Identification of a Sex Pheromone from a Spider

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The compounds (*R*)-3-hydroxybutyric acid and its dimer, (*R*)-3-[(*R*)-3-hydroxybutyryloxy]-butyric acid, have been identified as sex pheromones of a spider. These compounds elicit web reduction behavior by males of *Linyphia triangularis* (Clerck) on the webs of unmated adult females.

For a long time it has been postulated that spiders communicate by using pheromones (*1*). In spite of several biological studies that have proven their existence [for a review, see (*2*)], no sex pheromones have been identified. The Linyphiidae are the second largest spider family with over 3500 species in 400 genera. The females of many of these species build horizontal sheet webs. The males use two different mating strategies (*3*). In densely populated areas, penultimate female instars are guarded by a male until the final molt, after which copulation takes place. If no male is present, the adult female produces a pheromone that is emitted from the web to attract males (*4*). When a male reaches the web, it may start to cut threads of the web and to roll up large parts of it to form a small ball. This very specific male behavior is observed only on the webs of unmated females. Before cutting threads, the male walks actively around the web and adds silk to the web in a zigzag pattern (zigzag walking) all along the periphery a few centimeters inside the margin (*5*). The cutting of the web is done in an agitated way systematically along the margin of the web and the threads suspending the web are cut close to the attachment points. After completion of this procedure, courtship and copulation start, lasting up to 5 hours (*3*). The web reduction behavior is believed to reduce the rate of evaporation of the pheromone from the web (*4*). Thus, the male reduces the chances of another male reaching the web and interfering with him. Encounters between two males on a female web lead to termination of the courtship of the first male and to fighting between the males (*6*). After mating, females make new webs that are no longer attractive to the males (*4*).

We became interested in the identification of the sex pheromone of *Linyphia triangularis* (Clerck), a widespread European linyphiid that lives in forests and *Calluna* fields. Penultimate female instar spiders were collected in the field and kept in boxes equipped with plastic or glass frames (10 cm by 10 cm by 10 cm), between which the females built their webs. These frames were used with the web reduction behavior of the males as an assay to guide us in the identification of the sex pheromone. Adult males, collected from the field, were placed individually on a web from which the female had been removed. If the male started to cut threads within 5 min after introduction, the experiment was counted as positive for the presence of pheromone (*7*). The males often responded positively and cut threads of the webs of unmated adult females, whereas they never reacted on webs of mated females. Extracts of webs from unmated females were prepared with hexane, methylene chloride, methanol, or water. These extracts were sprayed onto the inactive webs of mated females, and the reaction of males toward such material was observed. The extracts made with methylene chloride, methanol, or water were able to induce web reduction, whereas hexane extracts proved inactive.

Methylene chloride extracts of silk from unmated and mated females (called U extract and M extract, respectively) were analyzed by gas chromatography (GC) and gas chromatography–mass spectroscopy (GC-MS). A large, tailing peak occurred in the chromatograms of the U extracts, whereas it was absent in the M extracts. Derivatization with diazomethane improved the peak shape and gave the chromatograms shown in Fig. 1, indicating that the large peak in the U extract is a carboxylic acid. In addition, a minor, early eluting peak occurred in the chromatograms of the U extracts only. By GC-MS, this compound was identified as methyl 3-hydroxybutyrate (MHB). There were similarities between the mass spectrum of the later eluting main component and the mass spectrum of MHB in the lower mass region, but the mass spectrum of the later eluting main component showed in addition characteristic ions at a mass-to-charge ratio (m/z) of 101, 128, 160, and 189. Therefore, this compound was interpreted to be the dimer of MHB, methyl 3-(3-hydroxybutyryloxy)-butyrate (MHBB). The ions were attributed to a loss of $CH_3CHOHCH_2COO$ (101), $CH_3CH{=}O$ (160), and in addition CH_3OH (128) and CH_3 (189) from the molecular ion. Comparison with a synthetic sample (*8*) confirmed our identification. Analyses by GC-MS of nonmethylated extracts proved that the naturally occurring compounds were the corresponding free acids, 3-hydroxybutyric acid (HBA) and 3-(3-hydroxybutyryloxy)-butyric acid (HBBA). Traces of methyl crotonate (MC) could also be detected in the chromatograms of the methylated U extracts. Presumably as a result of the small amounts and its eluting properties, free crotonic acid (CA) could not be detected in the underivatized extracts. Methylation of pure samples of HBA and HBBA with diazomethane showed that no MC was formed during this process, proving the presence of CA in the original samples. In addition, traces of the trimer 3-[3-(3-hydroxybutyryloxy)-butyryloxy]-butyric acid (HBBBA) could also be detected in the chromatograms of some U extracts.

The absolute configurations of HBA and HBBA were determined with methylated extracts in GC on a chiral Lipodex E phase (*9*). The natural products were represented by pure (*R*)-enantiomers (Fig. 2). Whereas the enantiomers of MHB were well separated, MHBB could only be separated into its diastereomers. To elucidate its configuration, a U extract was saponified with potassium hydroxide in methanol to yield MHB, which proved to be exclusively the (*R*)-enantiomer, thus establishing the (*R,R*)-configuration for the naturally occurring HBBA (Fig. 3).

Synthetic samples of the identified com-

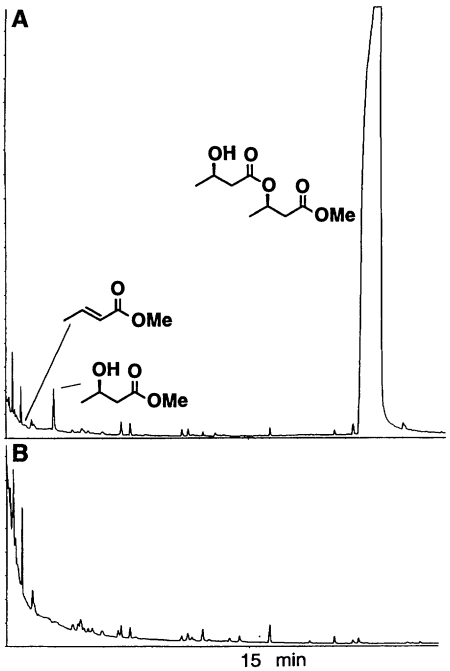

Fig. 1. Total ion chromatograms of methylated methylene chloride extracts of webs of (**A**) unmated and (**B**) mated *L. triangularis* females (30m Rt_x-5 fused-silica GC column).

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pounds (8) were tested in the web reduction experiment. Both (*R*)-HBA and (*R*,*R*)-HBBA were active, whereas CA and the nonnatural (*S*)-HBA were inactive (Table 1). These results show that (*R*)-HBA and (*R*,*R*)-HBBA are sex pheromones of female *L. triangularis* and that they can trigger the web reduction behavior of the males. In the same web reduction test series, males that had tested positively with (*R*)-HBA were tested with CA (30 individuals) and (*S*)-HBA (9 individuals). The lack of response in all cases indicates that the web reduction behavior is not dependent on sexual motivation.

The pheromones are also likely to play a role in the attraction of the males to the webs. Preliminary olfactometer experiments with another species, *L. montana*, indicate that (*R*)-HBA and CA may be attractive for males whereas (*R*,*R*)-HBBA is not, presumably because of its low vapor pressure (9).

The dimer HBBA occurs in relatively large amounts (up to 5 μg per web), but HBA (up to 0.05 μg per web) and CA are present in much smaller quantities. The dimer HBBA and the monomer HBA are relatively unstable compounds; HBBA disintegrates slowly upon standing into the monomer, HBA, and the trimer, HBBBA, presumably by autocatalysis. The monomer HBA can easily lose water to form CA; thus, HBBA may be the precursor of both compounds (*10*).

The polymer of (*R*)-HBA, poly-(*R*)-3-hydroxybutyrate, is a widespread storage form of carbon in microorganisms (*11*). Some microorganisms produce extracellular enzymes that can selectively cleave the polymer into (*R*,*R*)-HBBA, the same enantiomer that occurs on the spider webs (*8*). This coincidence suggests that microorganisms may be involved in the production of the dimer. Involvement of microorganisms in the production of pheromones has been proposed for some insects (*12*).

Whether by producing HBBA in relatively large quantities, either de novo or by use of microorganisms, the spider has an efficient pheromone delivery system. The female spider deposits HBBA on the silk, most probably during web construction, thus covering a large area with HBBA.

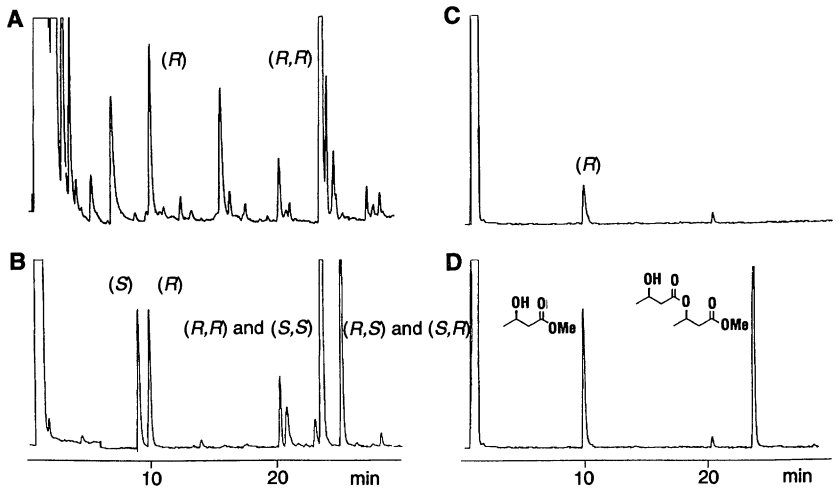
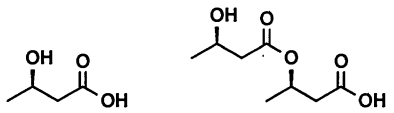


Fig. 2. Separation of enantiomers of MHB and MHBB on a chiral Lipodex E phase. (**A**) Natural extract as in Fig. 1. (**B**) Racemic mixture of MHB and MHBB. (**C**) Saponified (KOH, methanol) and methylated natural extract. (**D**) (*R*)-MHB and (*R*,*R*)-MHBB enantiomers.

Once on the silk, the dimer decomposes slowly to form HBA, which in turn breaks down into CA; the volatility of the compounds increases with each transformation. The advantage for the female of the use of such compounds would be an increased and constant rate of evaporation of the pheromone, independent of permanent activity. Upon arrival, the male immediately reduces the rate of pheromone evaporation by reduction of the web surface. After copulation, the female rebuilds its web without pheromone (*13*).

We also investigated the webs of five other linyphiid spiders that are members of the three closely related genera *Linyphia*, *Microlinyphia*, and *Neriene*. We found HBA and HBBA in webs of unmated females of *L. tenuipalpis*, *N. emphana*, *N. clathrata*, and *M. impigra*. At least one of the spiders, *L. tenuipalpis*, lives in the same habitat and develops during the same time of the year as *L. triangularis* (*14*). Web reduction in males of this species was also induced by (*R*)-HBA. Cross attraction to female webs of a related species would be possible if no other mechanisms of separation are involved, because both species seem to use the same attraction pheromone. Heterospecific males put in contact with webs of virgin females containing the natural pheromone sometimes perform web reduction, although generally at a lower frequency than in conspecific webs (Table 2). One out of 12 *L. tenuipalpis* males tested on mated *L. triangularis* webs sprayed with (*R*)-HBA performed web reduction.



(*R*)-HBA (*R*,*R*)-HBBA

Fig. 3. Sex pheromones of *L. triangularis*.

The reduced responsiveness of males on heterospecific webs may reflect the presence of species-specific chemicals on the web. We found that all webs of the linyphiids that we investigated were covered with a lipid layer. The lipids consist of hydrocarbons, carboxylic acids, aldehydes, amides, and wax esters, which are common in the cuticle of arthropods (*15*). In addition, long-chain methyl-branched alkylmethyl ethers could be identified, which form a new class of natural products (*16*). Whereas the pattern of the ubiquitous compounds mentioned above was more or less similar in the webs of all species examined, the alkylmethyl ethers

Table 1. Results of tests for the induction of web reduction behavior in males of *L. triangularis* by synthetic compounds (*18*).

Compound	Control		Test		Statistical test	
	Pos.	Neg.	Pos.	Neg.	χ^2	*P*
(*R*,*R*)-HBBA	0	26	16	22	12.44	0.0007
(*R*)-HBA	0	58	49	82	27.37	0.0000
(*S*)-HBA	0	20	0	17		
CA	0	35	0	50		

Table 2. Web reduction response of males in conspecific and heterospecific webs of unmated females.

Males	Webs			
	L. triangularis		*L. tenuipalpis*	
	Pos.	Neg.	Pos.	Neg.
L. triangularis	29	20	8	16
L. tenuipalpis	2	27	36	27
N. emphana	5	4	3	15

showed strong species-specific differences. It is unknown whether these compounds are involved in species recognition.

The above-mentioned volatile acids may form a less species-specific long-range pheromone that is embedded in a species-specific matrix that accounts for close-range recognition. In another linyphiid, *Frontinella pyramitela*, spiders are able to distinguish between sexes by use of chemical stimuli emitted from the cuticle (*17*).

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7. We did not follow web reduction to completion in order to test several males on each web. Cutting of threads has been observed in one other context: males walking on the upper side of the female's sheet may cut a small hole through which they can get to the underside. Web reduction is always done from below the sheet. Cutting single threads is very rarely observed and has never been accepted as a positive response. Our 5-min criterion was chosen to allow the males to recover from the disturbance due to experimental manipulation and to allow for the variable time spent in zigzag walking. We knew from experience that web reduction rarely started later.
8. (*R*)-HBA was synthesized from poly-(*R*)-3-hydroxybutyrate by solvolysis with methanol followed by saponification [D. Seebach and M. Züger, *Helv. Chim. Acta* **65**, 495 (1982)]. (*S*)-HBA was obtained by yeast reduction of ethyl acetoacetate followed by saponification [E. Hungerbühler, D. Seebach, D. S. Wasmuth, *Helv. Chim. Acta* **64**, 1467 (1981)]. (*R,R*)-HBBA was prepared starting from (*R*)-HBA by protection of the alcohol or acid function, respectively, followed by condensation of the two units and deprotection [D. Seebach, U. Brändli, P. Schnurrenberger, *Helv. Chim. Acta* **71**, 155 (1988); T. Tanio *et al.*, *Eur. J. Biochem.* **124**, 71 (1982)].
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13. If microorganisms are involved in the pheromone production, the spider must have a mechanism to stop them from producing HBBA.
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18. In the experiments we used empty webs of mated females. Before spraying, a series of males was tested on each web to ascertain lack of activity (control columns). Subsequently the webs were sprayed with a methylene chloride solution of 1 to 5 mg of each compound, and the males were added again after evaporation of the solvent (test columns). Because of the open structure of the web, only a small amount of the solution was actually applied on the silk by the spraying procedure. Positive responses were obtained only during the first 30 to 60 min after spraying, indicating rapid evaporation or deactivation of the applied compound. The webs were therefore resprayed at regular intervals, which usually reactivated the web. As a result of the deactivation of the pheromone, the chances of a positive response decrease with time and positive responses are therefore underrated in the test. Rather than reducing the data set to results obtained within a restricted period of time after spraying, we have analyzed the full data set, as this made it more difficult for us to obtain statistical significance. However, because the controls showed no positive responses, the statistical significance is rather insensitive to variations in the male response rate. The statistical test we used is the χ^2 test. The response frequency of the males seemed to depend also on uncontrolled environmental factors such as barometric pressure and air humidity. Evaluation of these factors is in progress.

19. We thank S. Westermann for preliminary experiments, K. Rosendahl for technical assistance, W. Francke for critical review of the manuscript, W. A. König for supplying the Lipodex E GC column, and D. Seebach for additional samples of HBBA.

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Structural Basis of Amino Acid α Helix Propensity

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The propensity of an amino acid to form an α helix in a protein was determined by multiple amino substitutions at positions 44 and 131 in T4 lysozyme. These positions are solvent-exposed sites within the α helices that comprise, respectively, residues 39 to 50 and 126 to 134. Except for two acidic substitutions that may be involved in salt bridges, the changes in stability at the two sites agree well. The stability values also agree with those observed for corresponding amino acid substitutions in some model peptides. Thus, helix propensity values derived from model peptides can be applicable to proteins. Among the 20 naturally occurring amino acids, proline, glycine, and alanine each have a structurally unique feature that helps to explain their low or high helix propensities. For the remaining 17 amino acids, it appears that the side chain hydrophobic surface buried against the side of the helix contributes substantially to α helix propensity.

Estimates of the α helix propensity of the different amino acids have been made on the basis of many different sources including statistical surveys (*1, 2*), host-guest analysis (*3*), model peptides (*4–7*), directed mutagenesis (*8, 9*), and molecular dynamics calculations (*10, 11*). To determine α helix propensity in the context of a folded protein and to obtain insights into its underlying physical basis, we made multiple amino acid replacements at each of two positions in α helices of T4 lysozyme.

Ideally, the substitution site for such an experiment would be in the middle of a relatively long, regular helix and would be exposed to solvent, remote from charged groups, devoid of possible contacts with neighboring side chains, and not close to a contact site in the crystals. Because all of these criteria cannot be met in T4 lysozyme, Ser^{44} and Val^{131} were chosen as compromises (*12*). All 19 alternative amino acids were substituted at position 44 (*13*), and the proteins were purified (*14*). For 13 replacements (Ala, Arg, Asn, Glu, Gly, Ile, Leu, Lys, Phe, Pro, Thr, Trp, and Val), crystals suitable for structural analysis were obtained (*15*). The coordinates have been deposited in the Brookhaven Protein Data Bank.

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With the exception of Pro, no substitution significantly distorted the α helix backbone. In the isomorphous structures (except for Pro) the backbone atoms within the α helix superimposed within 0.10 to 0.14 Å (Fig. 1A). For the nonisomorphous structures, the corresponding values were 0.19 to 0.33 Å but did not suggest a distortion of the helix (Fig. 1B). Thus, except for Pro there is no clear evidence that differences in helix propensity are caused by different amounts of strain introduced within the α helix. Similar structural results were observed for eight variants at position 131 (*16*).

We determined the thermostabilities of the mutant lysozymes (Table 1) by recording the circular dichroism as a function of temperature (*17*). Excluding those of Asp and Glu, the differences between the free energy of unfolding of each mutant and that of the Gly variant used for the site 131 replacements are remarkably similar to those at site 44 (correlation of 97%). In the crystal structure of the Asp^{131} variant, Asp^{131} forms a salt bridge with Arg^{154}, and we presume that electrostatic stabilization also occurs for Glu^{131}. The measured ΔΔG values were compared with other determinations of helix propensity (Table 1). The agreement with propensity scales that were derived from three studies of model peptides (*4–6*) was good (correlations of 71 to 93%), despite the different sequences involved, the differences