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Identification of a New Sex Pheromone from the Silk Dragline of the Tropical Wandering Spider *Cupiennius salei***

Mirjam Papke, Stefan Schulz,* Harald Tichy, Ewald Gingl, and Rudolf Ehn

Dedicated to Professor Wittko Francke on the occasion of his 60th birthday

The chemistry of pheromones of arachnids and particularly spiders has not received the same attention as that of the pheromones of insects,[1] despite the widespread distribution of spiders, their importance in many ecosystems, and their species-rich taxon. The silk is known to play a role as substrate for pheromones in various spiders,[2] but so far only one pheromone has been identified.[3] The large tropical wandering spider *Cupiennius salei* (Ctenidae) uses its silk dragline as a chemical signpost, which it leaves behind in the environment. A single thread of a female initiates courtship behavior in conspecific males. The males recognize the inherent chemical signal by examining the silk thread with their pedipalps and send vibratory signals through the substrate which are returned by the female.[4] In the current study we report on the identification and synthesis of the female courtship-inducing pheromone.

Silk can be obtained from the spiders by using an electrically driven reeling machine which draws the silk thread out of the silk glands of an immobilized, but awake spider. About 5 – 10 mg silk can be obtained in a week from a single individual. A bioassay was developed to guide in the identification of the active principle. In short, the spiders wandered along a glass duct covered with filter paper and encountered natural or chemically modified silk threads on their way. The characteristic vibratory courtship behavior could be elicited by silk samples from adult females.[5] In contrast, silk from males or juvenile females was not active. Active silk was extracted with different solvents and the extracts were then applied on inactive silk from male spiders to test their activity. Methanol extracts showed good responses and were used for further analyses.

By use of GC-MS methods usually employed in pheromone research only methyl pyroglutamate could be identified, but both enantiomers did not evoke any response in the bioassay. We then analyzed extracts by NMR spectroscopy. Surprisingly, active extracts prepared with deuterated methanol showed the presence of only one prominent compound, which was absent in inactive silk samples (Figure 1). The spectrum

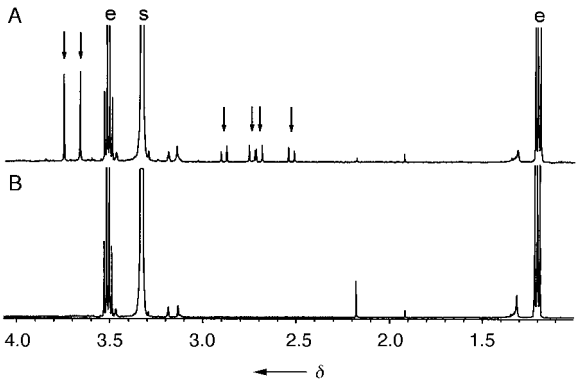

Figure 1. ^{1}H NMR spectrum of a silk extract (CD_3OD) from adult female (A) and male (B) *Cupiennius salei* spiders. Arrows indicate characteristic signals; e: ethanol (contaminant), s: solvent.

showed two different CH_3 groups and two different CH_2 groups, each of them with diastereotopic H atoms. The spectrum is consistent with the structure of the asymmetric dimethyl citrate **1**. This ester, to the best of our knowledge not known from nature before, was then synthesized as racemate to confirm the assignment.[6]

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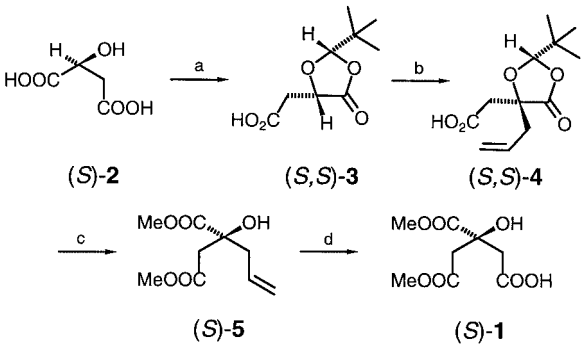
Comparison of the spectra of the synthetic asymmetric dimethyl citrate and the natural compound revealed some differences in chemical shift values of the CH_2 groups. Because these values depend on the pH value and owing to the presence of ions in the solution, mixing experiments were performed. A natural sample was blended with the same amount of synthetic material, using benzene as internal standard. The NMR spectrum showed only one set of signals, thus confirming that the natural compound is indeed the asymmetric dimethyl citrate **1**.

Both enantiomers were then synthesized for assignment of the absolute configuration of the natural compound (Scheme 1). Both (*R*)- and (*S*)-malic acids **2** were converted with pivalaldehyde into the respective dioxolanones **3**, which



Scheme 1. Synthesis of (*S*)-cupilure (*S*)-**1**. a) H^+, pivalaldehyde; b) Li-HMDS, then CH_2=CH-CH_2Br; c) 5 equiv $BF_3 \cdot Et_2O$, MeOH; d) $RuCl_3$, $NaIO_4$, CCl_4, MeCN, H_2O. HMDS = hexamethyldisilazanide.

were subsequently alkylated with allyl bromide, according to the method developed by Seebach et al.[7] The resulting allyl dioxolanones **4** exhibited a high enantiomeric ratio in excess of 96:4. Treatment with a large excess of $BF_3 \cdot OEt_2$ in methanol cleaved the acetal and esterified the acid groups in one step. Final oxidative cleavage of the double bond in **5** with RuO_4[8] yielded the desired enantiomers (*R*)- and (*S*)-**1** in a high enatiomeric ratio (96:4).

Comparison of the synthetic samples with the natural compound by gas chromatography on a chiral phase proved the predominant *S* configuration (e.r. = 95:5) of the natural ester (Figure 2). Separation was possible only after transformation of **1** into its isopropyl ester **6** with isopropyl alcohol/acetyl chloride. The conditions of the derivatization did not alter the enantiomeric ratio of the compounds. Only the cyclodextrin phase heptakis(6-*O*-TBDMS-2,3-di-*O*-acetyl)-β-cyclodextrin (in 50% OV1701)[9] was able to separate the racemate (TBDMS = *tert*-butyldimethylsilyl), while several other phases tested failed.

Behavioral experiments with the synthetic material applied to inactive silk samples proved that (*S*)-**1** stimulates male courtship behavior, while the respective *R* enantiomer is inactive.[5] The synthetic (*S*)-**1** enabled for the first time the electrophysiological identification of a pheromone sensilla in a spider.[5] These sensilla are contact chemoreceptors located on the pedipalps. Single-cell recordings were made by inserting an electrolytically sharpened tungsten wire into the shaft of the sensillum; the second electrode was introduced into the tip of the pedipalpus. The area of a single sensillum

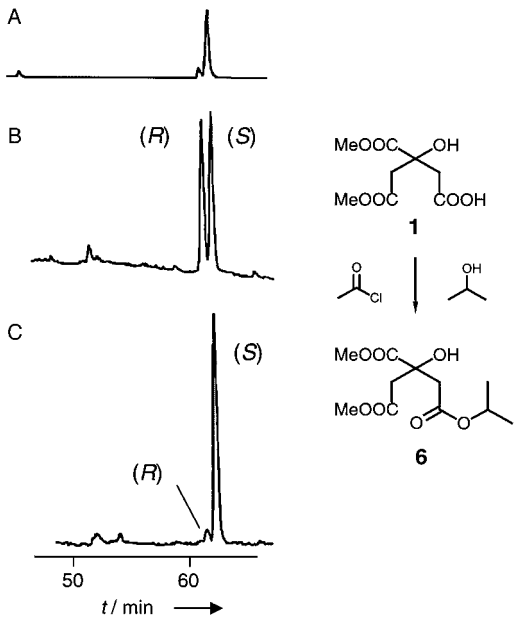


Figure 2. Gas chromatographic separation of dimethyl citrate **1** on a chiral phase (25 m 50% heptakis(6-*O*-TBDMS-2,3-di-*O*-acetyl)-β-cyclodextrin and 50% OV1701,[10] 110 °C isothermal) after transformation into the respective isopropyl esters **6**. Synthetic (*S*)-**1** (A), *rac*-**1** (B), and natural silk extract containing **1** (C). The stereochemical designators refer to the parent compound **1**.

that contacts the silk dragline is 0.03 μm^2. This area contains at most 170000 pheromone molecules, as we extracted a maximum of 1 μg pheromone from 20 m dragline silk.

Our results from chemical analysis, from electrophysiology of pheromone-receptive sensilla, and from the behavioral assay clearly demonstrate that **1**, for which we propose the name cupilure, is a contact sex pheromone bound to the silk of female *Cupiennius salei* spiders. Cupilure is a unique compound, structurally not related to known pheromones. The only other contact spider pheromone identified so far is (*R*,*R*)-3-(3-hydroxybutyryloxy)butyric acid, which induces web reduction by the males of *Linyphia triangularis*.[3] Both compounds are biosynthetically very close to the primary metabolism: cupilure can be easily formed by double methylation of ubiquitous citric acid, while the *Linyphia* compound is a simple condensation product of (*R*)-3-hydroxybutyric acid, an important intermediate of the fatty acid biosynthesis. Such close links to primary metabolites are not known from insect pheromones. Whether this is a general feature of spider pheromones, which reflects their carnivorous life style, will require further studies. Recently we have identified a volatile spider pheromone, 8-methyl-2-nonanone, which resembles known insect pheromones.[10]

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Analysis of the Silkworm Moth Pheromone Binding Protein – Pheromone Complex by Electrospray-Ionization Mass Spectrometry

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In the four decades since Butenandt et al. identified bombykol ((10*E*,12*Z*)-hexadeca-10,12-dien-1-ol) as the female sex attractant of the silkworm moth, *Bombyx mori*,[1] considerable progress has been made in our understanding of pheromone chemistry and biology. Throughout this time, the *B. mori* system has continued to serve as a useful model for unraveling the intricacies of chemical communication. Indeed, a complete picture is slowly emerging, from bombykol biosynthesis and regulation in females to olfactory detection and catabolism in male antennae.[2] The study of mechanisms involved in pheromone detection is particularly active, and has produced a detailed model of events at the molecular level. Lipophilic pheromone molecules enter the antennal sensilla (sensory hairs) through cuticular pores, where they come into contact with the aqueous lymph medium that surrounds the receptor (nerve) cells. Here they are solubilized by a pheromone binding protein (PBP; a small (< 20 kDa), water-soluble lipid binding protein),[3] and transported through the lymph to the nerve cell membrane. The pheromone receptor itself resides in this membrane, and there is strong evidence that it is G-protein-coupled.[4] Activation of the receptor, therefore, stimulates a secondary messenger cascade and ion-channel opening, thereby generating a receptor potential.

Progress towards total structural characterization of the *B. mori* PBP (BmPBP) has been rapid, from production of cDNA clones and deduction of the amino acid sequence,[5] to determination of the disulfide bridging pattern,[6] culminating in the elucidation of the X-ray crystal structure of BmPBP complexed with bombykol.[7] In an attempt to probe the interaction between pheromone and binding protein further, we examined the system by electrospray-ionization mass spectrometry (ESI-MS) under nondenaturing conditions. By careful instrumental optimization, use of near-neutral spraying buffer, and low desolvation gas temperature (30 °C), it is possible to observe a variety of noncovalently bound protein – ligand complexes in the gas phase.[8] Examples of bound ligands seen using this method include enzyme cofactors,[9] substrates,[10] and inhibitors.[11]

We postulated that it should be possible to extend this approach to the BmPBP – bombykol system, and thereby observe the noncovalent complex in the gas phase. Figure 1 shows the (transformed) native ESI mass spectrum of recombinant BmPBP alone, and the spectrum after exposure of BmPBP to bombykol (20 molar equivalents, overnight).[12] The largest peak in the upper trace (Figure 1a) displayed a mass of 15878 Da, a value identical to the theoretical mass deduced from the amino acid sequence of BmPBP, and to the measured mass using standard (denaturing) ESI-MS conditions (spraying solvent: water/acetonitrile (80/20) with 0.1% formic acid; data not shown). A second significant peak, at 16160 Da, only appeared under nondenaturing conditions and, therefore, was attributed to a noncovalent adduct of the BmPBP with an unknown contaminant of mass 282 Da. The impurity was probably introduced during expression, or purification, of the recombinant protein. It is interesting to note that oleic acid has a mass of 282 Da, and treatment of the PBP with an excess of this common lipid did lead to a significant increase in the 16160 Da peak. To date, however, we have not been able to confirm oleic acid as the contaminant.

When BmPBP was incubated with bombykol, a third ion was observed in the ESI mass spectrum (Figure 1b). In addition to the masses at 15878 (BmPBP) and 16160 Da (unknown BmPBP adduct), a large peak at 16116 Da was visible. This species was reproducibly 238 Da (= the molecular mass of bombykol) larger than the mass of BmPBP and was identified as the noncovalent BmPBP – bombykol complex [BmPBP+bombykol]. Lower molar excesses of bombykol produced a similar result (minimum excess measured was 5-fold).

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