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Introducing our AUTHORS

Kerry Zobel

Current position: Genentech, Inc., Department of Protein Engineering and Medicinal Chemistry, research associate with Dr. Kurt Deshayes

Education: University of Wisconsin–Madison, B.S. in chemistry, 1999

Nonscientific interests: Bicycling the streets of San Francisco, camping and backpacking in the redwood forests, and guitar playing

As an organic chemist, a major interest of mine has been designing and creating new organic compounds. My work at Genentech allows me to incorporate this passion into the study of biology that is crucial for understanding and developing therapeutics. For example, the regulation of apoptosis, which is the current focus of our lab. To me this paper is an exciting example of progressing from a protein target to specific tight binding small molecules through the use of rationally designed peptidomimetics. We demonstrate this through a class of [7,5] bicyclolactame compounds that bind to the baculoviral inhibitor of apoptosis protein (IAP) repeat (BIR) and BIR3 domains of melanoma IAP and X-chromosome-linked IAP. (Read Zobel's article on p 525.)

This project was my first chance to incorporate my passion for studying insects with my formal training in biochemistry. One aspect that I found most fascinating was that we were able to analyze venom from a single insect and discover unreported components of that substance. Such a discovery opens new doors to understanding arthropod chemistry. Indeed, only a tiny fraction of the total chemical biodiversity that exists in insects alone has been determined. I hope to continue exploring the large potential for discovery that exists in these creatures. Using cutting-edge technologies such as the microsample NMR used in our study of phasmid insect venom, we can now begin to push the frontiers of natural products chemistry. I am currently looking for future work involving medicinal and natural product discovery from invertebrates. (Read Dossey's article on p 511.)

Current position: University of Florida, College of Medicine, Department of Biochemistry and Molecular Biology, postdoctoral research associate with Prof. Arthur Edison

Education: Oklahoma State University, B.S. in biochemistry and molecular biology, *cum laude*, 2001; University of Florida, Gainesville, Ph.D. in biochemistry and molecular biology, with Prof. Art Edison, 2006

Nonscientific interests: Entomology, keeping and breeding invertebrates, comedy, playing trumpet, nature photography, travel, gardening, camping, fishing, and hiking through the wilderness

Aaron Dossey

Jacquin Niles

Current position: University of California, Berkeley, Department of Chemistry, postdoctoral research associate with Prof. Michael Marletta

Education: Massachusetts Institute of Technology, S.B. in chemistry, 1994; Massachusetts Institute of Technology, Ph.D. in toxicology with Dr. Steven Tannenbaum, 2001; Harvard Medical School, M.D., 2002

Nonscientific interests: Most sports—definitely cricket!

One of my interests is the development of versatile and easily accessible tools for addressing fundamental questions in biology. Aptamer technology has many attributes compatible with this goal, especially because diverse cellular processes can be targeted with exquisite specificity. In this paper, we have used heme-binding aptamers to specifically target the well-studied heme biosynthetic pathway in the model organism *Escherichia coli*, demonstrating the applicability of this technology in modulating a small-molecule-regulated pathway. In the long run, I am interested in extending these approaches to nonmodel organisms that are less amenable to traditional genetic methods for studying essential cellular processes. (Read Niles's article on p 515.)

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LETTER

ACS chemical biology

Single-Insect NMR: A New Tool To Probe Chemical Biodiversity

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ABSTRACT Because of analytical limitations, multiple animals or plants are typically required to identify natural products. Using a unique 1-mm high-temperature superconducting NMR probe, we directly examined the chemical composition of defensive secretions from walking stick insects. Individual milkings were dissolved in D_2O without purification and examined by NMR within 10 min of secretion. We found that *Anisomorpha buprestoides* secretes similar quantities of glucose and mixtures of monoterpene dialdehydes that are stereoisomers of dolichodial. Different individual animals produce different stereoisomeric mixtures, the ratio of which varies between individual animals raised in the same container and fed the same food. Another walking stick, *Peruphasma schultei,* also secretes glucose and a single, unique stereoisomer that we are naming "peruphasmal". These observations suggest a previously unrecognized significance of aqueous components in walking stick defensive sprays. Single-insect variability of venom demonstrates the potential importance of chemical biodiversity at the level of individual animals.

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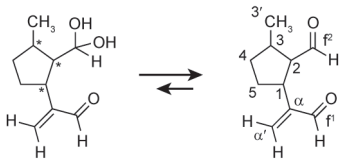
Individual organisms often produce natural products in very small quantities (*1*). Accordingly, their isolation and identification traditionally require large amounts of starting material and a significant effort in sample preparation. Analytical techniques such as mass spectrometry (MS), capillary electrophoresis, and fluorescence spectroscopy are now extremely sensitive and are being used to expedite this process. The use of NMR on the other hand, has lagged behind due to large sample requirements. Although notoriously insensitive, NMR is indispensable to natural product identification because it provides structural information that is not accessible with other techniques. Microcoil (*2–6*) and cryogenic (*7*) technology for NMR probes has significantly reduced sample mass requirements and enhanced several natural product studies (*3*, *8–12*). We recently combined the advantages of small-diameter samples with cryogenic technology in a 1-mm-diam NMR probe made from high-temperature superconducting (HTS) material to achieve ~25× greater sensitivity than a conventional probe (*13*). Here we have used this novel probe to characterize the defensive secretions of individual walking stick insects.

Anisomorpha buprestoides (order Phasmatodea) is common in the southeastern U.S. and is often found in pairs with the smaller male riding on the back of the female (*14*). When threatened, it accurately sprays a secretion at predators (*14*, *15*). Following the extraction of >1000 *A. buprestoides* "milkings" into methylene chloride, Eisner and Meinwald (*15*) identified its active component as a cyclopentanoid monoterpene dialdehyde that they named anisomorphal. At about the same time, Cavill and Hinterberger (*16*) identified a similar compound in ants that they named dolichodial. Anisomorphal had lower optical activity than dolichodial, suggesting that *A. buprestoides* secretions contained a mixture of isomers or an optically active impurity (*15*). Subsequently, two related stereoisomers were identified from a plant in the mint family, *Teucrium marum* (cat thyme) (*17–19*). The minor isomer from *T. marum* was assigned to anisomorphal (*17*). For clarity, we will refer to any of the stereoisomers with the covalent structure as "dolichodial-like" (Scheme 1); we will suggest specific assignments at the end of this work.

Without purification or additional preparation, we were able to collect the 1D ^{1}H NMR spectrum (Figure 1, panel a) within ~10 min following the milking of a single midsized *A. buprestoides* male. The spectrum was more complicated than expected for a compound with only 10 carbon atoms, so we extracted the sample with an equal volume of deuterated chloroform ($CDCl_3$) and collected ^{1}H NMR data on the respective aqueous (Figure 1, panel b) and organic (Figure 1, panel c) fractions.

We confirmed that the aqueous fraction contains essentially pure glucose by adding 0.9 μL of 50 mM D-glucose with 0.11 mM 3-(trimethylsilyl) propionate-*2,2,3,3-d*$_4$ (TSP) in D_2O to a similarly prepared sample. Only the peaks corresponding to those in the aqueous fraction increased in intensity (Figure 1, panel e), no additional resonances were detected, and the resonances observed within the aqueous fraction were identical with those of aqueous D-glucose (Figure 1, panel f). HPLC/MS of aqueous fractions supplemented with $^{13}C_6$ D-glucose also supports this conclusion (Supplementary Figure 1). Using HPLC and colorimetric (*20*) assays, we estimate that an *A. buprestoides* secretion contains between 140 and 280 mM glucose. By NMR, we find roughly equal amounts of glucose and dolichodial-like isomers (Figure 1), but the exact ratio varies between animals. We are unaware of any previous reports of glucose in phasmid insect secretions.



Scheme 1. Aqueous equilibrium between the dialdehyde (right) and diol forms (left) of dolichodial-like structures. Chiral carbons are identified by asterisks. The numbering scheme is according to Chemical Abstracts Service.

In order to assign the NMR resonances (Supplementary Table 1), 2D datasets were recorded from a single walking stick milking (Figure 2). We were able to collect high-quality COSY, TOCSY, ROESY, and natural abundance ^{13}C HMQC and HMBC datasets in the time typically used for conventional 600 μL samples (Supplementary Figure 2). From the 1D and 2D NMR data on *A. buprestoides*, we identified two major dolichodial-like isomers with the corresponding diols that are expected in aqueous solution (Scheme 1). Each major isomer could be fully assigned through $^1H–^1H$ and/or $^1H–^{13}C$ scalar coupling correlations. Diols were recognized by the disappearance of the formyl[2] (Scheme 1) aldehyde proton in water and were verified by extracting the sample into $CDCl_3$, which essentially eliminates the diol. We estimate that in water the diols are about 14% of the concentration of the dialdehydes based on integration of NMR peaks in the aldehyde and vinyl regions. Using gas chromatography (GC) with mass spectrometry detection (GC/MS) we also identified two major isomers as well as a minor isomer (Figure 3, panel c and Supplementary Figure 3).

The isomeric heterogeneity of dolichodial-like isomers led us to examine the composition of single milkings from different individual *A. buprestoides* as a function of time. We separated four half-grown males from our culture into their own containers, collected a sample from each, and analyzed them by 1D ^1H NMR. We similarly collected and analyzed milkings from the same four animals 2 and 8 d later. An expansion of the vinyl region of the NMR spectrum of each milking for each animal is shown (Figure 3). The chemical shifts of the vinyl protons are different for each isomer and thus provide a direct indication of the heterogeneity of the samples. To our surprise, different individual *A. buprestoides* raised under identical conditions produce different mixtures of dolichodial-like isomers. Furthermore, the composition of the isomeric mixture changed with time for some individuals.

Peruphasma schultei, a recently described walking stick from Peru, also produces a defensive secretion (*21*). We obtained a pooled sample of three *P. schultei* milkings and found by NMR that it also contains glucose but only one dolichodial-like isomer and corresponding diol; it was distinct from either of the two major isomers found in *A. buprestoides* based on a comparison of NMR chemical shifts (Figure 2 and Figure 3) and by GC (Figure 3, panel c).

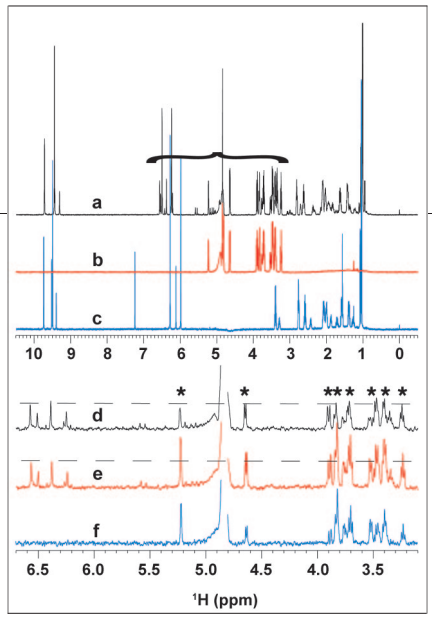


Figure 1. One-dimensional ^1H NMR spectra of single *A. buprestoides* milkings. All spectra were collected at 600 MHz using a 1-mm HTS probe, and sample temperatures were 27 °C. Each spectrum was collected with eight scans. a) About 1 μL was collected from a single insect on a glass pipet tip and added to 10 μL of D_2O containing 0.11 mM TSP. The sample was loaded into a 1-mm capillary NMR tube without purification, and the spectrum was obtained within ~10 min of the sample collection. Sample a was extracted with 15 μL of chloroform-*d*$_3$, and the aqueous b) and organic c) fractions were collected and recorded. d) Expansion of a second sample that includes the aqueous component and the vinyl organic region of the spectrum. e) 0.9 μL of pure 50 mM D-glucose was added to sample d. The region of the expansions in d–f is indicated by a bracket in spectrum a. The horizontal dashed lines in spectra d and e indicate the constant vinyl peak intensities, and the asterisks indicate peaks that increased in intensity. f) NMR spectrum of pure glucose.

To compare and name the different dolichodial-like isomers identified in this study, we performed GC/MS on chloroform extracts of walking stick secretions and *T. marum*, reported previously to produce dolichodial and a small amount of anisomorphal (*17*). The two *T. marum* isomers are consistent with the two major *A. buprestoides* isomers (Figure 3, panel c), and on the basis of assignments of Pagnoni and coworkers (*17*), we are assigning the *A. buprestoides* isomers at 11.95 and 12.15 min to "dolichodial" and "anisomorphal", respectively (Figure 3, panel c). These results are in apparent disagreement with more recent studies suggesting that *A. buprestoides* pro-

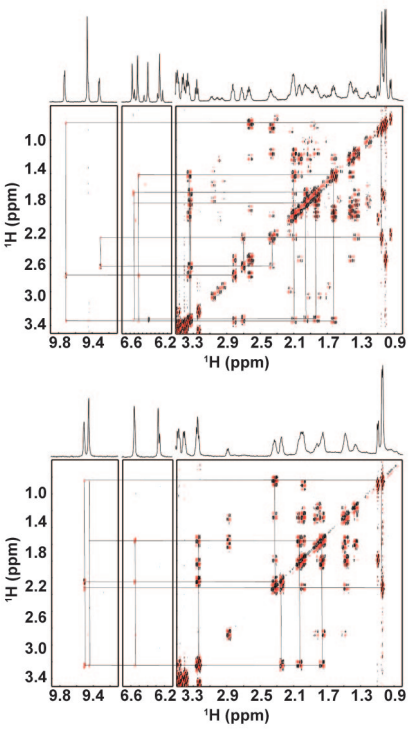


Figure 2. Two-dimensional expansions of COSY (right panels) and ROESY (left and center panels) from a single milking of *A. buprestoides* (top) and a pooled sample from three *P. schultei* milkings (bottom). One-dimensional ^{1}H spectra from the same samples are shown along the top. All data were collected at 600 MHz using the 1-mm HTS probe. The COSY experiments were collected in ~2.5 h with 8 scans and 512 complex indirect data points. The ROESY experiments were collected in ~9 h with 32 scans, 512 complex points, and a 400-ms mixing time.

duces a single dolichodial-like isomer (*22*). This could be due to improvement of analytical methods, genetic variability, or environmental factors. The *P. schultei* and minor *A. buprestoides* isomers at 11.78 min (Figure 3, panel c) appear to be the same and, we believe, are previously unreported. We are naming this isomer "peruphasmal".

Previous studies using MS, electrophoresis, or LC have reported individual variation in polypeptide toxins from snakes (*23–25*), cone snails (*26*), and a variety of arthropods (*27–30*). To our knowledge, it has never before been possible to perform a detailed molecular study of a mixture of natural products from an individual insect using NMR. This new capability provides the possibility of elucidating chemical variation, such as stereochemistry, in greater detail. Three major findings on walking stick defense secretions were enabled by high-sensitivity NMR (*13*): (i) the heterogeneity of defensive dolichodial-like stereoisomers that varies between *A. buprestoides* individuals and with time, (ii) a new dolichodial-like isomer called peruphasmal from *P. schultei*, and (iii) the identification of glucose in phasmid secretions. The quantity of glucose suggests a biological or chemical role in walking stick venom that merits further investigation.

METHODS

Insect Rearing and Sample Preparation. Adult *A. buprestoides* were collected at night in Gulf Hammock, FL, during the fall of 2005. Eggs produced by the insects were hatched in captivity. The young phasmids were fed a diet of only variegated *Ligustrum sinense* purchased from a local plant nursery. We were able to collect single milkings from half-grown males consisting of ~1 μL of a whitish fluid by gently touching the secretory duct with a glass pipet. To this we added 10 μL of D_2O containing 0.11 mM TSP as a chemical shift reference to the sample.

NMR. NMR experiments were done using a 600-MHz 1-mm triple-resonance HTS cryogenic probe that was developed through collaboration between the University of Florida, the National High Magnetic Field Laboratory (NHMFL), and Bruker Biospin (*13*). The total sample volume is ~8 μL, and each sample was loaded into a 1-mm × 100-mm capillary NMR tube (Norell, Inc.) using a 10-μL syringe with a fixed 110-mm × 30-gauge blunt needle. The capillary tube was held in a standard 10-mm spinner using a Bruker MATCH device, and the capillary–MATCH–spinner combination was lowered vertically into the magnet on an air column as usual. The sample temperature was regulated at 27 °C. The spectrometer was a Bruker Avance 600 with Xwin-NMR software, and all other data acquisition was done using standard technology. Two-dimensional datasets were processed using NMRPipe (*31*) and manually assigned using NMRView (*32*).

GC–Flame Ionization Detector. A Hewlett-Packard (Palo Alto, CA) 5890 series II gas chromatograph and a flame ionization detector (GC–FID) with nitrogen make-up gas (1.5 mL/min) and helium carrier gas (1.3 mL/min) were used. Cool on-column and splitless injections (1 μL) were at 40 and 200 °C, respectively; the detector was maintained at 260 °C. The oven program was as follows: isothermal for 5 min, heating from 40 to 200 °C at 11 °C/min, isothermal for 10 min, heating from 200 to 250 °C at 25 °C/min, and then isothermal for 15 min. GlasSeal connectors (Supleco) fused three silica columns in series: a primary deactivated column (8 cm long, 0.53 mm i.d.), an HP-1MS retention gap column (2 m long, 0.25 mm i.d., df = 0.25 μm), and a J&W DB-5 analytical column (30 m long, 0.25 mm i.d., df = 0.25 μm).

GC/MS. A Varian 3400 gas chromatograph and a Finnigan MAT Magnum ion trap mass spectrometer in electron impact ionization mode (70 eV) with a filament bias of 11765mV or chemical ionization mode (isobutane) were employed to acquire full-scan spectra over the ranges m/z 40–400 at 0.85 s per scan. Holox (Charlotte, NC) high-purity helium was used as a carrier gas (1.4 mL/min). Injection and oven conditions were as above. Transfer-line and manifold temperatures were 240 and 220 °C, respectively.

Acknowledgments: A sample of defensive secretions from *P. schultei* was kindly provided by O. V. Conle of Bolsterlang, Germany. We thank Drs. W. W. Brey (NHMFL) and R. S. Withers and R. E. Nast (Varian NMR) for the collaboration and support on the 1-mm HTS probe. Dr. P. Teal (USDA Laboratory, Gainesville, FL) provided helpful encouragement and discussions. Supported by NIH P41RR016105, the Human Frontier Science Program (ASE), and the NHMFL. NMR data were collected in the Advanced Magnetic Resonance Imaging and Spectroscopy Facility at McKnight Brain Institute of the University of Florida.

Supporting Information Available: This material is available free of charge *via* the Internet.

REFERENCES

1. Metcalf, R. L. (1998) Ultramicrochemistry of insect semiochemicals, *Mikrochim. Acta. 129,* 167–180.
2. Olson, D. L., Peck, T. L., Webb, A. G., Magin, R. L., and Sweedler, J. V. (1995) High-resolution microcoil H-1-NMR for mass-limited, nanoliter-volume samples, *Science 270,* 1967–1970.
3. Gronquist, M., Meinwald, J., Eisner, T., and Schroeder, F. C. (2005) Exploring uncharted terrain in Nature's structure space using capillary NMR spectroscopy: 13 Steroids from 50 fireflies, *J. Am. Chem. Soc. 127,* 10810–10811.
4. Peti, W., Norcross, J., Eldridge, G., and O'Neil-Johnson, M. (2004) Biomolecular NMR using a microcoil NMR probe—new technique for the chemical shift assignment of aromatic side chains in proteins, *J. Am. Chem. Soc. 126,* 5873–5878.
5. Li, Y., Logan, T. M., Edison, A. S., and Webb, A. (2003) Design of small volume HX and triple-resonance probes for improved limits of detection in protein NMR experiments, *J. Magn. Reson. 164,* 128–135.

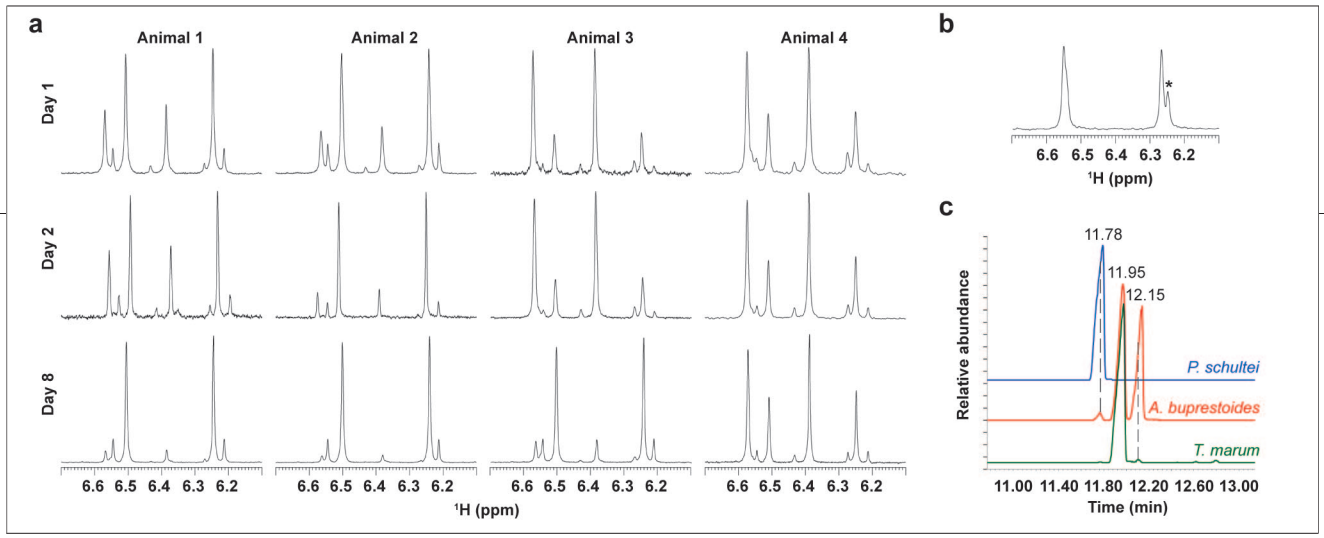


Figure 3. Isomeric variation of venom. a) Expansions of the vinyl region of NMR spectra of single milkings from individual *A. buprestoides* collected on different days. Samples were dissolved in 10 μL of D_2O + TSP without further purification. b) Same expansion from a mixture of three milkings from *P. schultei*. The shoulder marked with an asterisk corresponds to one of the vinyl peaks of the diol, and the broad peak at 6.54 ppm is an overlap of the diol and dialdehyde isomers (Scheme 1). c) GC analysis of chloroform extracts of *A. buprestoides* (red) and *P. schultei* (blue) secretions, and *T. marum* (green). With both cool on-column (shown) and splitless injection, all isomers ionized with comparable efficieny (FID), fragmented similarly (electron impact MS), and had identical masses of 166 Da (chemical ionization MS).

6. Li, Y., Webb, A. G., Saha, S., Brey, W. W., Zachariah, C., and Edison, A. S. (2006) Comparison of the performance of round and rectangular wire in small solenoids for high-field NMR, *Magn. Reson. Chem. 44,* 255–262.
7. Kovacs, H., Moskau, D., and Spraul, M. (2005) Cryogenically cooled probes—a leap in NMR technology, *Prog. Nucl. Magn. Reson. Spectrosc. 46,* 131–155.
8. Rogers, E. W., and Molinski, T. F. (2005) A cytotoxic carotenoid from the marine sponge Prianos osiros, *J. Nat. Prod. 68,* 450–452.
9. Wolters, A. M., Jayawickrama, D. A., and Sweedler, J. V. (2005) Comparative analysis of a neurotoxin from *Calliostoma canaliculatum* by on-line capillary isotachophoresis 1H NMR and diffusion 1H NMR, *J. Nat. Prod. 68,* 162–167.
10. McPhail, K. L., France, D., Cornell-Kennon, S., and Gerwick, W. H. (2004) Peyssonenynes A and B, novel enediyne oxylipins with DNA methyl transferase inhibitory activity from the red marine alga *Peyssonnelia caulifera*, *J. Nat. Prod. 67,* 1010–1013.
11. Russell, D. J., Hadden, C. E., Martin, G. E., Gibson, A. A., Zens, A. P., and Carolan, J. L. (2000) A comparison of inverse-detected heteronuclear NMR performance: conventional vs cryogenic microprobe performance, *J. Nat. Prod. 63,* 1047–1049.
12. Saman, D., Cvacka, J., Svatos, A., Bouman, E. A. P., and Kalinova, B. (2006) Structural identification of an anthrasteroid hydrocarbon from the sheep tick *Ixodes ricinus*. *J. Nat. Prod.* DOI. 10.1021/np0680127.
13. Brey, W. W., Edison, A. S., Nast, R. E., Rocca, J. R., Saha, S., and Withers, R. S. (2006) Design, construction, and validation of a 1-mm triple-resonance high-temperature-superconducting probe for NMR, *J. Magn. Reson. 179,* 290–293.
14. Eisner, T. (1965) Defensive spray of a phasmid insect, *Science 148,* 966–968.
15. Meinwald, J., Chadha, M. S., Hurst, J. J., and Eisner, T. (1962) Defense mechanisms of arthropods. 9. Anisomorphal, the secretion of a phasmid insect, *Tetrahedron Lett.* 29–33.
16. Cavill, G. W., and Hinterberger, H. (1961) Chemistry of ants. 5. Structure and reactions of dolichodial, *Aust. J. Chem. 14,* 143–149.
17. Pagnoni, U. M., Pinetti, A., Trave, R., and Garanti, L. (1976) Monoterpenes of teucrium-marum, *Aust. J. Chem. 29,* 1375–1381.
18. Eisner, T., Eisner, M., Aneshansley, D. J., Wu, C. L., and Meinwald, J. (2000) Chemical defense of the mint plant, *Teucrium marum* (Labiatae), *Chemoecology 10,* 211–216.
19. Ricci, D., Fraternale, D., Giamperi, L., Bucchini, A., Epifano, F., Burini, G., and Curini, M. (2005) Chemical composition, antimicrobial and antioxidant activity of the essential oil of *Teucrium marum* (Lamiaceae), *J. Ethnopharmacol. 98,* 195–200.
20. Hendrix, D. L. (1993) Rapid extraction and analysis of nonstructural carbohydrates in plant-tissues, *Crop Sci. 33,* 1306–1311.
21. Conle, O. V., and Hennemann, F. H. (2005) Studies on neotropical Phasmatodea I: A remarkable new species of Peruphasma Conle & Hennemann, 2002 from northern Peru (Phasmatodea: Pseudophasmatidae: Pseudophasmatinae), *Zootaxa 1068,* 59–68.
22. Eisner, T., Morgan, R. C., Attygalle, A. B., Smedley, S. R., Herath, K. B., and Meinwald, J. (1997) Defensive production of quinoline by a phasmid insect (*Oreophoetes peruana*), *J. Exp. Biol. 200,* 2493–2500.
23. Menezes, M. C., Furtado, M. F., Travaglia-Cardoso, S. R., Camargo, A. C., and Serrano, S. M. (2006) Sex-based individual variation of snake venom proteome among eighteen *Bothrops jararaca* siblings, *Toxicon 47,* 304–312.
24. Francischetti, I. M., Gombarovits, M. E., Valenzuela, J. G., Carlini, C. R., and Guimaraes, J. A. (2000) Intraspecific variation in the venoms of the South American rattlesnake (*Crotalus durissus terrificus*), *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. 127,* 23–36.
25. Monteiro, R. Q., Yamanouye, N., Carlini, C. R., Guimaraes, J. A., Bon, C., and Zingali, R. B. (1998) Variability of bothrojaracin isoforms and other venom principles in individual jararaca (*Bothrops jararaca*) snakes maintained under seasonally invariant conditions, *Toxicon 36,* 153–163.
26. Jakubowski, J. A., Kelley, W. P., Sweedler, J. V., Gilly, W. F., and Schulz, J. R. (2005) Intraspecific variation of venom injected by fish-hunting Conus snails, *J. Exp. Biol. 208,* 2873–2883.
27. Borges, A., Garcia, C. C., Lugo, E., Alfonzo, M. J., Jowers, M. J., and Op den Camp, H. J. (2006) Diversity of long-chain toxins in *Tityus zulianus* and *Tityus discrepans* venoms (Scorpiones, Buthidae): molecular, immunological, and mass spectral analyses, *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. 142,* 240–252.
28. Lai, C. C., and Her, G. R. (2000) Analysis of phospholipase A2 glycosylation patterns from venom of individual bees by capillary electrophoresis/electrospray ionization mass spectrometry using an ion trap mass spectrometer, *Rapid Commun. Mass Spectrom. 14,* 2012–2018.
29. Pimenta, A. M., de Lima, M. E., De Marco Almeida, F., Martin-Eauclaire, M. F., and Bougis, P. E. (2003) Individual variability in *Tityus serrulatus* (Scorpiones, Buthidae) venom elicited by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, *Rapid Commun. Mass Spectrom. 17,* 413–418.
30. Escoubas, P., Corzo, G., Whiteley, B. J., Celerier, M. L., and Nakajima, T. (2002) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and high-performance liquid chromatography study of quantitative and qualitative variation in tarantula spider venoms. *Rapid Commun. Mass Spectrom. 16,* 403–413.
31. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) Nmrpipe—a multidimensional spectral processing system based on Unix pipes, *J. Biomol. NMR 6,* 277–293.
32. Johnson, B. A., and Blevins, R. A. (1994) NMR View—a computer-program for the visualization and analysis of NMR data, *J. Biomol. NMR 4,* 603–614.