males from long distances, and the efficacy of the male's web-reduction behavior in reducing the pheromone's detection by rivals were documented in experiments testing the attraction of males to three types of caged females with webs: (i) virgin females with intact webs, (ii) mated females with intact webs, and (iii) virgin females with reduced webs. The testing apparatus is shown in Fig. 2A.

In each trial, a single male was released atop the central vertical support of the T-rod and exposed to a breeze reaching him through the cage. Two sets of data were recorded: (i) the percentage of the male's total exposure time spent within each of the two 10-cm terminal sections of the T-rod near and far from the cage and (ii) the section of the T-rod from which the male finally descended, by means of a dragline, to the table surface (*14*).

Only when exposed to a virgin female on an intact web did males spend significantly more time at the 10-cm portion of the T-rod near the cage than at the 10-cm portion of the T-rod far from the cage. In addition, under this condition males spent a greater percentage of their total exposure time within the 10-cm section of the T-rod near the cage than males exposed to mated females with intact webs or virgin females with reduced webs (Mann-Whitney test; $P = 0.002$ and $P = 0.008$, respectively). The percentage of exposure time spent by males near the cage did not differ significantly between these latter two conditions (Mann-Whitney test; $P = 0.279$) (Fig. 2B). Males exposed to intact webs of virgin females descended from the 10-cm section of the T-rod near the cage more often than those exposed to nonvirgins with intact webs or virgins with reduced webs (Table 1; $P = 0.046$) (*15*).

Thus, oriented searching behaviors were exhibited by males exposed to virgin females with intact webs, while in the other two conditions males wandered about the T-rod apparatus without obvious orientation. The data show that male Sierra dome spiders are attracted to the webs of mature virgin females by an airborne emission, but web reduction neutralizes the attractiveness of these webs.

These results illustrate the complex blend of mutual and conflicting interests that can exist in intersexual mating strategies. Female Sierra dome spiders probably benefit by producing a male attractant pheromone to ensure mating in circumstances where rates of male visitation are very low. Competition among attracted males could further benefit females by resulting in a higher quality mate. But, the first male to enter the web of a pheromone-producing female avoids combat with other males by quickly canceling the female's signal through web reduction. This cost of web reduction to the female is added to that associated with the destruction of a large portion of her web, which represents a nonrecoverable protein investment (*16*) and the female's sole means of foraging and defense against predators.

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6. The density of adult females on my study site during the middle of the breeding season was about one individual per 6 m^2.
7. This finding is based on a five-locus electrophoretic paternity exclusion analysis using the mates and progeny of 20 Sierra dome females. These females were allowed multiple matings at will in the field under natural circumstances.
8. Females are easily introduced onto webs, where they behave normally. A female promptly adds silk of her own to the web onto which she has been introduced.
9. The average male visitation rate is a minimum estimate based on a total of 57 hourly scans of the webs of each class of female. The significance level is based on a two-tailed Wilcoxon test ($T = 45$; $n = 27$).
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12. Web reduction would have this effect because the male's matting of the silk drastically decreases the concentration gradient of pheromone molecules between the silk and open air.
13. Since the mean male visitation rate at the webs of mature virgin females is approximately one arrival per 3-hour period, and copulations last 3 to 6 hours, many matings would be disrupted by one or more rivals if the early-arriving male failed to perform web reduction.
14. Females constructed normal webs within their cages (cage size: 30 by 30 by 40 cm, all sides screened). Each female was tested for attractiveness with three different males, but males were used in only one trial each. Tests on females with reduced webs were arranged by releasing a male into the cage of a mature virgin female, allowing completion of web reduction, and then removing the male from the cage. Such trials were begun minutes after web reduction was complete and involved only webs that clearly had been attractive to males immediately before web reduction.
15. Separate 2 × 3 analyses show that frequency distributions differ between virgins with intact webs versus mated females with intact webs ($P = 0.020$), and virgins with intact webs versus virgins with reduced webs ($P = 0.048$), but not between nonvirgins with intact webs versus virgins with reduced webs ($P = 0.338$). The average distance from the tip of the T-rod near the cage at which males in the "center" category descended was 60 cm (SEM = 4.0).
16. Linyphiid spiders do not ingest web silk.

17. I thank M. Apple, V. Demas, D. Glaser, B. Jacobs, D. Lee, E. Smith, and D. Watson for assistance with observations; J. Stanford and the staff of the University of Montana Biological Station for hospitality during my time in the field; S. T. Emlen, P. W. Sherman, T. Eisner, R. Charif, and J. Crawford for discussions and comments; and M. Miyakado for performing web extractions. Study supported in part by NSF Dissertation Improvement Grant BSR-8311331 and NIH grant 5T32MH15793.

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A Toxic Dipeptide from the Defense Glands of the Colorado Beetle

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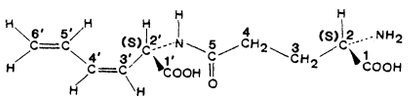
The Colorado beetle is protected against predators by the secretions of defensive glands located on the pronotum and the elytra. The single major compound of the secretion was identified as γ-L-glutamyl-L-2-amino-3(*Z*),5-hexadienoic acid by spectroscopic and chemical methods. This compound, which contains a nonprotein β,γ-unsaturated amino acid, is toxic to ants (*Myrmica rubra*) at a concentration 10^{-2} molar, which is less than its estimated concentration in the secretion (1.8×10^{-1} molar).

THE COLORADO BEETLE (*LEPTINOTARSA DECEMLINEATA*), a notorious pest of potato plants, is chemically well protected against predators (*1, 2*). Like many toxic insects, it has a bright coloration, which provides a visual signal for predators that hunt by sight, such as birds (*3*). The beetle produces a secretion from which we have isolated a toxic dipeptide, γ-L-glutamyl-L-2-amino-3(*Z*),5-hexadienoic acid (**1**).



1

The discovery of such a dipeptide containing a nonprotein amino acid in the defensive secretion of the Colorado beetle was unexpected on the basis of the known defensive chemistry of adult chrysomelids. The compounds so far isolated constitute a biosynthetically diverse group that includes cardenolides, from several *Chrysolina* spp. (*4–6*); polyoxygenated steroidal glucosides, from *Chrysolina hyperici* (*7*); and isoxazolinone glucosides, from several species be-

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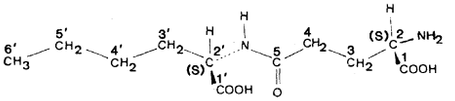
longing to the subtribe Chrysomelina (*6*).

In the Colorado beetle, as in many other adult chrysomelids (*8*), chemical protection is ensured by a secretion released from pronotal glands located along the anterior and lateral margins of the pronotum and from the elytral glands located beneath the black lines of the elytra. Each gland is made up of a group of secretory cells ending in a common duct. There is no extracellular reservoir, but the secretion is stored in large vacuoles of the glandular cells, which become greatly inflated in mature glands (*2*). When the beetle is disturbed, the secretion oozes from the gland pores and covers the integument. By gently squeezing the insect, we were able to collect about 0.3 μl (*9*) of secretion. The beetle refills its glands in 1 week, after which it can be "milked" again.

Adult beetles (n = 1200) were milked (*4*), and the secretion, collected on bits of filter paper, was stored in methanol. Thin-layer chromatography (SiO_2 plates; eluent, *n*-butanol:acetic acid:water = 8:2:2) showed the presence of a single major compound at R_F = 0.5 that gave a pink color on visualization with ninhydrin. Evaporation of the solvent yielded 22 mg of crude extract, which was submitted to reversed-phase (RP-18) column chromatography (eluent, water:methanol = 8:2). Lyophilization of the ninhydrin-positive fractions yielded 16 mg of **1** as a foamy solid [specific rotation at 579 nm ($[\alpha]_{579}$) of +59° (concentration, 0.47 g per 100 ml in water)], homogeneous in thin-layer chromatography.

The structure of **1** was determined by a combination of spectroscopic methods and chemical degradation. The infrared spectrum indicated the presence of amino (3400 to 2500 cm^{-1}), amide (1660 cm^{-1}), and carboxylate (1590 and 1400 cm^{-1}) groups. The presence of an acyclic conjugated diene was deduced from the ultraviolet spectrum (wavelength λ_{max} = 230 nm; molar absorptivity ϵ = 8000 in water). Fast atom bombardment mass spectrometry [mass-to-charge ratio (*m/z*) of 257, $(M + H)^+$] together with nuclear magnetic resonance (NMR) data (Table 1) led to an assignment of the molecular formula $C_{11}H_{16}N_2O_5$. The ^{13}C and ^1H NMR spectra of **1** showed that it is a dipeptide containing glutamic acid and a dienoic amino acid (Table 1). The latter was identified as 2-amino-3(*Z*),5-hexadienoic acid on the basis of extensive ^1H NMR double-irradiation experiments (Table 1). This identification was confirmed by comparison of the low-field region of the ^1H and ^{13}C NMR spectra of **1** with the data reported for (*Z*)-9,11-dodecadienyl acetate (*10*). Further evidence of the structure of **1** came from its catalytic hydrogenation (Pd-C, ethanol), which yielded γ-glutamylnorleucine, **2** [chemical ionization mass spectrum (NH_3): *m/z* 261 $(M + H)^+$]



2

Acid hydrolysis of **2** (6*N* HCl, 12 hours) yielded glutamic acid and norleucine in nearly equal quantities (*11*). Mass spectrometry and ^1H NMR showed that the linkage of the two amino acids of **2** (and thus of **1**) is through the γ-carboxyl group of glutamic acid. The chemical ionization mass spectrum (NH_3) of **2** showed prominent fragment ions at *m/z* 130 and 147 (pyroglutamic acid plus H^+ and plus NH_4^+, respectively) and *m/z* 132 and 149 (norleucine plus H^+ and plus NH_4^+, respectively). This type of fragmentation is characteristic for a γ-glutamyldipeptide (*12*). The deshielding of the α proton of glutamic acid observed in the ^1H NMR spectrum of **2** when the solvent was changed from D_2O to a mixture of D_2O and trifluoroacetic acid [3.81 parts per million (ppm) versus 4.14 ppm] also indicated a γ-glutamyl link (*13*). This conclusion was further confirmed by treatment of **2** with 2,4-dinitrofluorobenzene followed by acid hydrolysis (6*N* HCl, 12 hours, 100°C). Only norleucine could be detected in the hydrolysis mixture, which showed that the amino group of glutamic acid had reacted with 2,4-dinitrofluorobenzene. Finally, the absolute configurations of glutamic acid and norleucine obtained from **2** were established by methylation of the hydrolysis mixture (methanol, $SOCl_2$, room temperature, 12 hours) followed by derivatization with *N*-trifluoroacetyl-L-prolyl chloride (*14*). Gas chromatographic comparison of these derivatives with authentic samples of similarly derivatized D- and L-glutamic acid and D- and L-norleucine (*15*) demonstrated that they belong to the L series. This result indicated that compound **1** is γ-L-glutamyl-L-2-amino-3(*Z*),5-hexadienoic acid.

γ-Glutamyl dipeptides are well known from plants (*16*); L-2-amino-3(*Z*),5-hexadienoic acid is a nonprotein amino acid; and closely related β,γ-unsaturated amino acids have been reported, especially from fungi (*17*), but never in insects. These substances have potent biological activity, both as enzyme inhibitors (*18*) and as antibiotics (*19*). Preliminary experiments suggested that **1** exhibits toxic properties against the ant *Myrmica rubra* (*20*). Whereas the ants were not deterred by a $10^{-2}M$ solution of **1** in $10^{-1}M$ sucrose (*21*), our experiments indicated this solution was toxic when ingested orally (*22*). We do not yet know the mode and site of action of **1**. Since the concentration of the dipeptide in the secretion is estimated to be 1.8 $10^{-1}M$ (*23*), the secretion is certainly toxic to ants. More experiments are needed to fully assess the ecological significance of the secretion as a chemical defense.

Table 1. ^1H and ^{13}C NMR spectral data for **1** and glutamic acid, recorded with D_2O as solvent and 3-(trimethylsilyl)-propanesulfonic acid sodium salt (DSS) as the internal standard. Assignment of signals in the ^1H NMR spectrum were made by extensive decoupling experiments. Abbreviations: δ, chemical shift (ppm); M, multiplicity; *J*, coupling constant in hertz; s, singlet; d, doublet; dd, doublet of doublets; t, triplet; ddd, triplet of doublets; and m, multiplet.

Site	^1H NMR*; δ, M (*J*)		^{13}C NMR†; δ, M	
	1	Glutamic acid	**1**	Glutamic acid
C-1			175.4‡, s	175.6, s
C-2	3.74, t (6)	3.79, t (6)	54.9, d	55.7, d
C-3	2.13, m	2.24, m	28.0, t	28.1, t
C-4	2.45, t (7.5)	2.54, t (7.5)	33.4, t	34.5, t
C-5			175.6‡, s	182.3, s
C-1′			178.6, s	
C-2′	5.06, d (9.6)		56.0, d	
C-3′	5.43, dd (9.6, 10.2)		135.1 ‖, d	
C-4′	6.26, dd (10.2, 10.2)		127.7, d	
C-5′	6.75, ddd (16.7, 10.2, 10.2)		133.2 ‖, d	
C-6′	5.29, dd (10.2, 1.8)		122.4, t	
	5.38, dd (16.7, 1.8)			

*Recorded at 250 MHz. †Recorded at 62.8 MHz. ‡‖ These assignments may be reversed.

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20. The limited amount of natural compound available did not allow us to test it on a wide range of natural potential predators, vertebrates and invertebrates. *Myrmica rubra* was chosen for convenience.
21. A choice between 50 μl of sucrose solution ($10^{-1}M$) in distilled water and 50 μl of **1** ($10^{-2}M$) in the same sucrose solution was given to 12 groups of 50 ants each, isolated in petri dishes. The number of ants feeding on each solution was counted 3 minutes after the beginning of the experiment. After this delay, the ants recovered from the disturbance. The distribution of ants on both solutions was analyzed for significance by the Walsh test. About half of the ants (52%, 12 replications pooled) fed on the solution of **1** ($P >> 0.05$, not significant).
22. Drops of 5 μl of either pure water, sucrose ($10^{-1}M$) solution in water, or solution of **1** ($10^{-2}M$) in the sucrose solution were given to three groups of 50 ants isolated in petri dishes until they were satiated. Care was taken to give about the same amount of liquid to each of the three groups. Each day the dead ants were counted and the live ants were refed. After 3 days, more than 50% mortality was reached in the group fed with **1**. At this stage, the ants had ingested a mean of 2.7 μl of the solution per ant—that is, about 7 μg of **1** per ant. The mortality in the group fed with **1** was significantly higher ($P < 0.001$, χ^2 test corrected for continuity) than that in those fed with either pure water or the sugared solution. Mortality in these two control groups did not differ significantly ($P > 0.7$).
23. The concentration was estimated on the basis of the following figures: volume of secretion, 0.29 μl per beetle (*9*); the secretion of 1200 beetles yielded 16 mg of **1**; and molecular weight 256.
24. We thank R. Ottinger for the ^1H and ^{13}C NMR spectra, C. Moulard for the mass spectra, and J. André for the amino acid analysis.

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Study of Aldose Reductase Inhibition in Intact Lenses by ^{13}C Nuclear Magnetic Resonance Spectroscopy

W. F. WILLIAMS AND J. D. ODOM

Carbon-13 nuclear magnetic resonance spectroscopy has been used in the study of glucose metabolism, specifically aldose reductase inhibition, in intact rabbit lenses maintained in organ culture. This technique provides an effective method of screening potential inhibitors of aldose reductase under conditions that more closely approximate in vivo conditions than do earlier methods. The aspirin substitutes acetaminophen and ibuprofen were studied as aldose reductase inhibitors and were found to be effective in reducing sorbitol accumulation in lenses exposed to high glucose stress. Results of this work with various inhibitors of aldose reductase are discussed in terms of lens metabolism and implications regarding diabetic complications such as cataract formation.

CATARACT FORMATION IS ONE OF many secondary complications associated with diabetes mellitus. It has been estimated that in the United States alone over 10 million people suffer from diabetes and over 75,000 diabetic cataract extractions are performed each year (*1*). Cataracts are five times as likely to develop in a diabetic as in a nondiabetic of the same age and sex (*2*). Unfortunately, neither the disorder, diabetes mellitus, nor the causes of cataract formation are well understood. Glucose metabolism in the lens, via the sorbitol pathway and the enzyme aldose reductase (E.C. 1.1.1.21), appears to be directly related to the higher incidence of cataracts in diabetics. In this investigation we used a nondestructive technique, ^{13}C nuclear magnetic resonance (NMR) spectroscopy, to study glucose metabolism, glycolysis, and the sorbitol pathway in intact rabbit lenses and also the effectiveness of various aldose reductase inhibitors.

The normal physiological role of the sorbitol pathway remains unknown. Under the conditions of high plasma-sugar concentrations encountered in diabetics, hexokinase apparently becomes saturated and excess glucose is converted to its sugar alcohol, sorbitol, by aldose reductase with reduced nicotinamide adenine dinucleotide phosphate (NADPH) acting as the cofactor. In the second reaction of the sorbitol pathway, sorbitol is oxidized to its keto sugar, fructose, by polyol dehydrogenase with nicotinamide adenine dinucleotide (NAD^+) as the cofactor. Sorbitol is not readily metabolized and does not penetrate cell membranes easily. Once formed, sorbitol is trapped intracellularly. Numerous animal studies (*3, 4*) and limited research with human lenses (*5*) have suggested that the accumulation of sorbitol initiates a sequence of osmotic changes that leads to the formation of cataracts. In order to maintain osmotic equilibrium as sorbitol accumulates, water is drawn into the lens fibers, which causes swelling. Eventually the lens fibers rupture, and a lenticular opacity is observed. It has been suggested that osmotic effects are the causative factor in cataract formation under high glucose stress. Sorbitol pathway activity has various effects on lens metabolism that may be important in cataract development. Recently, research has suggested that a primary effect of sorbitol pathway activity is altered redox ratios of pyridine nucleotides (*6*). Strong evidence supporting the importance of the sorbitol pathway in cataract formation has come from the discovery of various compounds that are effective in blocking sorbitol formation and in delaying or preventing resulting cataractogenic changes (*7*). Moreover, the enzyme aldose reductase and high sorbitol levels have been found in other tissues involved in such secondary diabetic complications as retinopathy, nephropathy, and neuropathy (*8, 9*). The development of an orally active inhibitor of aldose reductase effective in human tissues could lead to a pharmacological approach for the treatment or even prevention of diabetic complications such as cataract formation.

Multinuclear magnetic resonance spectroscopy (^1H, ^{31}P, and ^{13}C) has been used in the study of the lens (*6, 10–13*). Use of ^{13}C NMR spectroscopy has been limited to the study of lenses exposed to various levels of glucose stress (*11, 12*) and to the determination of polyol pathway flux rates (*6, 13*). We now report that ^{13}C NMR spectroscopy provides an excellent means of studying the activity of pharmacological agents, such as aldose reductase inhibitors. The incubation of intact rabbit lenses in a high-concentration [1-^{13}C]glucose medium with and without inhibitors has been studied; with this procedure it is possible to study the utilization of glucose in the lens as changes occur. By monitoring the distribution of the ^{13}C it is possible to follow glycolytic and sorbitol pathway activities. The ^{13}C NMR spectrum

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