Sex Attractant of Sugar Beet Wireworm: Identification and Biological Activity

Abstract. *The sex attractant produced by adult females of the sugar beet wireworm,* Limonius californicus *(Mannerheim) has been isolated and identified as valeric acid. In the laboratory, male wireworm beetles are repelled by the pure attractant but are drawn with intense sexual excitement to its dilute solutions; in the field, male beetles are lured from a distance of 12 meters. The pheromone occurs in unusually large amounts in the female's body.*

The sugar beet wireworm, *Limonius californicus* (Mannerheim), is a pest attacking sugar beets, potatoes, corn, lettuce, onions, and other crops grown in the irrigated fields of the Pacific Northwest and Western Canada. The larvae develop in the soil where they feed on plant roots and cause considerable damage. The adults are commonly called click beetles because they can spring from a supine position several inches into the air (at the same time making a loud click). The beetles emerge from the soil in early spring; after mating, the females deposit their eggs in the ground.

Shirck (*1*) observed that adult males of *L. californicus* were attracted in numbers to each newly emerged female; Lilly (*2*) later showed that this attraction was caused by a chemical sex pheromone produced by virgin females. An extract prepared by crushing dead females in 70 percent ethyl alcohol lured numerous male beetles into an infested field within 10 seconds from as far away as 12 meters. Paper chromatograms of an extract developed with ethanol-ammonium hydroxide (95 : 5) could be bioassayed by exposing them to male beetles; the active zone had an R_F value of approximately 0.85. This became the basis for a very useful olfactometric method developed to determine sexual attraction for this insect and other species of click beetles in the laboratory (*3*).

Lilly and McGinnis (*4*) found that the natural sex pheromone obtained from females of *L. californicus* retained two-thirds of its attractancy after exposure to 100°C for 24 hours in a sealed container. However, only one-ninth of the activity was retained when the pheromone was exposed to the same time-temperature conditions in an open container. In stoppered vials at room temperature, ethanol extracts of the attractant could be stored for at least 5 years.

Laboratory tests conducted 35 years ago by Lehman (*5*) with 150 chemical compounds indicated that lactic, butyric, and caproic (hexanoic) acids were sexually attractive to male *L. californicus* and *L. canus* LeConte (Pacific Coast wireworm) (in Washington); his further field tests confirmed these results, especially with butyric and caproic acids, and showed that valeric acid was attractive to *L. canus* and, to a lesser extent, to *L. californicus*. Recent tests (*6*) showed conclusively that valeric acid is highly attractive sexually to male *L. californicus* from Alberta, both in the laboratory and in the field, but butyric and caproic acids did not attract. Also, a number of samples of additional organic compounds of several classes were placed every 7.5 meters in the field, tested for 10 to 15 minutes, and failed to draw males (Table 1). We now report the successful isolation and identification of the natural sex attractant of female *L. californicus*, and show that it is, in fact, valeric acid.

An ether extractive (50 mg) prepared by continuous (Soxhlet) extraction of 18 female abdomens for 3 hours was dissolved in a small volume of hexane, chromatographed on a column (35 by 1.5 cm) of silicic acid (*7*), and eluted successively with hexane, 10- and 25-percent ethyl ether in hexane, ether, and 10-percent methanol in ether (*8*). The activity of resulting solutions was then determined by a method similar to that of Lilly and McGinnis (*3*). Ten male beetles were placed in each of the two outer compartments of a rectangular olfactometer (dead air-space separated the two compartments). The responses of the beetles to 0.1 ml of each fraction spotted on a strip of Whatman No. 1 filter paper were assessed quantitatively by counting the numbers of males exhibiting sexual excitement at the site over a 10-minute period. After a short rest period the beetles in the compartments were interchanged, and they were used for a second test. A minimum of four sets of counts were obtained for each fraction. The results were compared by the *t*-test when necessary.

Only 25-percent ether in hexane removed the active material; this chromatographic fraction was then separated into neutral (14.2 mg) and acidic (13.0 mg) portions by dissolving it in 5 ml of ether and shaking the solution with three 0.5-ml portions of an ice-cold solution of 5-percent potassium hydroxide. The acidic portion was further separated into solid (10.5 mg) and liquid acids (2.4 mg) by cooling the combined alkaline extracts in an ice bath, acidifying to Congo Red paper with ice-cold, 5-percent hydrochloric acid, filtering off the separated white solid, and extracting the filtrate for 24 hours with ether in a continuous extractor. In laboratory bioassays, the neutral fraction was completely inactive; the solid fraction evoked a slight, indefinite response; the liquid acid fraction was active (Fig. 1). Crystallization of the solid fraction from dilute methanol gave the theoretical amount of colorless needles, m.p. 63° to 64°C, that consisted solely of palmitic acid, as shown by comparing the mixed melting point and infrared spectrum with those of authentic material (*9*).

The highly active liquid acid fraction (2.4 mg) was pale yellow and possessed a strong odor similar to that of valeric acid. It was dissolved in 15 ml of hexane-ether (75 : 25) and filtered through a 5-g layer of silicic acid (*7*) on a fritted-disc glass funnel; the removal of the solvent and microdistillation of the residue gave 2.0 mg of colorless liquid (b.p. 190°C, bath temperature; 760 mm) having the same odor, showing a refractive index (n_D^{25}) of 1.4063. Ascending chromatograms on Whatman No. 1 filter paper strips developed at room temperature for 5 hours with an ethanol–ammonium hydroxide (87 : 13) system (*10*) were sprayed with 0.1 percent bromcresol green in butyl alcohol. The resulting visible single spot (R_F 0.87) was identical to that of valeric acid. Also, a microsample of the natural acid failed to absorb hydrogen, and its infrared spectrum was identical to that of valeric acid. The acid was converted with boron trifluoride-methanol (14 percent) to its methyl ester; gas chromatograms (*11*) of this substance showed a single peak with a retention time of 2.0 minutes, identical to that of methyl valerate and different from those of the methyl esters of the remaining three isomeric 5-carbon saturated acids (Table 2) (*12*). Thus, the natural attractant is valeric acid.

The response of male beetles in the laboratory to diluted synthetic valeric acid and to the natural attractant was identical. A spot of concentrated

Table 1. Organic compounds that failed to attract male *Limonius californicus* in the field. After each compound was tested, a wad of absorbent cotton soaked in the natural sex attractant was placed at the site to prove that male beetles could be attracted.

Acids	*Alcohols continued*	*Esters*
Acetic	Geraniol	Ammonium valerate
Benzoic	Glycerol	Ammonium isovalerate
Formic	Guaiacol	Benzyl isobutyrate
DL-Glyceric	2-Methoxyethanol	Ethyl acetate
Isobutyric	2-Octanol	Ethyl nonanoate
Lactic	Octyl alcohol	Ethyl valerate
Lauric	Pentyl alcohol	Heptyl valerate
Linoleic	*tert*-Pentyl alcohol	Hexyl valerate
4-Methylvaleric	Propyl alcohol	Isobutyl hexanoate
Octanoic	Tetrahydrofurfuryl alcohol	Isobutyl isovalerate
Palmitic	*Aldehydes*	Isopentyl acetate
Stearic	2-Furaldehyde	Methyl butyrate
Alcohols	Isovaleraldehyde	Methyl isobutyrate
Benzyl alcohol	*m*-Nitrobenzaldehyde	Pentyl acetate
Butyl alcohol	*Amines*	Pentyl caproate (hexanoate)
tert-Butyl alcohol	Allyl	*Miscellaneous*
Dodecyl alcohol	2-Hydroxyethyl	Camphor
Eucalyptol	Isopentyl	Cyclohexane
Eugenol	Triethyl	

Table 2. Comparative physical, chemical, and spectral properties of the sex attractant of *Limonius californicus*, the isomeric valeric acids, and their respective methyl esters. B.P., boiling point; R.I., refractive index; R.T., retention time.

Acids and methyl esters	B.P. (°C) (*9*)	R.I. (n_D^{25})	R_F (*10*)	R.T. (min) (*11*)
		Pheromone		
Acid	190	1.4063	0.87	
Ester		1.3937		2.0
		Valeric		
Acid	186	1.4063	0.87	
Ester	126	1.3937		2.0
		Isovaleric		
Acid	176	1.4008	0.84	
Ester	117	1.3900		1.4
		dl-2-Methylbutyric		
Acid	175	1.4030	0.84	
Ester	105	1.3828		1.4
		Pivalic		
Acid	164	*	0.85	
Ester	104	1.3852		1.2

* Solid, m.p. 36°C.

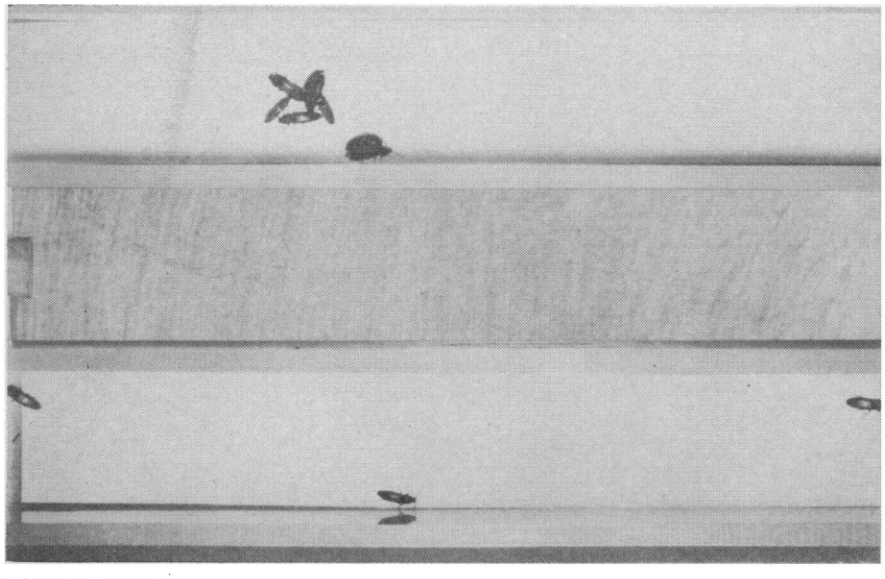

Fig. 1. Detection of active pheromone fraction by males of *Limonius californicus* in an olfactometer. In the upper compartment, the ten beetles respond to 0.1 ml of the liquid acid fraction by exhibiting typical sexual reactions, namely, waving their antennae, extruding their genitalia, and mounting each other repeatedly. In the lower compartment, no beetles are attracted to an equal amount of the neutral acid fraction.

valeric acid caused immediate sexual excitement among the bettles but as they approached the spot, they became increasingly confused and were repelled. Many rubbed their antennae as though experiencing irritation. The beetles then gathered 7 or 8 inches (7.5 to 20 cm) downwind from the spot and clustered there in typical response. As the acid was increasingly diluted, the responses of the beetles became more localized, until they grouped on the spot with 0.5 ml of a 0.1 percent solution. However, further dilution of the acid to 0.01 percent caused a marked reduction in response. Negative results were obtained with various concentrations of the isomeric acids—*dl*-2-methylbutyric, isovaleric (3-methylbutyric), and pivalic (trimethylacetic)—and with crotonic (*trans*-2-butenoic) and sorbic (*trans*-2,*trans*-4-hexadienoic) acids.

This is the first instance of the identification of a sex attractant from only a few insect specimens. The large amount (> 100 μg) obtained from each female suggests that the pheromone is stored in the female's body, possibly in bound form, and released in the free form as needed. This hypothesis is supported by the fact that a strong odor of valeric acid was detected only after the insect fraction was treated with alkali and subsequently acidified. Lilly and McGinnis (*4*) reported that for at least 4 hours after mating the female beetle contained the sex attractant in an amount equal to that of an unmated receptive female. However, with increasing postcopulatory time the amount recovered decreased fairly rapidly.

The stability, volatility, and ready availability of valeric acid make it a potentially valuable material for surveying for infestations of *L. californicus* and as a bait in traps for population control (*2*).

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References and Notes

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2. C. E. Lilly, *Can. Entomologist* **91**, 145 (1959).
3. C. E. Lilly and A. J. McGinnis, *ibid.* **97**, 317 (1965).
4. ———, in preparation.

5. R. S. Lehman, *J. Econ. Entomol.* **25**, 949 (1932).
6. C. E. Lilly, unpublished experiment.
7. Bio-Sil HA, minus 325 mesh, obtained from Bio-Rad Laboratories, Richmond, Calif.
8. The hexane used in these investigations was purified to the equivalent of spectral grade by percolation of reagent-grade hexane through silica gel and distillation. The ethyl ether was distilled and stored over sodium. The methanol was reagent-grade that was distilled before use.
9. Melting points are corrected; boiling points are uncorrected.
10. R_F values obtained by developing chromatograms with the ethanol-ammonium hydroxide system (95:5) were frequently not reproducible. This difficulty was overcome by increasing the ammonia in the developing solvent.
11. The vapor phase chromatography was carried out on an F & M instrument equipped with a Model 1609 flame-ionization attachment by using a stainless steel column (3.7 m by 0.31 cm diameter) packed with 10-percent diethyleneglycol succinate on acid-washed Chromosorb W; column temperature, 95°C; nitrogen flow rate, 20 ml/min.
12. Commercially available valeric acids almost always contain varying percentages of the structural isomers as contaminants, but the pure isomers were obtained by slow distillation through a long, straight-tube column. The methyl esters were prepared by refluxing the respective acids for 4 to 5 hours with methanol containing 5 percent of gaseous hydrogen chloride, boiling off the methanol, washing an ether solution of the ester with cold water, and fractionally distilling the product at atmospheric pressure through a straight-tube assembly.

26 October 1967

Protein Conformation in Solution: Cross-Linking of Lysozyme

Abstract. *The cross-linking of lysozyme by reaction with phenol-2,4-disulfonyl chloride has been effected. The cross-linked protein retained enzymatic activity, has approximately the same molecular weight as native lysozyme, and has essentially the same conformation as native lysozyme as judged by optical rotatory dispersion analysis. The positions of sulfonylation were assigned by a standard degradation sequence; the presence of sulfonamide bonds was confirmed by infrared spectroscopy. Cross-links may thus be introduced without incurring major structural changes in the protein, and certain intramolecular distances that are allowed in the active enzyme may be deduced.*

The primary structure of many proteins has now been worked out (*1*). A few of these have been studied by means of x-ray crystallographic techniques. The position of each atom heavier than hydrogen can be deduced from the x-ray data, and this has been done in the case of myoglobin (*2*). In other proteins the position of the backbone polypeptide has been determined, even though the exact positions of the side chains of the polypeptide chain (or chains) remain largely unknown.

It is important to determine whether a protein has the same three-dimensional structure in solution as it does in the crystalline form. Lysozyme is a protein that is amenable to such a comparison. Egg-white lysozyme has been studied in great detail; its primary structure has been determined (*3*), and a partial description of the three-dimensional structure has been set forth (*4*). Many physical methods have been utilized to study various aspects of the structure of lysozyme; frequently the information determined by physical methods can indicate the nature of various combinations of certain functional groups in juxtaposition, that is, hydrogen bonding, helical structure, but these methods cannot, in general, determine the position of functional groups in the amino acid sequence nor necessarily to structures proposed on the basis of x-ray diffraction studies.

The cross-linking of globular proteins with reagents of fixed geometry appears to be a tool of potential value in determining certain spacings within a protein molecule; studies with various reagents have been reported, although few assignments of cross-linking positions have been made (*5*).

We now report a method for assigning certain allowed distances within the lysozyme molecule and to establish minimum spacings for other groups. Lysozyme was subjected to cross-linking by phenol-2,4-disulfonyl chloride under essentially isoionic conditions. The isoionic conditions were maintained during the time of the cross-linking by suspending both the cation-exchange resin Dowex-50W-X12 (H form) and the anion exchange resin Dowex-1-X12 (OH form) in a solution of lysozyme (1 mg/ml). Lysozyme was not significantly adsorbed nor denatured by this treatment. Lysozyme was recovered in good yield and with complete retention of enzymatic activity, in control experiments. S^{35}-Labeled phenol-2,4-disulfonyl chloride dissolved in acetone was added slowly (approximately over an 8-hour period) to the gently stirred slurry of the resin particles in the lysozyme solution, which was essentially neutral throughout the process (approximately *p*H 7), hydrolyzed excess reagent was removed from the solution quantitatively during the run as demonstrated by the very low radioactivity in

Table 1. Amino acid composition of lysozyme peptides and assignment to primary structure. The letter T (last column) means tryptide; the subscript designates the position in the primary sequence (*3*). The abbreviations of the amino acid residues are: Lys, lysine; Arg, arginine; Cmc, carboxymethyl cysteine; Asp, aspartic acid; Thr, threonine; Ser, serine; Glu, glutamic acid; Pro, proline; Gly, glycine; Ala, alanine; Val, valine; Met, methionine; Ile, isoleucine; Leu, leucine; Phe, phenylalanine; and Tyr, tyrosine.

Peptide *	Presence of amino acids †																Assignment ‡
	Lys	Arg	Cmc	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met§	Ile	Leu	Phe	Tyr	
A	+	+	+	+	+	+	+	○	+	+	+	○	+	+			$T_3T_4T_{11}T_{12}T_{13}$
A_{11}	+		○				+			+		○		+			T_3
A_{12}		+															T_4
B	+	+	+	+	○	+	+	+	+	+	+	○	+	+	○	+	$T_6T_7T_{11}T_{12}T_{13}$
B_{22}	+		+	+		○			+	+	○			+			T_6
B_{24}		○		+	+	○	○			+				●	+	○	T_7
B_{31}	+		+	○	+	+		○		+	+		+	+			T_{11}
C	+	+	+	+	+	+	+		+	+	+	○	+	+	○	+	$T_6T_7T_{15}T_{16}$
C_{21}	+		○	○		○			+	+	○			+			T_6
C_{22}		○		+	+	+	○			○					+	○	T_7
C_{51}	+	+	+	○	+		+		○	+	+		+				$T_{15}T_{16}$
C_{52}	+		○														T_{15}

* The letters A, B, and C represent radioactive peptides derived by tryptic digestion of one of the fractions of cross-linked lysozyme. Each peptide (A, B. . .) was treated with hydrobromic acid (HBr) to give A_1, A_2, A_3. . . . Each of these was subjected to a second tryptic digestion: $A_1 \rightarrow A_{11}$ and A_{12}, and others, according to the procedure described (*6*). † Meaning of symbols: +, found and predicted from the Canfield sequence; ○, not found but predicted; ●, found but not predicted. ‡ The compositions observed by thin-layer chromatography were matched to the reported tryptide. Compositions reported by Canfield (*3*). § Methionine apparently modified to sulfoxide or sulfone (or both) by released bromine in HBr cleavage. It was not positively identified.