one another give the chemist much important information about the molecular structure of the chemicals in the sample.

Mass spectrometry (mass spec) is another technique often essential to determining the identity and molecular structures of chemicals in a sample. Mass spec uses chemistry or high-energy beams to break molecules into parts. The mass of these parts can be measured based on the time it takes them to fly through a vacuum chamber. The masses of the fragments, like pieces of a puzzle, can be used to reconstruct the original arrangement of atoms in the molecules. Probably the best known use of mass spec, Gas Chomatography coupled mass spectrometry, on GC-MS, is sometimes even used by police detectives in forensic analysis of crime scene evidence. Mass spec databases are often used to make very rapid identifications of commonly known chemical substances – but study of unknown substances usually takes much longer.

As described earlier, there are multiple methods one can use to obtain ant venom. I have personally put the "ant stinging porous material" method to the test (Fig. 2). First, I mounted three precleaned pieces of foam culture flask stopper on the top of clean glass Pasteur pipettes with forceps by inserting about 1/3 of each foam piece into the large end of the pipette. Then, I located a red imported fire ant (*Solenopsis* invicta) mound, stepped on it to agitate the ants, and stuck the three pipettes into the area where the emerging ants were in highest density. After 10 min, I removed the foam pieces with forceps and flicked the remaining ants off the material. One of the first indications that venom alkaloids were present on the foam piece was that, when rubbed onto pH indicator paper, the paper would turn blue, which indicates presence of an alkaline (basic) substance. The foam was extracted with methylene chloride (CH_2Cl_2) and the resulting residue was analyzed by NMR and mass spec. Indeed NMR and mass spec signals corresponding to previously published values for fire ant venom alkaloids were observed (MacConnell et al. 1970, 1971) – along with few other minor signals (Fig. 2). Thus, the method was a success!

3.3 Sprays and Secretions

As stated earlier, the vast majority of animals on earth are insects. Beetles alone make up the group (Order Coleoptera) with the largest number of described species of any other group of animals on earth. There are approximately 400,000 named species so far, comprising about 25% of all named species of organisms (Hammond 1992). The plethora of chemical compounds they produce for defense is similarly as vast and diverse (Blum 1981; Dettner 1987; Eisner 2003; Eisner et al. 2005). Aldehydes and ketones are common functional groups used by insects for chemical defense (Scheme 1). Aldehydes are a portion of a molecule consisting of a carbon atom double bonded to an oxygen atom and single bonded to a hydrogen atom and to another carbon atom (aldehydes are highlighted in red in Scheme 1 and Fig. 3). Aldehyde containing compounds tend to cause a burning sensation. Ketones are portions of a molecule consisting of a carbon atom double bonded to an oxygen atom and single bonded to two other carbon atoms (ketones are highlighted in blue in Scheme 1 and Fig. 3). Carbon atoms almost always have exactly four chemical bonds attached to them. Beetles, as well as many other insects and even plants, also tend to produce monoterpenes as active components of their chemical weapons. For example, chrysomelidial (Scheme 1, compound **16**) produced by leaf beetles (Family Chrysomelidae) is an isomer of anisomorphal (Scheme 1, compound **23**) – with the only difference being placement of the double bond (Meinwald et al. 1977). Dolichodial, anisomorphal, and peruphasmal (Scheme 1, compounds **22**, **23**, and **24**, respectively) are diastereomers (compounds with the same chemical formula, but different configurations at one or more of the functional groups, resulting in compounds that are not mirror images) and are the active components of several walkingstick insect defensive sprays (Dossey et al. 2006, 2008; Eisner 1965; Meinwald et al. 1962), as well as some ants (Cavill and Hinterberger 1961; Cavill et al. 1976; Cavill and Whitfield 1964; Pagnoni et al. 1976). Probably the best-known beetle defensive substance is cantharidin (Scheme 1, compound **10**), a compound from the blister beetles (Family Meloidae) that has a long history of medicinal use as discussed previously. Blister beetles have a very interesting way of deploying this chemical weapon. They can spontaneously bleed through the joints in their legs a blood concoction enriched in cantharidin (Eisner 2003; Eisner et al. 2005) (Fig. 3a). In some types of beetles, such as “fire-colored beetles” (Family Neophyrochroa), cantharidin is actually passed from males to females during mating (Eisner et al. 2005).

One of the most famous chemical defense mechanisms used by a beetle is that of the Bombardier beetle, characterized by the work of Eisner and Meinwald (Eisner 2003; Eisner et al. 2005) (Fig. 3b). The rather spectacular spray given off

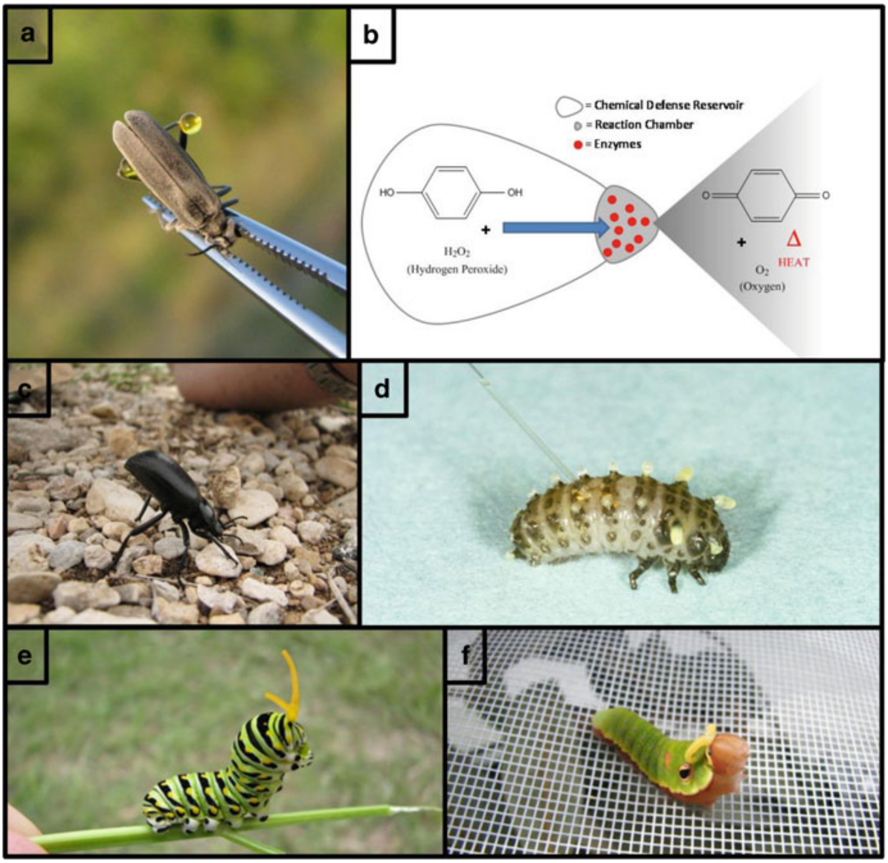


Fig. 3 Insects and their chemical defenses. (**a**) A blister beetle (*Epicauda* sp., Family Meloidae) deploying its typical defensive bleeding of cantharidin (Scheme 1, Compound **10**), (**b**) Bombardier beetle chemical defense mechanism (*Brachinus* sp., Family Carabidae, Order Coleoptera). (Figure adapted from diagrams in (Eisner, 2003) and (Eisner et al., 2005). This apparatus is in the posterior (rear) end of the beetle. In this diagram the rear end of the beetle is to the right, (**c**) darkling beetle (Genus *Eleodes*, Family Tenebrionidae) in its typical defensive posture – Carlsbad Caverns, New Mexico, USA, August 15, 2006, (**d**) Larva of leaf beetle species (*Phaedon cochleariae*, Family Chrysomelidae, Order Coleoptera) shown with drops of defensive secretion on protruded glands after being agitated. Glass capillary is being used to milk the secretion, (**e–f**) swallowtail butterfly caterpillars shown with chemical defense glands (osmeteria) extruded after having been agitated: (**e**) Eastern Black Swallowtail (*Papilio polyxenes*) and (**f**) Spicebush Swallowtail (*Papilio troilus*). Photographs in (**a**), (**b**), (**e**), and (**f**) by Dr. Aaron T. Dossey. Photograph in (**c**) by Nhu Nguyen. Photograph in (**d**) by Dr. Michael Hoscovec. Caterpillars are from Shady Oak Butterfly Farm in Brooker, FL, USA

by bombardier beetles when attacked is the result of a violent chemical reaction, which occurs upon the mixture of reactants and enzymes in the animal’s defensive apparatus (Fig. 3b). Specifically, hydroquinone and hydrogen peroxide, stored ready and waiting in the beetle’s defensive reservoirs, are brought together by enzymes in a high-energy oxidation/reduction reaction to form benzoquinone (Scheme 1, compound **11**), water, and heat. The reaction creates temperatures up to 100°C (212°F). During the reaction, pressure builds up in the defense gland reservoir until the substance can no longer be contained. At that point a rapid-fire series of pulsed sprays of hot toxic chemicals are deployed directly at the offending stimulus. Thus, it is the boiling of the mixture of these components and water which builds pressure and causes the explosion of toxins in the face or mouth of a predator unlucky enough to select a bombardier beetle as their next meal – or simply an unfortunate passerby who gets too close for the beetle's comfort.

The bombardier beetle is a member of the group known as ground beetles (Family Carabidae). Other examples of chemically armed ground beetles are species in the genus *Calosoma*. In the USA, probably the most familiar is *Calosoma scrutator*, also known as the Fiery Searcher or Caterpillar Killer because of its predatory nature. These beetles very often emit an indescribably nasty smelling concoction, which persists on one's skin even after hand washings and the passing of several hours. The main ingredients in the chemical defense sprays of *Calosoma* are methacrylic acid (Scheme 1, compound **12**) and salicylaldehyde (Scheme 1, compound **13**), which is emitted from the end of their abdomens. In fact, most adult beetles spray their defensive secretions from the end of their abdomen (the back end). To collect these secretions for analysis, one can simply cause the beetle to spray into a glass vial. However, since their abdomens often point downward, an alternative is to get the beetle to spray onto an inert surface such as a glass slide and collect the secretion off of that. In the case of the blister beetles or other insects that produce more of an ooze than a spray, a glass capillary is a very useful tool for collecting their secretions.

Another interesting example of beetle chemical defense occurs in darkling beetles (Family Tenebrionidae), which is the fifth largest family of beetles. These beetles are very common in the more arid regions of the southwestern USA, and as both adults and larvae they are scavengers, feeding on both live and decaying plant matter. Some of the larger darkling beetles, such as those in the genus *Eleodes*, are well known for their chemical defense. They can spray a large amount of benzoquinones when disturbed, similar to other beetles. However, many darkling beetles have a warning behavior associated with this defense – before launching their chemical spray, they will raise the rear end of their abdomen and stand on their head (Fig. 3c). This is a warning to back off before you get sprayed, equivalent to the coiling and rattling of a rattlesnake before it strikes. In fact, the chemical weaponry of benzoquinone-weilding darkling beetles is so successful that beetles in the genus *Moneilema* mimic their defensive head-standing posture when disturbed (Eisner 2003; Eisner et al. 2005; Evans and Bellamy 2000). This mimicry is pure deception because *Moneilema* spp. have no chemical defense spray of their own. For some beetles, it is the chemical defense of the larval stage for which the beetle is best known. The life cycle of beetles, as well as butterflies and moths (Order Lepidoptera), is called holometabolous (complete metamorphosis). This means that they have larval stages that look very different from the adults.

The larvae later become a pupa, an immobile stage like the cocoon of a moth or the chrystalis of a butterfly, which subsequently morphs into the adult. Other types of insects, such as walkingsticks (Order Phasmatodea) and grasshoppers (Order Orthoptera), are hemimetabolous (incomplete metamorphosis). This means that instead of a larval stage they have a nymph stage which resembles the adult, and lack a pupal stage. The larvae of many leaf beetles (Chrysomelidae) have pores along their body, which secrete droplets of liquid (Fig. 3d) containing repellant compounds such as chrysomelidial (Scheme 1, compound **16**) when the insect is disturbed. Leaf beetle species that make chrysomelidial have been shown to obtain this substance one of two ways (1) make it themselves using sugars or other metabolites that they consume or (2) access chemical precursors directly from the plants they feed on (Kunert et al. 2008). They control their resources so efficiently that, in the event of a false alarm, they can bring the liquid back in for later use.

There are thousands of other insects besides ants, beetles, and walkingsticks that make impressive and potent defensive chemicals and their methods of deployment are as diverse as the chemicals they make. For example, some termites, such as *Nasutitermes exitiosus* from Australia, will surround an offending creature and spray a sticky adhesive – a glue – at their opponent (Eisner 2003; Eisner et al. 2005). Often the termites will work as a team, especially for larger opponents, spraying this glue to immobilize their attacker. Butterflies (Order Lepidoptera, which also includes moths), particularly their larval stage (caterpillars), can also wield chemical weapons to keep from being eaten. Swallowtails (Family Papilionidae) have a particularly spectacular display associated with their chemical defense; a brightly colored defensive gland just behind their head (Fig. 3e, f). Unlike many other insects, swallowtail caterpillars do not spray their defensive chemicals. Rather, these caterpillars will rear back and wipe their pungent smelling defensive gland, wet with defensive secretion, onto the offending stimulus or predator. The most spectacular aspect of this defensive mechanism is the gland itself. When the insect is disturbed, the gland shoots out of the area behind their head, seemingly out of nowhere. The glands of these caterpillars are often covered with small pungent short-chain fatty acids, such as isobutyric acid and 2-methylbutyric acid and (Scheme 1, compounds **17** and **18**), whose smells have been described as rancid butter or gym sock. These acids are produced by several North American species such as the Eastern Black Swallowtail (*Papilio polyxenes*) (Fig. 2e), Spicebush Swallowtail (*Papilio troilus*) (Fig. 2f), and the Giant Swallowtail (*Papilio cresphontes*). The gland of the Giant Swallowtail is deep red and can be ejected particularly violently and extends past the rear of the body. However, other species of swallowtails, such as the more tropical Polydamas swallowtail (*Battus polydamas*), which occur in the southern USA (Florida and Texas) and through Central and South America to Argentina, make very different compounds – sesqueterpenes, such as selinene (Scheme 1, compound **19**) (Eisner et al. 1970, 1971, 2005; Eisner 2003). Indeed, even among these closely related butterfly species, this impressive example of chemical biodiversity exists.

3.4 Significance of Tropical Species for Chemical Biology Exploration

Most chemical biology and our overall understanding of the natural world come from studies done in temperate zones, and many of the examples represented in this chapter are describing the defenses of temperate insects. This is because historically most of the scientifically advanced nations occur in the temperate zone (Europe, North America, Central and Eastern Asia), while tropical regions are mainly in countries that lack the scientific infrastructure necessary to study the vast wealth of chemical biodiversity which exists within their borders. Thus, chemical ecology and other scientific studies of temperate species are disproportionately represented. The word disproportionate is particularly applicable, because most of the world's diversity and species richness is found in the tropics (Erwin 1997; Groombridge and Jenkins 2002; Hester and Harrison 2007). Accordingly, predation pressures and the need to fend off attack have led to added diversity of chemical strategies in insects (Eisner 2003; Eisner et al. 1995, 2005; Meinwald and Eisner 1995). In fact, there are very few nontropical species of walkingstick insects that produce any sort of defensive secretion. There is much to be explored and discovered in the tropics, especially in the realm of insect chemical biology.

3.4.1 Defensive Chemical Ecology of Walkingstick Insects (Order Phasmatodea)

Walkingstick insects (also called "phasmids") are best known for their nonchemical defensive mechanism, camouflage (Brock 1999). Their common name, walkingstick, indicates one of the ways in which some species blend into their environment, by mimicking sticks, twigs, or leaves to avoid predators. However, many species of phasmids also emit irritating chemical substances, which they deploy against potential attackers (Bedford 1978; Bouchard et al. 1997; Carlberg 1981, 1985a, b, 1986, 1987; Chow and Lin 1986; Dossey et al. 2006, 2007, 2008; Dossey 2009; Eisner 1965; Eisner et al. 1997; Ho and Chow 1993; Meinwald et al. 1962; Schmeda-Hirschmann 2006; Smith et al. 1979). In fact, many species of walkingstick insects do not blend into their background; they display bright colors as a warning to predators, a defense mechanism known as aposematism. Most walkingsticks deploy their chemical weapons from the front, in contrast to beetles, many of which spray from the back end. However, males of at least one type of phasmid, the New Guinea Spiny Stick (Genus *Eurycantha*), have a defense gland at the rear end of their abdomen. They use this gland in an elaborate defensive behavior – when threatened, the males will raise their abdomen in the air, repeatedly expose their scent gland, which gives off a skunk-like odor, and prepare their spiny rear legs to jab at the offending stimulus (Bedford 1975, 1978; Brock 1999). These insects are large, strong, robust creatures and their spines can inflict minor cuts on human skin. However, *Eurycantha* are exceptional in this mode of defense. Most species of phasmids produce their noxious defensive spray in a pair of laterally symmetric glands in their prothorax which has openings on each side just behind the head (Chow and Lin 1986; Dossey 2009, 2010; Eisner et al. 1997; Eisner 1965; Happ et al. 1966). All the examples of walkingstick sprays given in this chapter are tropical species, with the exception of *Anisomorpha buprestoides*, which ranges from subtropical to temperate USA. In fact, only two temperate species of phasmids have been reported to make defensive sprays, *A. buprestoides* and *A. ferruginea* (both from the USA).

4 Early Work on Phasmid Defensive Secretions

Some of the earliest work examining insect defensive sprays was conducted using walkingsticks (Meinwald et al. 1962; Schneider 1934) (Fig. 4a–c). The earliest chemical analysis on walkingstick defensive chemistry was conducted in 1934 on *Agathemera crassa* (formerly *Paradoxomorpha crassa*) from Chile (Schneider 1934). Phasmids in the genus *Agathemera* are known locally in Chile by the names "chinchemoyo" (Schneider 1934), "chinchemolle", "chinchimol", or "tabolango" (Schmeda-Hirschmann 2006). The 1934 publication on *A. crassa*, published in Spanish by Chilean researchers, utilized the technology available at that time (before NMR and mass spec had been invented), and the chemical structure reported is rather unusual (Scheme 1, compound **20**) and likely incorrect. Later, in 2006, analysis of the defensive chemistry from a closely related species, *A. elegans* (also from Chile), revealed a more likely chemical structure (Scheme 1, compound **21**) (Schmeda-Hirschmann 2006), showing a substance that is extremely persistent and often retained for years in dead and dried specimens.

It was not until the pioneering work of Eisner and Meinwald in 1962 that another walkingstick insect defensive secretion was analyzed and published. Meinwald et al. collected thousands of milkings from hundreds of individuals of *Anisomorpha buprestoides*, the "Southern Two-Striped Walkingstick Insect" from the southeastern USA, extracted an oily pungent substance, and analyzed it using various analytical techniques available at the time including NMR and mass spec. From these data they were able to determine the structure of the active component, which they named anisomorphal (Scheme 1, compound **23**). Shortly after the discovery of anisomorphal, Eisner demonstrated experimentally that the chemical spray of *A. buprestoides* was actually quite potent in repelling predators such as beetles, ants, and bluejays (Eisner 1965). Later, Ulf Carlberg performed quantitative experiments demonstrating the effectiveness of *A. buprestoides* chemical defense against Norwegian rats (*Rattus norvegicus*) (Carlberg 1985a). However, *A. buprestoides* is not always able to keep from being eaten. Black bears in Florida have been known to make a meal of these insects (Eisner et al. 2005; Roof 1997) – perhaps they even enjoy the spicy flavor of the spray.

The next species of phasmid whose defensive chemistry was published was the coconut stick insect, *Graeffea crouani*, from the South Pacific region (Smith et al. 1979). At the time this insect was reported to be a major pest of coconut palms. The work by Smith et al. determined that the secretion of these species contains at least

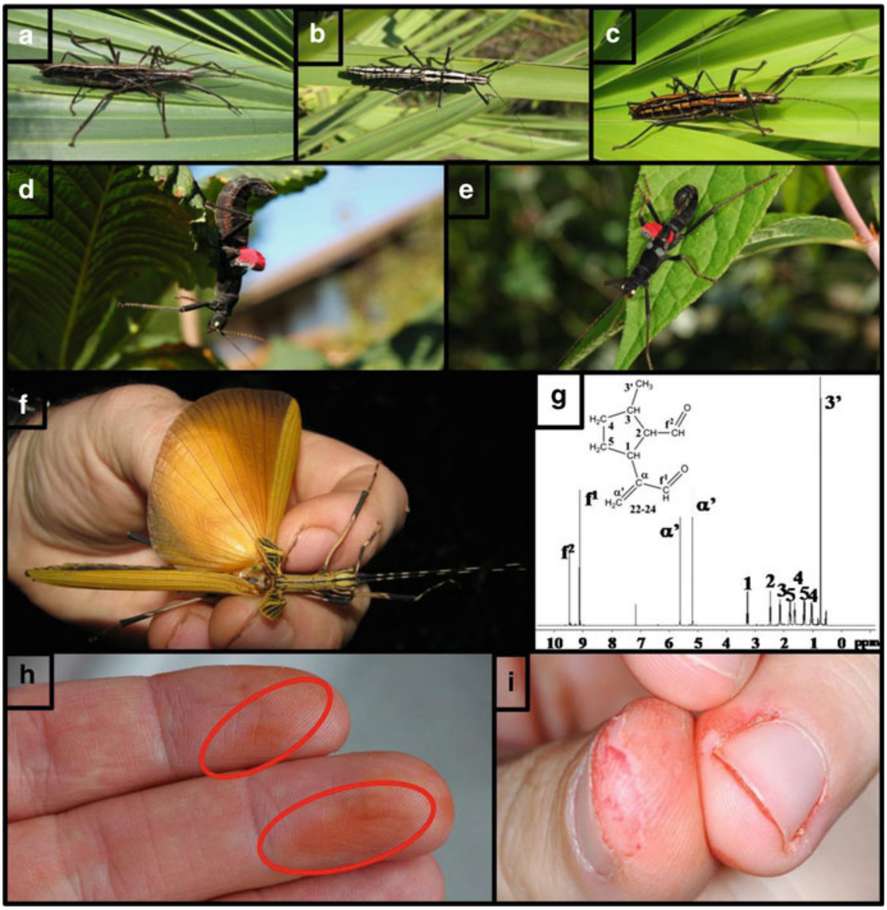


Fig. 4 Walkingstick insects and their chemical weapons. (**a–c**) *Anisomorpha buprestoides*, (**a**) Brown form (Gainesville, FL), (b) Black and White form ["Skeleton Stick" (McMonogle and Dossey, 2007), Ocala National Forest, FL], (**c**) Black and Orange form (Archbold Research Station, FL), (**d**) *Peruphasma schultei* female, (e) *P. schultei* male (captive reared in Germany by Oskar V. Conle), (**f**) *Pseudophasma annulipes* adult female found in Tambopata National Reserve, Peru (12°50′32″ S, 60°17′ 18″ W), December 7, 2007. Species identified by Oskar V. Conle, (**g**) one-dimensional ^{1}H NMR spectrum of *Pseudophasma annulipes* defensive spray dissolved in benzene-d6 and structure of anisomorphal. Numbered hydrogens in the structure correspond to the numbered peaks in the NMR spectrum, (**h–i**) effect of defensive spray from *Parectatosoma mocquerysi* on the hand of Oskar V. Conle (**h**) minutes and (**i**) several hours after sprayed by *P. mocquerysi*. *Red circles* indicate affected areas. Photographs (**a**), (**b**), (**c**), and (**f**) by Dr. Aaron T. Dossey. Photographs (**d**), (**e**), (**h**), and (**i**) by Oskar V. Conle

two isomers of iridodial (Scheme 1, compound **14**) and nepetalactone (Scheme 1, compound **31**). Interestingly, nepetalactone (McElvain et al. 1941; McElvain and Eisenbraun 1955) is also the compound in catnip (*Nepeta cataria*) responsible for the behavioral effects on cats (Eisner 1964). A few years after the coconut stick insect's spray was studied, other researchers began analyzing and studying the chemical defense of *Megacrania tsudai* (Chow and Lin 1986; Ho and Chow 1993) from Taiwan. In the Chow and Lin publication, *M. tsudai* was erroneously identified as *M. alpheus* (*M. tsudai* is the only species in this genus present in Taiwan) (Hsiung 2007). In general, members of the genus *Megacrania* (Hsiung 2007) are referred to as "peppermint stick insects" due to the characteristic odor of their defensive spray. In the studies by Ho and Chow, it was first determined that actinidine (Scheme 1, compound **15**) was the major component of *M. tsudai* defensive spray. In that same paper, they demonstrated that experimental nymphs of *M. tsudai* whose defense glands had been emptied by milking were less likely to survive in the wild than control nymphs that had not been milked. In addition to actinidine, the 1993 paper by Ho and Chow also identified several minor components in the spray, which are illustrated in Scheme 1 (compounds **25–29**). Notice that most of those compounds are very similar to actinidine (Scheme 1, compound **15**), and that actinidine and nepetalactone (Scheme 1, compound **31**) have similar structures. It turns out that some plants containing actinidine have also been shown to elicit similar behavioral responses in cats to that induced by nepetalactone (Tucker and Tucker 1988).

Another phasmid species to have the chemical composition of its defensive spray identified was *Sipyloidea sipylus* (the "pink-winged stick insect") (Bouchard et al. 1997). The chemical spray of this species contains a rather complicated mixture; probably the most chemically diverse phasmid chemical spray analyzed to date (Scheme 1, compounds **32–36**). Among the various components are acetic acid (Scheme 1, compound **33**) and benzaldehyde (Scheme 1, compound **34**). Both of these can be rather effective irritants in high doses. Benzaldehyde and acetic acid are found in the chemical defenses of various other insect species as well. Interestingly, the most abundant compound in *S. sipylus* spray as reported by Bourchard et al. was diethyl ether (Scheme 1, compound **32**) (about 70% of the total volatiles measured), a chemical commonly used as a laboratory solvent. Bouchard et al. also examined the effectiveness of the five identified compounds from *S. sipylus* defensive spray (Scheme 1, compounds **32–36**) as pest control against rats (*R. norvegicus*), and found that a test mixture of these substances was effective in repelling the rats. Earlier experiments performed by Ulf Carlberg using live *S. sipylus* insects also demonstrated the effectiveness of their chemical defense against predation by the same rat species (Carlberg 1986). Based on experimental comparisons between *S. sipylus*, *Anisomorpha buprestoides*, and *Extatosoma tiratum*, the chemical defense of *S. sipylus* was the most effective of the three.

Oreophoetes peruana from Peru also has a defensive secretion that was characterized by Eisner et al. They discovered that the major nonaqueous component of *O. peruana* defensive spray was quinoline (Scheme 1, compound **30**) (Eisner et al. 1997). Various derivatives of quinoline have been found in several other insect species; however, this was a very unusual finding because quinoline itself occurs very rarely in nature. In fact, *O. peruana* was the first animal species ever shown to produce quinoline (Eisner et al. 1997). Once quinoline was shown to be the chemical component in *O. peruana* defensive spray, Eisner et al. was able to demonstrate that it is indeed effective against a variety of potential predators such as ants, spiders, cockroaches, and frogs. Interestingly, quinoline is very similar to the active insect-repelling component of moth balls, naphthalene. A collaborator on this study was Randy Morgan from the Cincinnati Zoo. This is important to point out because live insect collections such as at zoos and insectariums are very valuable resources for scientific research. Often permits to transport exotic insects can be difficult for researchers to obtain, but many exotic animals are already legally being reared at zoos. It is also important to acknowledge the contributions of nonchemists to the field of chemical biology and of amateur entomologists to science. In addition to the work by Eisner et al., the actual spraying mechanism of *O. peruana* was recently analyzed by high-speed photography and structure of the gland was analyzed by electron microscopy (Bein and Greven 2006).

Both male and female Australian Spiny Sticks (*Extatosoma turatum* – also known as the Australian Stick or Macleay's Specter) produce a secretion which smells a bit like toffee (Bedford 1978). Carlberg, who has probably done the most extensive studies of the effectiveness of phasmid chemical defense of any researcher, tested whether the secretions of *E. turatum* would repel frogs (*Xenopus laevis* and *Rana esculenta*) or rats (*R. norvegicus*). According to his study, the chemical defense of this species was rather poor against these predators (Carlberg 1985b). Interestingly, the *X. laevis* frogs refused to eat the abdomens of the insects that did not contain the defense glands. Although *E. tiratum* clearly emits a chemical secretion when disturbed, the small amount and pleasing odor of this substance seems to suggest it may have some function besides warding off predators for which it is better suited. Additionally Dossey et al. has also found that the leaf insect species phyllium westwoodii produces only a tiny amount of spray when disturbed (Dossey 2009). This spray was found to contain dimethyl alkyl pyrazines, which not only seem ineffective as innitants, but are common components in the odor/escence of chocolate, coffee and roasted food (Dossey 2009). In fact, it has been postulated that the chemical defenses of some walkingsticks may function as pheromones, yet this hypothesis has not yet been tested (Dossey et al. 2008; Tilgner 2002).

5 Continuing the Tradition of Discovery Using New Technologies

My studies of phasmid defensive chemistry were inspired by my life-long hobby and passion for studying insects. In 2005–2006 I was keeping a few *A. buprestoides* in captivity. When I first read the work by Meinwald (Meinwald et al. 1962) and Eisner (Eisner et al. 1997) on *A. buprestoides,* I was intrigued by this report that the defensive secretion contained only a single compound. It is not often that nature produces single substances so pure and in such large quantity. At the same time, the laboratory I worked in as a PhD graduate student had just received and installed a new cryogenically cooled micro NMR probe which was reported to be the most sensitive in the world (Brey et al. 2006) – being able to detect very small quantities of organic substances in timeframes shorter than possible using other NMR probes. In February 2006, the *A. buprestoides* I was keeping were small, about an inch or so long. I thought, considering the reported purity and apparent quantity of spray produced by these animals, I would use them to put this new NMR probe to the test. Because the original studies on the defensive chemistry of *A. buprestoides* and other phasmids were done using organic solvent extracts, I decided to use deuterated water to determine if there were any additional aqueous components in the secretion (Dossey et al. 2006; Amato 2006; Dossey 2006), which turned out to be a fruitful endeavor. In addition to the previously known anisomorphal (Scheme 1, compound **23**), I discovered that the defensive sprays of young *A. buprestoides* can also contain dolichodial (Scheme 1, compound **22**), peruphasmal (Scheme 1, compound **24**), and, because I used water rather than organic solvent, I also observed glucose (Scheme 1, compound **37**) in the mixture.

At the same time as I was studying *A. buprestoides* defensive spray, Oskar Conle had just described a new species of walkingstick insect, which he named *Peruphasma schultei* (after Rainer Schulte, a German herpetologist in northern Peru) (Fig. 4d, e) (Conle and Hennemann 2005). When I read about this new species I emailed Oskar and asked for a sample of the secretion of *P. schultei*. It turned out that *P. schultei* spray also contained glucose and an isomer of dolichodial that was differed from the anisomorphal or dolichodial found in young *A. buprestoides* defensive spray. Following the tradition of compound nomenclature established by Eisner and Meinwald of incorporating the genus of the walkingstick insect in the chemical name, I named this new compound peruphasmal (after the genus *Peruphasma*). Peruphasmal was originally found in ants but was referred to only as an isomer of dolichodial (Cavill et al. 1976) rather than given its own name. Since that first analysis of defensive spray from *P. schultei* adults, I have found that samples from young *P. schultei*, unlike those of young *A. buprestoides*, also contain only glucose and peruphasmal, and not anisomorphal or dolichodial.

Some specific variables in *A. buprestoides* ecology correspond to the dolichodial isomeric variability in their defensive spray. Specifically (1) different populations produce different isomers in different ratios, and (2) at different life stages, the young of some populations produce a varying ratio of isomers in their defensive spray which switches drastically when they reach sexual maturity (Dossey et al. 2008). By systematically analyzing single milkings of individual insects, wild caught from various locations around the state of Florida and captive-reared individuals at various life stages through sexual maturity, I found that populations of the black and white and the black and orange color forms produce only anisomorphal as adults in the wild, whereas populations of the brown color form in Gainesville, Sanibel Island, and Gulf Hammock produced only anisomorphal, only peruphasmal, or some combination of the two. The young examined at different life stages were progeny of a peruphasmal-producing population in Gainesville. As young, they produced variable mixtures of anisomorphal and dolichodial and only traces of peruphasmal. However, upon reaching sexual maturity, all animals studied switched to producing only peruphasmal, as had their parents (Dossey et al. 2008).

These studies on the defensive sprays of *A. buprestoides* and *P. schultei*, my first in insect chemistry, demonstrated several important points. First, the work was aided tremendously by modern high-sensitivity NMR technology (Amato 2006; Brey et al. 2006; Dossey et al. 2006). Being able to analyze material from single young insects allowed the comparisons between individuals and within an individual over time possible. In general, modern analytical chemistry technology and instrumentation has improved greatly in recent years, making much more of nature's chemistry amenable to analysis and, thus, potentially useful to humans. Second, simple and direct sampling of substances such as defensive secretions and venoms is very beneficial in these sorts of studies. When the substance comes directly from the gland of the animal that produces it and it is not chemically manipulated or modified, there is greater confidence that the observations made are biologically relevant. Third, it is important to consider all variables and sources of material in natural products chemistry. In looking at the aqueous phase of phasmid defensive secretions, I was able to find glucose, which was previously not known to exist in these substances. I was also able to find proteins in *A. buprestoides* defensive spray. It is important to look everywhere and use a variety of techniques and experimental conditions in bioprospecting to surveying the chemical complexities of a natural substance. In studying different insects on different days, I was able to determine that, in fact, the defensive secretions varied in their chemical composition (Amato 2006; Dossey 2006; Dossey et al. 2006, 2008). Finally, it is important to not be afraid to pursue new ideas, even if it appears that everything is already known about a particular system. In *A. buprestoides* defensive spray, in addition to the presence of glucose, I was able to determine that in fact there are three possible isomers of dolichodial produced (Amato 2006; Dossey 2006; Dossey et al. 2006, 2008) rather than only one (anisomorphal) as previously reported (Eisner et al. 1997; Meinwald et al. 1962). As technologies improve or new minds approach questions in science, new discoveries are very likely just around the corner.

In December 2007, I had the opportunity to travel outside of the USA for the first time. I spent some time in Lima, Peru, and then headed off for several days at Explorer's Inn, an eco-lodge in Peru's Tambopata National Reserve (TNR). Immediately upon arrival, the immense level of biodiversity in this area became clear. Over ten species of butterflies were clustered at the edge of the Tambopata River drinking from run-off puddles. Walking toward the lodge from the boat I saw many millipedes, beetles, butterflies, and ants galore. Ants are very much an omnipresent feature of the forest; they can be found on any tree or plant or few square yards of forest floor. This was very exciting, but I was there mostly focused on collecting defensive secretions from insects. Among my primary targets: walkingstick insects.

On the first day of hiking, I saw several walkingsticks; however, these were different than walkingsticks in the USA. They had wings and were strong fliers so that one could hardly get a few feet from them without them fluttering off into the dark forest undergrowth. From a photograph I sent him shortly after the trip, a colleague Oskar Conle was able to identify this insect as *Pseudophasma annulipes*

(Fig. 4f). This is the first published record of this species occurring in Peru, although it was previously reported from Bolivia. During the same trip, several males and females of this species were observed flying in the daytime within about 20 ft of ground level. One mating pair was also observed resting on a fern. A defensive spray sample collected was from the female in Fig. 4f which I was finally able to catch landing on the ground where I could successfully milk her. My milking method requires that I put a slight pressure against the insect with a vial to keep it firmly on the gland opening to collect a maximum amount of spray. A couple of months later I received the sample I had collected in the mail from our collaborators in perv. Immediately I extracted a small portion of it in deuterated benzene and acquired an NMR spectrum (Fig. 4g), which matched exactly to that of anisomorphal (Scheme 1, compound **23**). Gas chromatography and mass spectrometry experiments also verified that the substance was anisomorphal. This is the first study on the defensive secretion chemistry of this species and the first report that it contains anisomorphal. It demonstrates a continuing trend of dolichodial-like isomers as chemical defenses in North and South American walkingsticks closely related to the genus *Anisomorpha*.

One of the most exciting discoveries for me in my walkingstick defensive chemistry project was a novel compound, parectadial (Scheme 1, compound **38**), which I discovered from the species *Parectatosoma mocquerysi* of Madagascar (Burks 2007; Dossey et al. 2007). That discovery began as part of my continuing collaboration with Oskar Conle whom, as you may recall, I began collaborating with on the paper which included the analysis of *P. schultei*. Subsequent to that publication I asked Oskar which would be the next most exciting walkingstick species defensive secretion to analyze. That is when he told me about this interesting secretion from *P. mocquerysi* that causes reddening and peeling of the skin (Fig. 4h, i). As soon as I got the sample in the mail from Oskar I was able to analyze it by NMR and mass spec. First I examined the water soluble components, which again contained glucose. There appeared to be only a small number of other peaks besides glucose. The mass spec analysis confirmed that indeed there was only one other component. Upon further analysis of the NMR data, I was able to easily determine the structure of this component. It also turned out to be a compound completely new to science. Thus, in the aforementioned tradition of naming walkingstick defensive compounds, I named this new compound parectadial after the genus *Parectatosoma* from which it was isolated (Burks 2007; Dossey et al. 2007). For the publication of parectadial's discovery and structure. I was awarded the Jack L. Beal award for best paper of the year in the Journal of Natural products for 2007 (Kinghorn, 2008).

The effects parectadial has on skin suggest it may have value as a medicinal compound for conditions such as cancer or psoriasis. Additionally, parectadial is very similar in structure to perillyl alcohol (POH) and perillaldehyde, which have both been explored for use against cancer in a number of studies (Elegbede et al. 2003; Fernandes et al. 2005; Yeruva et al. 2007). POH has even been the subject of several clinical trials for treatment of cancer (da Fonseca et al. 2008; Ripple et al. 1998). Thus, it is logical to believe that parectadial may also have significant efficacy against cancer. However, further investigation is needed to test such a hypothesis.

6 Biosynthesis: New Discoveries for Pathway Elucidation

Phasmids have several advantages which make them useful for biosynthesis studies, foremost among them is their large size, and for chemical biology and biosynthesis bigger is often better. Other advantages are that they are easily cultured in the laboratory and are long lived, with many species living well over a year. Some species are also parthenogenic, which facilitates genetic studies. Finally, they are a very diverse group of insects that produce a wide variety of chemicals for defense and other purposes.

In my first insect chemistry paper I discovered that glucose, the most fundamental nutrient and carbon source for all living systems (Scheme 1, compound **37**), was present in the defensive secretions of *A. buprestoides* and *P. schultei* (Dossey et al. 2006). Since that paper, the defensive sprays of nearly every phasmid (as well as those of other insects) I have investigated have contained glucose (Dossey et al. 2007, 2008, 2009, 2010). One other group has also found glucose in the defense spray of yet another phasmid species, *megacrania nigrosulfurea* (Prescott 2009). This observation turns out to provide possible clues into how the defensive compounds of walking-stick insects are made and transported to their glands. As mentioned earlier, larval beetles in the family Chrysomelidae make similar compounds for chemical defense, and the research teams of Profs Wilhelm Boland and Jacques Pasteels have done extensive work characterizing the biosynthesis of chrysomelid defensive chemicals (Kunert et al. 2008), including the use of glucose. They have shown that glucose-conjugated precursors are transported into the glands of these beetles (Kuhn et al. 2007). Once inside the gland reservoir, the glucose is removed and various other chemical steps, such as oxidations and cyclizations (Veith et al. 1996), are performed on the precursor to give rise to the final product which is secreted or sprayed by the animal upon being disturbed or attacked (Oldham et al. 1996; Feld et al. 2001). This means that all of the required machinery to make these chemical transformations exists in the gland reservoir and are quite possibly released in the defensive secretion itself. In fact, it has been shown in some chrysomelid beetles that components of this biosynthetic machinery can be found in their defensive secretions (Feld et al. 2001; Kunert et al. 2008; Oldham et al. 1996).

For biosynthesis studies, the goal is to understand how the chemical compounds of interest are made by the organism(s). The primary tools by which chemistry is done in nature are enzymes. One of the important discoveries I made recently for biosynthesis in phasmids is that there are several proteins present in the defensive spray of *A. buprestoide*. The proteins were extracted individually and analyzed by mass spec. This will provide information on their sequences. Since all protein sequences are coded for by genes (DNA), such information will aid in cloning the genes from which those proteins came. In a process known as recombinant

DNA technology, these genes can be put into and expressed in various types of cells such as bacteria, insect cells, or even live plants (Voet and Voet 1995). Theoretically, if those genes are expressed in those new cells properly, the compounds made by those genes will also be made. The genes can possibly even be mixed and matched to make new compounds other than the actual ones made by the insects! Since proteins are the direct products of genes, the previously mentioned benefit of parthenogenic walkingstick insects is also an attribute, which makes some species beneficial to biosynthesis studies.

7 The Future of Insect Chemistry: From Biosynthesis to Drugs

By now it should be clear that insects are among the most important organisms on earth for many aspects of science and human existence – from biosynthesis to agriculture to disease (Dossey 2010). In these later sections, a discussion is given of some specific examples of how natural compounds from insects and other invertebrates can be important for technological advances, particularly in drug discovery. While reading this section, use your imagination and consider the even broader implications of insects and discovery.

8 Insects as a Source of Drugs

8.1 Toxins

Some of the most commonly sought types of natural substances are toxins (Fox and Serrano 2007). Natural products in general are attractive sources of material for drug discovery. Over 70% of drugs on the market are derived from or based on natural compounds (Newman and Cragg 2007). Compounds produced by nature are optimized for their functions, such as binding to specific target proteins in cells (Ortholand and Ganesan 2004). There are two major types of toxins that are attractive as drug lead compounds, those that can kill cells and those that function as neurotoxins. Cell killing or cytotoxic substances are generally sought for use as anticancer chemotherapeutics (Lodish 2000). For example, cantharidin (Scheme 1, compound **10**) from blister beetles and its chemical derivatives have already been explored for use as anticancer therapeutics (Sakoff et al. 2002; Sagawa et al. 2008). It is also often used in the treatment of warts (Moed et al. 2001). Cantharidin causes blistering of the skin, a common property of compounds known as vesicants. Blistering is the result of cell dying, thus being a property suggestive of usefulness as an anticancer compound. Venoms of stinging insects and other arthropods often possess properties that give them great potential as lead compounds for drug discovery. The previously mentioned solenopsins from red imported fire ants have also been pursued for a variety of medicinally relevant applications due to their ability to illicit necrosis in human tissue. Solenopsin, an alkaloid found in the venom of fire ants (Brand et al. 1973; MacConnell et al. 1970, 1971), has been investigated for its ability to inhibit angiogenesis (Arbiser et al. 2007), as an inhibitor of nitric oxide production (Yi et al. 2003), for its effect on the nervous system and on cardiosuppression in humans (Howell et al. 2005). In addition to venoms, other substances from insects can prove to be potent toxins useful for fighting cancer. For example, in 2005 three novel antineoplastic agents were isolated from preserved grasshopper specimens from Texas (*Brachystola magna*) (Pettit et al. 2005). It is fascinating to imagine that even an old jar of grasshoppers could potentially hold the cure for cancer.

Neurotoxins are often studied for their use as pain killers. In fact, one pain medication from a sea snail, Prialt® (Ziconotide) derived from the species *Conus magus*, has been approved for use by the USA Food and Drug Administration (FDA) and is already on the market as an alternative to morphine to treat chronic pain (Amstutz et al. 1998; Justice et al. 1994; FDA and UFADA 2006). This compound was discovered by an undergraduate researcher, in the laboratory of Baldomero Olivera (McIntosh et al. 1982; Machalek 2002), one of the most famous cone snail toxin laboratories in the world. Arthropods also produce a vast array of neurotoxic substances that merit study as possible drugs. Spider venoms in particular are the most common arthropods that have been studied for a variety of reasons oops. Most spider venoms contain neurotoxins. This is because they are most often used to paralyze prey rather than for defense. In insects, venoms can also contain neurotoxic substances of interest. For example, the venom of the bullet ant previously mentioned contains a protein called Poneratoxin. This protein blocks voltage-dependent ion channels in insects. It has even been proposed as a potential insecticide if expressed in an insect virus (Szolajska et al. 2004). There is no medical application yet for this toxin, so it could represent yet another opportunity for discovery in medical science.

8.2 Antibiotics

With the emergence of various antibiotic-resistant microbes, the search for new and novel antibiotics is a particularly important goal of modern drug discovery. Insects, as with many organisms, are susceptible to infection by microorganisms. In fact, many insects have dormant immobile stages (pupae), which are particularly vulnerable to a number of harmful elements. However, insect pupae can often sit dormant and immobile in such environments as soil or rotten logs without becoming infected. This protection against infection is partly due to antimicrobial substances. They are often peptides, but in some cases antimicrobial secondary metabolites have been found (Bexfield et al. 2004; Huberman et al. 2007b; Meylaers et al. 2004) (Dossey 2010). These substances are found in the hemolymph (blood) of the insects rather than sprayed or secreted. Nonetheless, they are a form of chemical defense against microbial attack. For example, two families of antimicrobial peptides have been discovered in the pupae of cecropia moths (*Hyalophora cecropia*) from North America: the cecropins and the attacins (Boman et al. 1991; Boman and Hultmark 1987; Hultmark et al. 1980, 1983). Cecropins and defensins have subsequently been found in a number of other insect species (Cociancich et al. 1994), as well as other organisms (Pillai et al. 2005).

Additionally, fly larvae have been successfully used in hospitals to remove dead tissue from wounds quickly simultaneously protecting against infection (Whitaker et al. 2007). In relatively recent studies, antibiotic substances have been discovered in the larvae of flies ranging from small organic metabolites such as lipids to antibacterial peptides and thus far unidentified compounds (Bexfield et al. 2004; Huberman et al. 2007a, b; Meylaers et al. 2004; Natori 1994; Whitaker et al. 2007). In fact, substances from larvae of one species of diptera used in wound healing medicine, *Lucilia sericata*, have already been shown to be effective against methicillin-resistant *Staphylococcus aureus* popularly known as "MRSA" (Bexfield et al. 2004). Thus, flesh eating flies provide a potential source of chemical substances which are likely beneficial to humans in use as antibiotics. However, these compounds are used out of their natural context in treating human ailment. Flesh flies usually feed on dead animals in the wild. Thus, they may not be defensive chemicals per se – I am unaware of any study demonstrating what benefit they provide to the fly or fly larvae. It could be defensive against microbial pathogens, selective for certain microbes to help the larvae digest the flesh, or even in slowing the flesh rotting process as to give the larvae sufficient time to feed on the carcass.

Insects themselves are only the tip of the iceberg as far as the biodiversity they represent. Many species of insects also harbor symbiotic fungi and bacteria, which aid them in a variety of functions such as food digestion and warding off pathogens. A very recent study demonstrated chemical warfare that occurs between fungi on the body of the beetle, *Dendroctonus frontalis*, a common pest of pine trees in the USA (Berenbaum and Eisner 2008; Scott et al. 2008). The larvae of the beetle depend on two species of pine-dwelling fungi for food, *Entomocorticum* sp. and *Ceratocystiopsis ranaculosus*. A third species of fungus, *Ophiostoma minus*, can help protect the beetles against the tree's chemical defenses but can also inhibit the growth of *Entomocorticum* sp., which the beetle larvae depend on for food. Scott et al. found that a fourth species, an actinomycete, produces an antibiotic compound new to science called mycangimycin. This antibiotic inhibits the growth of *O. minus*, thus allowing the beetle's larval food bacteria to thrive in a fascinating example of multispecies interaction. In addition to insects, their associated microbes or fungi can also prove to be a rich source of promising toxins. For example, two novel cyclopeptides called hirsutatins were discovered in 2005 from the insect pathogenic fungus *Hirsutella nivea*. These peptides showed activity in killing the microbial (protozoan) parasite *Plasmodium falciparum* and the causative agent of tuberculosis (*Mycobacterium tuberculosis*) (Isaka et al. 2005). These examples demonstrate that not only insects, but also their associated microbes (symbiotic, parasitic, etc.) have a vast potential in the future of new antibiotic discovery.

9 Biosynthesis: Toward New Methods of Chemical Production

Recent interest in useful chemical substances from natural sources has created a need to understand how these compounds are made by their originating organisms (Ajikumar et al. 2008). Thus, it is important to be aware of opportunities to characterize biosynthetic enzymes and pathways of natural products as they arise. With my recent findings of chemical biodiversity in walkingstick insects, as well as the presence of proteins in their defensive secretions, it is clear that these organisms represent a good model system for discovery of biosynthetic mechanisms of a variety of substances. The proteins found in walkingstick defensive secretions are likely to have valuable use as components in bioengineering combinatorial approaches in the overall biosynthesis of many sorts of compounds, even ones not produced by walkingsticks or possibly not produced by nature at all. In general, insects and other invertebrates represent one of the greatest resources available for the broad field of chemical biology that exists in the natural world. Mankind would be well advised to simultaneously protect and capitalize on our access to this rich resource.

10 Suggested Reading and Viewing

Here is a list of books and television programs, most of which were referred to earlier in this chapter, for suggested reading. These will be very useful and enjoyable for anyone interested in, involved with, or simply intrigued by the natural world, chemical defense, or the fascinating creatures we call insects:

- *For the Love of Insects* – Thomas Eisner, 2003
- *Secret Weapons: Defenses of Insects, Spiders, Scorpions, and Other Many-Legged Creatures* – Thomas Eisner, 2005
- *Life in the Undergrowth* – David Attenborough, a television series by the BBC Natural History Unit, also available on DVD.
- *Chemical Ecology: The Chemistry of Biotic Interaction* – Thomas Eisner and Jerrold Meinwald (Editors), 1995
- *World Atlas of Biodiversity: Earth's Living Resources in the 21st Century*, Brian Groombridge and Martin D. Jenkins, 2008
- *Biodiversity Under Threat*, R. E. Hester and R. M. Harrison, 2007
- *Biodiversity II*, Marjorie L. Reaka-Kudla, Don E. Wilson, and Edward O. Wilson, 1997
- *Chemical Defenses of Arthropods* – Murray Sheldon Blum, 1981
- *The Amazing World of Stick and Leaf-Insects* – Paul D. Brock, 1999
- *Stick Insects of the Continental United States and Canada* – Chad Arment, 2006
- *An Inordinate Fondness for Beetles* – Arthur V. Evans and Charles L. Bellamy, 2000

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