A Journal of the Gesellschaft Deutscher Chemiker
Angewandte Chemie
International Edition
www.angewandte.org

Accepted Article

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To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201702864
Angew. Chem. 10.1002/ange.201702864

Link to VoR: http://dx.doi.org/10.1002/anie.201702864
http://dx.doi.org/10.1002/ange.201702864

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 10.1002/anie.201702864

The chemical web of a spider orchid – Sulfurous semiochemicals seduce male wasp pollinator

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Abstract: One of the most intriguing natural observations is the pollination of orchids by sexual deception. Here, we identify floral semiochemicals from *Caladenia* (spider orchids) for the first time. We demonstrate that *C. crebra,* attracts its single pollinator species with a unique system of (methylthio)phenols, three of which are new natural products. Furthermore, the same compounds constitute the sex pheromone of the pollinator, the thynnine wasp *Campylothynnus flavopictus*, representing the first occurrence of sulfurous sex pheromones in Hymenoptera.

While studies of plant-pollinator communities show that many plant species attract multiple pollinators,[1] numerous highly specialized pollination systems have been discovered that are underpinned by chemistry.[2a-c] Pollination by sexual deception is a highly specialized pollination strategy, primarily known from orchids, where chemical mimicry of courting female insects leads to attraction of male pollinators.[2] So far there have only been a few studies where the compounds involved have been elucidated and confirmed with field bioassays. These include either blends of alkanes and alkenes, or hydroxy- and keto acids in *Ophrys* orchids, cyclohexane-1,3-diones in *Chiloglottis* orchids and pyrazines in *Drakaea* orchids.[3]

Caladenia is a diverse genus of Australian terrestrial orchids, comprising over 360 species.[4] The genus is unique in that it employs multiple pollination strategies including food-reward, food-deception and more than 100 cases of sexual deception.[5]

Until now, the identity of the semiochemicals involved in sexual deception have remained elusive for any *Caladenia* species, although given that many different wasp genera are exploited, multiple chemical systems are expected to operate. Thus, detailed studies of the chemistry of this genus, beyond the promise of revealing new natural products, also offers a unique opportunity to better understand the role of floral volatiles in the evolution of sexual deception as a pollination strategy.

Here we investigate the chemistry of sexual deception in *Caladenia crebra* A.S.George. By combining chemical ecology, analytical chemistry and organic chemistry methods, we demonstrate that in *C. crebra*, pollination by the thynnine wasp *Campylothynnus flavopictus* (Smith, 1859) requires a specific blend of unique aromatic sulfurous compounds in precise ratios. In the exceptional mating behavior of thynnine wasps, the larger, winged males carry the smaller, wingless females *in copula* to a food source for mating and feeding.[3a] While conducting field bioassays with artificially presented orchid flowers, on three occasions thynnine wasp pairs were observed *in copula* arriving at the flower, a behavior not previously reported from sexually deceptive orchids. Furthermore, in one of these cases, which was captured on video (S3), the female was released during vigorous attempted copulation by the male with the flower. These unprecedented observations, despite the limited number of observations, confirm the extreme sexual attractiveness of the flower, *via* its semiochemicals, to the male pollinators.

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10.1002/anie.201702864

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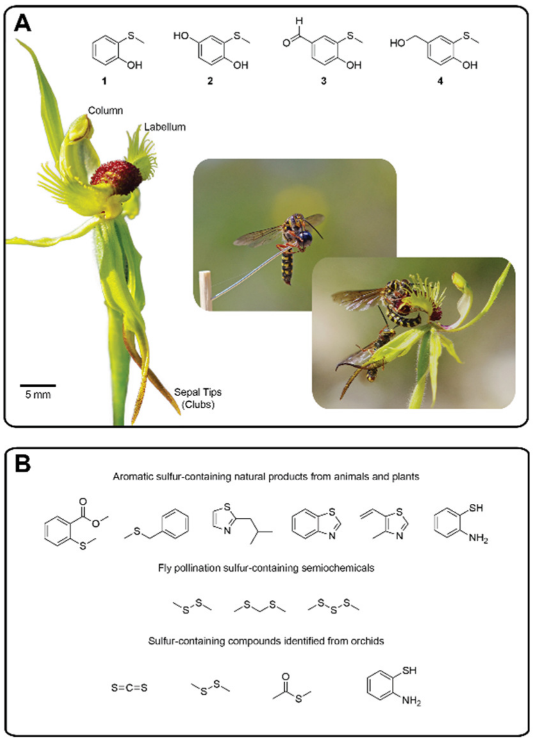


Figure 1. *Caladenia crebra*, *Campylothynnus flavopictus* and semiochemicals: A: *C. crebra* with floral parts labelled, the pollinator *C. flavopictus* pseudocopulating with a pin spiked with synthetic semiochemicals and a flower. The structures of the four (methylthio)phenol semiochemicals are shown; B: Structurally or biologically related sulfur-containing natural products in other species.[10-13, 15-16, 19]

Despite success in other thynnine systems,[3a-b] gas chromatography/electroantennography detection (GC-EAD), where insect antennae are employed as the chemical detector, failed to detect any electrophysiologically active compounds in the *C. flavopictus/C. crebra* system. Variation of GC conditions, columns and floral extraction protocols did not aid detection, indicating that the limitation is either the suboptimal chromatographic properties for these compounds (broad peaks) or problems related to the specific antennal receptors. Furthermore, GC-MS screening for cyclohexane-1,3-dione- and pyrazine-based compounds known in other Australian orchid/thynnine wasp pollination systems,[2] as well as structurally related compounds, revealed none of these compounds. A final set of semiochemical candidates was defined by their presence in both the active lateral sepal tips (clubs, Figure 1) and female wasp extracts, but not in the inactive floral remains. High resolution GC-MS (GC-HRMS) revealed compounds with molecular formulae C_7H_8OS (*m/z*: 140.0296, calcd. 140.0296), $C_7H_8O_2S$ (*m/z*: 156.0251, calcd. 156.0245), $C_8H_8O_2S$ (*m/z*: 168.0240, calcd. 168.0245) and $C_8H_{10}O_2S$ (*m/z*: 170.0399, calcd. 170.0402), all of which were more abundant in polar extracts (methanol) than in intermediately polar extracts (dichloromethane).

Mass spectra and molecular formulae indicated that all four candidates were phenols with sulfur-containing substituents. The identification process was expedited by the occurrence of the mass spectrum of our simplest candidate in the NIST-11 mass spectrum library. This compound **1** (Figure 2), previously found in bacteria,[6] was confirmed by co-injection of the biological extracts with a commercially available reference compound. Based on similarities in molecular formulae and mass spectra, we proposed several structurally related compounds for the remaining three candidates, including hydroxy, hydroxymethyl- and formyl compounds, which were all prepared synthetically and from which the matching compounds were successfully identified. Compounds **2**-**4** (Figure 2), all *ortho*-(methylthio)phenols, were confirmed to be the natural products by co-injection with extracts.[7]

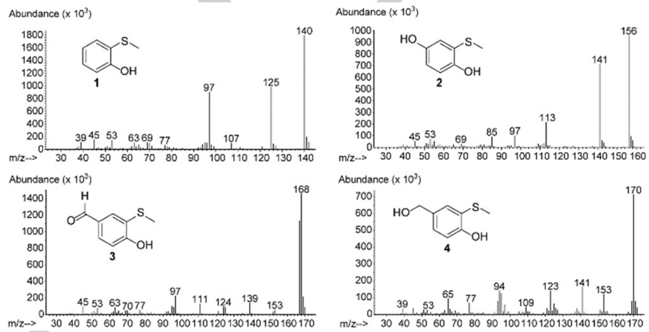
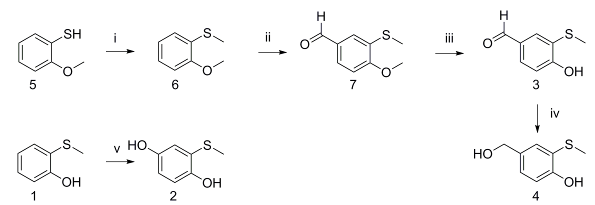


Figure 2. Mass spectra of the four (methylthio)phenols **1**-**4** in *C. crebra and C. flavopictus.*

In the analysis of solvent extracts of wasp body parts, compounds **2** and **3** were successfully extracted from heads with ethanol or dichloromethane as solvents. The ratios of compound **2** and **3** differed between the ethanol flower extracts (ca. 10-fold excess of **3**) and the two ethanol female wasp extracts examined (ca. 10-fold excess of **2**).

Compound **3** was prepared from **5** via an *S*-methylation, Vilsmeier-Haack formylation and *O*-demethylation (Scheme 1).[8] The hydroxymethyl compound **4** was prepared from **3** *via* a sodium borohydride reduction while the hydroquinone **2** could be prepared by oxidizing the commercially available phenol **1** using Fremy's salt in a buffered system (Scheme 1).[9]



i) MeI, Et_3N, DCM; ii) *N*-methylformanilide, $POCl_3$; iii) HBr, HOAc; iv) $NaBH_4$, v) Fremy's salt, Phosphate-buffer

Scheme 1. Synthesis of (methylthio)phenols **1**-**4** identified from *C. crebra* and *C. flavopictus*.

Across two years of field bioassays with these compounds, a total of 923 *C. flavopictus* male wasp responses were recorded. By a combination of additive and subtractive bioassays we show that the two thiophenols **2** and **3** are required to achieve strong sexual attraction in this system, including frequent attempted copulations at simplistic female dummies. Neither compounds **1** or **2** on their own were attractive, while compound **3** did elicit weak attraction. Compounds **1** and **4** appear to be unnecessary for eliciting strong sexual behavior, but not inhibitory in blends with **2** and **3** (Figure 3 and S1).

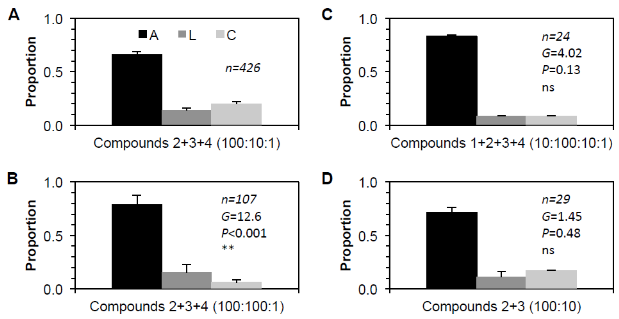


Figure 3. Outcomes of bioassays showing the response of male *Campylothynnus flavopictus* thynnine wasps to four different blends of synthetic thiophenols (**1**-**4**). Exp 1. A: Compounds **2**-**4** at 100:10:1 as control blend; B: Compounds **2**-**4** at 100:100:1; C: Compounds **1**-**4** at 10:100:10:1; D: Compounds **2** & **3** at 100:10 (control blend without **4**). Wasp responses are shown as mean proportions of the total (±se), further partitioned into approaches (A, black bars), lands (L, dark grey bars) and attempted copulations (C, light grey bars). G-test results shown are relative to the control blend shown in A. Note that there was no response in replicate experiments with a blend of **2** & **4** at 100:1 (control blend without **3**) and a blend of **3** & **4** at 10:1 (control blend without **2**). See S1 for details and the outcomes of additional bioassays.

Compounds **2**, **3** and **4** have not been previously found in nature. Sulfur-containing phenols are to our knowledge only recently discovered from marine bacteria.[6] Other aromatic sulfurous compounds identified from plants or animals, apart from orchids, are limited to thiophenes,[10] thiazoles[11] and benzyl methyl sulfide (Figure 1).[12] Sulfurous floral volatiles are typically rare[13] and their function often unknown.[14]. Within orchids the only examples are carbon disulfide,[15] dimethyl disulfide,[13] *S*-methyl thioacetate,[16] and 2-aminothiophenol,[13] each only reported once (Figure 1). Furthermore, we appear to have discovered the first volatile sulfur-containing pheromone in Hymenoptera[17] and the second in any animal[18] after methyl 2-(methylthio)benzoate was identified as a sex pheromone for two beetle species (Figure 1).[19]

The challenges to detect, identify, synthesize and confirm function of new and unusual semiochemicals, suggest that there is likely to be a strong bias towards reporting pollination studies involving common compounds. Accordingly, it is also very likely that pollination systems employing novel compounds are underrepresented in the literature, as advanced chemistry studies capable of identifying new, unusual and EAD-challenging compounds are rarely conducted. Prioritizing such studies would certainly not only lead to new semiochemical discoveries, but also to new insights into the multiple routes to specialization and the biosynthetic pathways involved.

Acknowledgements

BB, GRF, RDP, RAB and RP acknowledge the Australian Research Council for funding (DE160101313, FT110100304, DE15010720 and LP130100162). The authors acknowledge the facilities, and the scientific and technical assistance of the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy, Characterization & Analysis, The University of Western Australia, a facility funded by the University, State and Commonwealth Governments. Pollinators were identified by Graham Brown from the Northern Territory Museum and Art Gallery. Alyssa Weinstein is thanked for laboratory assistance.

Keywords: Sexual deception • (methylthio)phenol • pheromones • natural products • pollination

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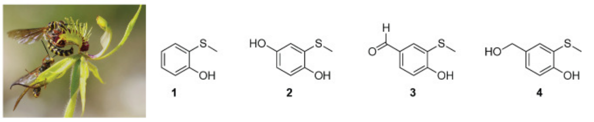

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The chemical web of a spider orchid – Sulfurous semiochemicals seduce male wasp pollinator

Sulfurous seduction: Four (methylthio)phenols **1**-**4**, where **2**-**4** are novel natural products, are extraordinary strong pollinator attractants in the sexually deceptive spider orchid *Caladenia crebra* and sexual pheromone components for the pollinator *Campylothynnus flavopictus*.