Pheromones (1)

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The Sex Pheromone of the Wasp Spider *Argiope bruennichi***

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Current biochemical research on spiders focuses on the two characteristic traits common to spiders, namely silk and toxins. Nevertheless, chemical communication over longer distances is also vital: potential mates that are solitary and predatory need to find members of the same species (conspecifics) over large distances during the reproductive season. The production of a volatile signal that helps to attract mating partners is vital, but to date only few of the respective pheromones are known (see the following Communication).[1] Herein we report the identification of the first pheromone from an orb weaver (Araneidae), namely the wasp spider, *Argiope bruennichi*, and show for the first time that a spider pheromone can be used to trap spiders in the field.

Orb weavers are one of most successful spider families worldwide, with over 2600 known species. Early experiments showed that *Cyrtophora cicatrosa* emits a volatile pheromone that attracts males and induces courtship in higher concentrations.[2] Cages with females of *Araneus trifolium* and *Argiope trifasciata* were able to attract conspecifics.[3] The latter also attracted *Argiope aurantia* males, suggesting the presence of a common sex pheromone in both species.[4] Sex pheromones that attract conspecific males over a distance to the web of a partner has so far only been reported from the desert spider *Agelenopsis aperta* (Agelenidae) that uses 8-methyl-2-nonanone.[5]

We investigated the pheromone system of the very characteristic wasp spider *Argiope bruennichi*, which lives on meadows in the Mediterranean but occurs now also in the temperate zone of Middle Europe. When adult, the large females build webs in the grass, whilst the much smaller males wander through the meadow to find a mate, probably guided by a female pheromone. Herein, we report on the identification, synthesis, and activity of this pheromone in the field.

Headspace sampling of odors emitted by individual female spiders was performed with activated charcoal traps using glass chambers containing the spiders in three developmental and reproductive states (subadult, virgin, and mated). The adsorbent was extracted with dichloromethane and the extracts analyzed by GC-MS. Only the extract of virgin adult spiders contained a compound **A** that was absent from subadult or mated females (Figure 1).

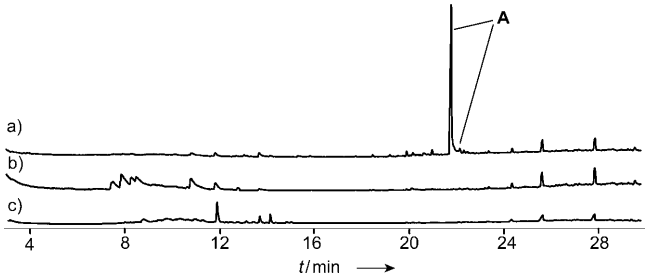

Figure 1. Gas chromatograms from headspace analysis of *Argiope bruennichi*. a) Virgin female; b) adult female; c) sub-adult female.

Extracts of webs were also analyzed, and these again showed the presence of **A** in webs of virgin females only, but additionally a second, female specific compound (**B**; Figure 2). Although **A** was present in all the samples of virgin females, compound **B** did not occur in all samples, and was also found in those from mated females. Therefore, we concentrated on the biological activity of compound **A** in the field experiments.

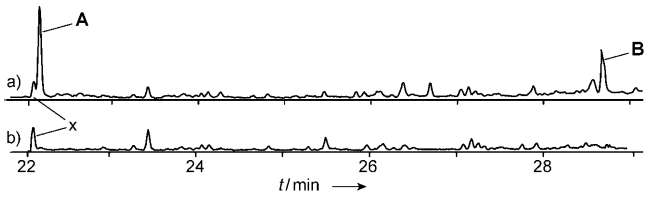

Figure 2. Gas chromatograms of silk extracts of *Argiope bruennichi*. a) Virgin female; b) sub-adult female. × = impurity.

The mass spectrum of compound **A** is similar in appearance to that of trimethyl citrate (Figure 3), but each major fragment ion is 14 mass units larger, thus indicating the presence of an additional CH_2 group at an unknown position. The derivatization with MSTFA (*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide) provided evidence for the presence of

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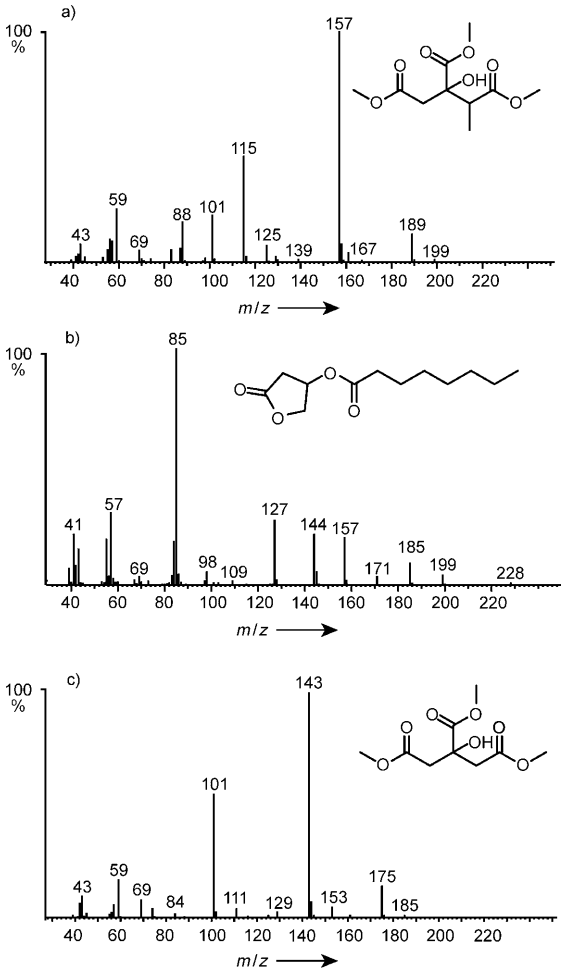


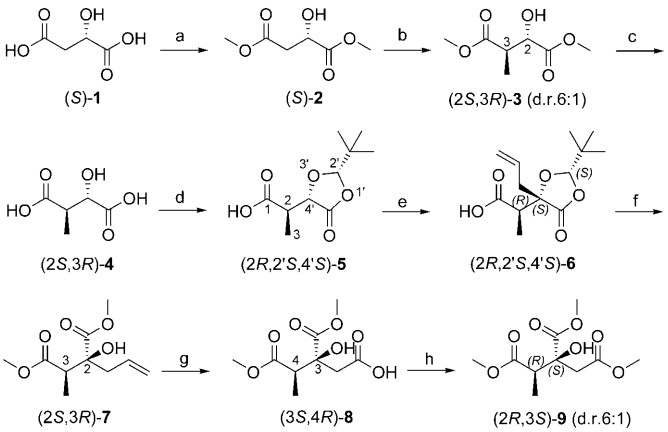
Figure 3. Mass spectra of a) trimethyl methylcitrate (**A**); b) 3-octanoyloxy-4-butanolide (**B**); c) trimethyl citrate.

one labile hydrogen atom in the molecule and indicated the molecular ion of the parent compound **A** to be *m*/*z* 248. Mixed ethyl methyl esters of citric acid have completely different mass spectra, leaving only two structures with an additional carbon atom, one being trimethyl methylcitrate (by methylation of one of the methylene groups), and the other requiring extension of the carbon chain that seems to be biosynthetically unlikely.

The mass spectrum of compound **A** is in full agreement with the trimethyl methylcitrate structure and is closely related to that of trimethyl citrate. The fragmentation can be explained by α-cleavage of the methoxycarbonyl group to form the ion *m*/*z* 189. An elimination of methanol provided the base ion *m*/*z* 157; elimination of methyl acetate (74 amu) or methyl propionate (88 amu) by a McLafferty rearrangement and loss of the methoxycarbonyl furnished the ions *m*/*z* 101 and 115 from the molecular ion (not observed) at *m*/*z* 248 (Figure 3).

Compound **A** occurred in a diastereomeric ratio of between 6:1 and 25:1 in all extracts of virgin females. The major diastereomer showed a gas chromatographic retention index (RI) of 1518 on an apolar GC phase, whereas the minor diastereomer had a RI of 1527. As trimethyl methylcitrate contains two stereogenic centers, it was necessary to synthesize stereoisomers to prove our structural proposal, to clarify the stereochemistry of the natural compound, and to provide material for biological testing. It is well-known that stereochemical aspects play an important role in pheromone research,[6] and that unnatural stereoisomers can suppress reactions in bioassays (see the following Communication).[1]

The chiral building block (*S*)-malic acid (**1**) was used as starting material (Scheme 1). After conversion into the methyl ester **2**, methylation using LDA as base[7] furnished



Scheme 1. Synthesis of trimethyl (2*R*,3*S*)-methylcitrate (**9**). a) HCl, MeOH, 80 °C; b) LDA, MeI, −78 °C; c) 2 M KOH, THF/MeOH (1:1); d) H_2SO_4, pivalaldehyde; e) LiHMDS, −78 °C, allyl bromide; f) $BF_3{\cdot}OEt_2$, MeOH; g) $RuCl_3$, $NaIO_4$, CCl_4, MeCN, H_2O; h) EDC, MeOH. EDC = *N*′-(3-dimethylaminopropyl)-*N*-ethyl carbodiimide, LDA = lithium diisopropylamide, LiHMDS = lithium hexamethyldisilazanide.

methylated dimethyl malate **3** in a diastereomeric ratio of 6:1 in favor of the 2*S*,3*R* isomer.[8] Hydrolysis of **3** gave the respective acid **4**,[9] which was then treated with pivalaldehyde to convert it into the thermodynamically favored *cis*-dioxolanone **5**[10] that was subsequently alkylated with allyl bromide, yielding **6** according to the chiral relay method developed by Seebach et al.[11] Treatment with $BF_3{\cdot}OEt_2$ in methanol cleaved the acetal group and simultaneously esterified the acid to give **7**. Oxidative cleavage of **7** with RuO_4 furnished the acid **8**,[12,13] which was esterified[14] with methanol to yield trimethyl (2*R*,3*S*)-methylcitrate as the major enantiomer. Because the Seebach method used here is highly stereoselective,[11] the minor stereoisomer present was assigned the 2*S*,3*S* configuration. The major stereoisomer synthesized proved to be identical in mass spectrum and gas chromatographic retention time to the major natural product.

Chiral gas chromatography was then performed to clarify the absolute configuration of the natural product. The separation of the four enantiomers of trimethyl methylcitrate was partly possible on a chiral hydrodex-6-TBDMS column (TBDMS = *tert*-butyldimethylsilyl; Figure 4). The analysis of natural extracts showed that the 2*R*,3*S* and 2*S*,3*S* enantiomers occurred naturally in a ratio between 6:1 to 25:1. The major isomer proved to be identical to the major synthesized compound, trimethyl (2*R*,3*S*)-methylcitrate, whilst the minor

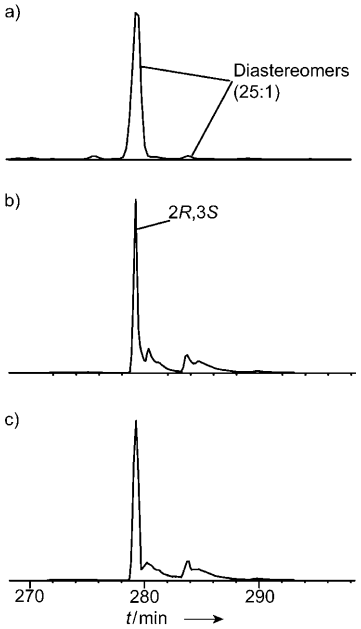


Figure 4. Gas chromatographic separation of trimethyl methylcitrate on a chiral hydrodex-6-TBDMS phase. Temperature program: 50 °C for 5 min, then with 0.2 °C min^{-1} to 200 °C min^{-1}. a) Natural extract; b) synthetic **9**; c) co-injection.

isomer is the minor synthetic product, trimethyl (2*S*,3*S*)-methylcitrate. Compound **B** exhibited a molecular ion at *m*/*z* 228. Its mass spectrum (Figure 3) was characterized by the ion *m*/*z* 85, which is characteristic for a butyrolactone ring. Other characteristic ions at *m*/*z* 127 (octanoyl), 144 (McLafferty rearrangement product), and 157 (β-cleavage to carbonyl group) and comparison with synthetic reference material showed that this compound is 3-octanoyloxy-γ-butyrolactone.

The synthesized 6:1 mixture of (2*R*,3*S*)- and (2*S*,3*S*)-trimethyl methylcitrate was then evaluated for its activity in the field. Tripod traps containing trimethyl methylcitrate and control traps were placed in an open meadow field during a dry and hot summer period. The test compound was highly attractive to males of *A. bruennichi*: during the 30 minute test period for each trial, 34 males were attracted to 10 traps containing 25 μg per trap. These males also showed typical courtship behavior, such as application of silk strands from the trap to the vegetation, jerking, and abdomen vibration. Control traps were never approached. The attractivity of the pheromone tended to decrease with the concentration; response to less than 1 μg was low, but still observable (Table 1). Higher concentrations were more attractive than lower ones, as shown by competition experiments (see Experiment 2 in the Supporting Information). We also investigated whether the ratio of the two enantiomers present in the natural pheromone is important for attraction. Both a 6:1 and a 2:1 mixture of (2*R*,3*S*)- and (2*S*,3*S*)-trimethyl methylcitrate were equally attractive to the spiders (see Experiment 3 in the Supporting Information).

The results clearly demonstrate that trimethyl methylcitrate is the sex pheromone of female *Argiope bruennichi* spiders that is used to attract males. This compound is closely

Table 1: Results of bioassays performed in the field.[a]

Amount [μg]	Trials	Successful trials (%)	Attracted males
25.00	10	10 (100)	34
12.50	10	8 (80)	19
2.25	5	3 (60)	7
1.25	10	5 (50)	6
0.625	4	4 (100)	5
0.30	10	4 (40)	4
0.15	7	1 (14)	1

[a] Traps containing differing amounts of the 6:1 mixture of (2*R*,3*S*)- and (2*S*,3*S*)-trimethyl methylcitrate (**9**) was tested against traps with dichloromethane only (control). The number of test trials, number of trials in which attraction occurred, and total number of males attracted over all trials are given. Control traps never attracted spiders.

related to the female sex pheromone of the tropical wandering spider *Cupiennius salei*,[15] unsymmetrical (*S*)-dimethyl citrate, that induces courtship behavior in males. Attraction of spiders to traps in the field by use of a pheromone has been shown herein for the first time; 8-methyl-2-nonanone, the sex pheromone of *Agenelopsis aperta*,[5] is the only other highly volatile spider pheromone known to date. Methylcitric acid occurs naturally in many organisms; the 2*R*,3*S* and 2*S*,3*S* isomers are usually formed by action of a *si*-citrate synthase in animals, such as pigs and humans,[16] whereas the other two isomers are formed by bacteria and yeasts using *re*-citrate synthase.[17] The use of derivatives of citric acid, a typical primary metabolite, as pheromones has not been reported from other animals. Whether this close connection to primary metabolites is typical for pheromones of spiders will need further exploration.

In summary, we have identified and synthesized the sex pheromone of the wasp spider *Argiope bruennichi* as a mixture of the 2*R*,3*S* and 2*S*,3*S* isomers of trimethyl methylcitrate. This pheromone is able to attract male spiders in the field in a concentration-dependent manner. Another female specific spider compound, 3-octanoyloxy-γ-butyrolactone, has been identified, but its function needs to be established.

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